REPUBLIC OF CAMEROON
Peace-Work-Fatherland
****
UNIVERSITY OF YAOUNDE 1
****
POST GRADUATE AND TRAINING SCHOOL OF LIFE SCIENCES-HEALTH AND ENVIRONMENT
****
DOCTORATE TRAINING UNIT OF LIFE SCIENCES

RÉPUBLIQUE DU CAMEROUN
Paix-Travail-Patrie
****
UNIVERSITÉ DE YAOUNDE 1
****
CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE –SANTE ET ENVIRONNEMENT
****
UNITE DE RECHERCHE SCIENCE DE LA VIE

LABORATORY FOR MOLECULAR MEDICINE AND METABOLISM

POTENTIAL MECHANISMS OF ACTION OF PESTICIDES ON FACTORS RELATED TO TYPE 2 DIABETES

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF A DOCTOR OF PHILOSOPHY (PhD) DEGREE IN BIOCHEMISTRY

OPTION: BIOTECHNOLOGY AND DEVELOPMENT

BY:

NDONWI Elvis NGWA

Master of Science (MSc.) in Biochemistry

Registration Number: 05S330

SUPERVISORS

MBANYA Jean-Claude
Professor
University of Yaounde 1

ATOGHO TIEDEU Barbara
Associate Professor
University of Yaounde 1

2021 – 2022 ACADEMIC YEAR

UNIVERSITE DE YAOUNDE I
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FACULTE DES SCIENCES
****
CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE, SCIENCE DE LA VIE, SANTE ET ENVIRONNEMENT
****
UNITE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE
****
DEPARTEMENT DE BIOCHIMIE

UNIVERSITY OF YAOUNDE 1
****
FACULTY OF SCIENCES
****
CENTER FOR RESEARCH AND GRADUATE STUDIES IN LIFE, HEALTH AND ENVIRONMENTAL SCIENCES
****
UNIT FOR RESEARCH AND GRADUATE STUDIES IN LIFE SCIENCES
****
DEPARTMENT OF BIOCHEMISTRY

ATTESTATION DE CORRECTION DE LA THÈSE DE DOCTORAT/PHD

Nous, soussignés, Prof. MOUNDIPA FEWOH Paul, Président du jury et Prof. FOKOU Elie, Membre du Jury attestons que Mr. NDONWI ELVIS NGWA, Matricule 05S330 a effectué les corrections conformément aux exigences du Jury de soutenance de la thèse de Doctorat/PhD en Biochimie, option Biotechnologie et Développement avec pour thème "Potential mechanisms of action of pesticides on factors related to type 2 diabetes". Cette thèse a été soutenue le 04 Janvier 2022.

En foi de quoi, la présente attestation lui est établie et délivrée pour servir et valoir ce que de droit.

04 FEV 2022

Examinateurs

Pr. Elie Fokou
Dr d'Etat en Biochimie
Nutrition et Sciences Alimentaires

Président du Jury

Mounthipa Fewou Paul
Professeur
Enzymologie - Toxicologie

Chef de Département

Mounthipa Fewou Paul D3C,D. Etat
Professor
Enzymology-Toxicology

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University of Yaounde 1

2021 – 2022 ACADEMIC YEAR

UNIVERSITÉ DE YAOUNDÉ I **Faculté des Sciences** Division de la Programmation et du Suivi des Activités Académiques		THE UNIVERSITY OF YAOUNDE I **Faculty of Science** Division of Programming and Follow-up of Academic Affaires
LISTE DES ENSEIGNANTS PERMANENTS		**LIST OF PERMANENT TEACHING STAFF**

ANNÉE ACADEMIQUE 2021/2022

(Par Département et par Grade)

DATE D'ACTUALISATION 22 septembre 2021

ADMINISTRATION

DOYEN : TCHOUANKEU Jean- Claude, *Maître de Conférences*
VICE-DOYEN / DPSAA : ATCHADE Alex de Théodore, *Maître de Conférences*
VICE-DOYEN / DSSE : NYEGUE Maximilienne Ascension, *Professeur*
VICE-DOYEN / DRC : ABOSSOLO Monique, *Maître de Conférences*
Chef Division Administrative et Financière : NDOYE FOE Marie C. F., *Maître de Conférences*
Chef Division des Affaires Académiques, de la Scolarité et de la Recherche DAASR : AJEAGAH Gideon AGHAINDUM, *Professeur*

1- DÉPARTEMENT DE BIOCHIMIE (BC) (38)

N°	NOMS ET PRÉNOMS	GRADE	OBSERVATIONS
1	BIGOGA DAIGA Jude	Professeur	En poste
2	FEKAM BOYOM Fabrice	Professeur	En poste
3	FOKOU Elie	Professeur	En poste
4	KANSCI Germain	Professeur	En poste
5	MBACHAM FON Wilfried	Professeur	En poste
6	MOUNDIPA FEWOU Paul	Professeur	Chef de Département
7	NINTCHOM PENLAP V. épse BENG	Professeur	En poste
8	OBEN Julius ENYONG	Professeur	En poste
9	ACHU Merci BIH	Maître de Conférences	En poste
10	ATOGHO Barbara Mma	Maître de Conférences	En poste
11	AZANTSA KINGUE GABIN BORIS	Maître de Conférences	En poste
12	BELINGA née NDOYE FOE M. C. F.	Maître de Conférences	Chef DAF / FS
13	BOUDJEKO Thaddée	Maître de Conférences	En poste
14	DJUIDJE NGOUNOUE Marcelline	Maître de Conférences	En poste
15	EFFA NNOMO Pierre	Maître de Conférences	En poste
16	EWANE Cécile Anne	Maître de Conférences	En poste

17	MOFOR née TEUGWA Clotilde	Maître de Conférences	Inspecteur de Service MINESUP
18	NANA Louise épouse WAKAM	Maître de Conférences	En poste
19	NGONDI Judith Laure	Maître de Conférences	En poste
20	NGUEFACK Julienne	Maître de Conférences	En poste
21	NJAYOU Frédéric Nico	Maître de Conférences	En poste
22	TCHANA KOUATCHOUA Angèle	Maître de Conférences	En poste

23	AKINDEH MBUH NJI	Chargé de Cours	En poste
24	BEBEE Fadimatou	Chargée de Cours	En poste
25	BEBOY EDJENGUELE Sara Nathalie	Chargé de Cours	En poste
25	DAKOLE DABOY Charles	Chargé de Cours	En poste
26	DJUIKWO NKONGA Ruth Viviane	Chargée de Cours	En poste
27	DONGMO LEKAGNE Joseph Blaise	Chargé de Cours	En poste
28	FONKOUA Martin	Chargé de Cours	En poste
29	KOTUE KAPTUE Charles	Chargé de Cours	En poste
30	LUNGA Paul KEILAH	Chargé de Cours	En poste
31	MANANGA Marlyse Joséphine	Chargée de Cours	En poste
32	MBONG ANGIE M. Mary Anne	Chargée de Cours	En poste
33	Palmer MASUMBE NETONGO	Chargé de Cours	En poste
34	PECHANGOU NSANGOU Sylvain	Chargé de Cours	En poste

35	MBOUCHE FANMOE Marceline Joëlle	Assistante	En poste
36	OWONA AYISSI Vincent Brice	Assistant	En poste
37	WILFRIED ANGIE Abia	Assistante	En poste

2- DÉPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE ANIMALES (BPA) (46)

1	AJEAGAH Gideon AGHAINDUM	Professeur	*DAARS/FS*
2	BILONG BILONG Charles-Félix	Professeur	Chef de Département
3	DIMO Théophile	Professeur	En Poste
4	DJIETO LORDON Champlain	Professeur	En Poste
5	DZEUFIET DJOMENI Paul Désiré	Professeur	En Poste
6	ESSOMBA née NTSAMA MBALA	Professeur	*Vice Doyen/FMSB/UYI*
7	FOMENA Abraham	Professeur	En Poste
8	KAMTCHOUING Pierre	Professeur	En poste
9	KEKEUNOU Sévilor	Professeur	En poste

10	NJAMEN Dieudonné	Professeur	En poste
11	NJIOKOU Flobert	Professeur	En Poste
12	NOLA Moïse	Professeur	En poste
13	TAN Paul VERNYUY	Professeur	En poste
14	TCHUEM TCHUENTE Louis Albert	Professeur	*Inspecteur de service Coord.Progr./MINSANTE*
15	ZEBAZE TOGOUET Serge Hubert	Professeur	*En poste*

16	BILANDA Danielle Claude	Maître de Conférences	En poste
17	DJIOGUE Séfirin	Maître de Conférences	En poste
18	JATSA BOUKENG Hermine épse MEGAPTCHE	Maître de Conférences	En Poste
19	LEKEUFACK FOLEFACK Guy B.	Maître de Conférences	En poste
20	MEGNEKOU Rosette	Maître de Conférences	En poste
21	MONY Ruth épse NTONE	Maître de Conférences	En Poste
22	NGUEGUIM TSOFACK Florence	Maître de Conférences	En poste
23	TOMBI Jeannette	Maître de Conférences	En poste

24	ALENE Désirée Chantal	Chargée de Cours	En poste
25	ATSAMO Albert Donatien	Chargé de Cours	En poste
26	BELLET EDIMO Oscar Roger	Chargé de Cours	En poste
27	DONFACK Mireille	Chargée de Cours	En poste
28	ETEME ENAMA Serge	Chargé de Cours	En poste
29	GOUNOUE KAMKUMO Raceline	Chargée de Cours	En poste
30	KANDEDA KAVAYE Antoine	Chargé de Cours	En poste
31	MAHOB Raymond Joseph	Chargé de Cours	En poste
32	MBENOUN MASSE Paul Serge	Chargé de Cours	En poste
33	MOUNGANG Luciane Marlyse	Chargée de Cours	En poste
34	MVEYO NDANKEU Yves Patrick	Chargé de Cours	En poste
35	NGOUATEU KENFACK Omer Bébé	Chargé de Cours	En poste
36	NGUEMBOK	Chargé de Cours	En poste
37	NJUA Clarisse Yafi	Chargée de Cours	Chef Div. UBA
38	NOAH EWOTI Olive Vivien	Chargée de Cours	En poste
39	TADU Zephyrin	Chargé de Cours	En poste
40	TAMSA ARFAO Antoine	Chargé de Cours	En poste
41	YEDE	Chargé de Cours	En poste

42	BASSOCK BAYIHA Etienne Didier	Assistant	En poste
43	ESSAMA MBIDA Désirée Sandrine	Assistante	En poste
44	KOGA MANG DOBARA	Assistant	En poste
45	LEME BANOCK Lucie	Assistante	En poste
46	YOUNOUSSA LAME	Assistant	En poste

3- DÉPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE VÉGÉTALES (BPV) (33)

1	AMBANG Zachée	Professeur	Chef Division/UYII
2	BELL Joseph Martin	Professeur	En poste
3	DJOCGOUE Pierre François	Professeur	En poste
4	MBOLO Marie	Professeur	En poste
5	MOSSEBO Dominique Claude	Professeur	En poste
6	YOUMBI Emmanuel	Professeur	Chef de Département
7	ZAPFACK Louis	Professeur	En poste

8	ANGONI Hyacinthe	Maître de Conférences	En poste
9	BIYE Elvire Hortense	Maître de Conférences	En poste
10	KENGNE NOUMSI Ives Magloire	Maître de Conférences	En poste
11	MALA Armand William	Maître de Conférences	En poste
12	MBARGA BINDZI Marie Alain	Maître de Conférences	CT/ MINESUP
13	NDONGO BEKOLO	Maître de Conférences	*CE / MINRESI*
14	NGODO MELINGUI Jean Baptiste	Maître de Conférences	En poste
15	NGONKEU MAGAPTCHE Eddy L.	Maître de Conférences	En poste
16	TONFACK Libert Brice	Maître de Conférences	En poste
17	TSOATA Esaïe	Maître de Conférences	En poste

18	DJEUANI Astride Carole	Chargé de Cours	En poste
19	GOMANDJE Christelle	Chargée de Cours	En poste
20	MAFFO MAFFO Nicole Liliane	Chargé de Cours	En poste
21	MAHBOU SOMO TOUKAM. Gabriel	Chargé de Cours	En poste
22	NGALLE Hermine BILLE	Chargée de Cours	En poste
23	NGOUO Lucas Vincent	Chargé de Cours	En poste
24	NNANGA MEBENGA Ruth Laure	Chargé de Cours	En poste
25	NOUKEU KOUAKAM Armelle	Chargé de Cours	En poste
26	ONANA JEAN MICHEL	Chargé de Cours	En poste

27	GODSWILL NTSOMBAH NTSEFONG	Assistant	En poste

28	KABELONG BANAHO Louis-Paul-Roger	Assistant	En poste
29	KONO Léon Dieudonné	Assistant	En poste
30	LIBALAH Moses BAKONCK	Assistant	En poste
31	LIKENG-LI-NGUE Benoit C	Assistant	En poste
32	TAEDOUNG Evariste Hermann	Assistant	En poste
33	TEMEGNE NONO Carine	Assistant	En poste

4- DÉPARTEMENT DE CHIMIE INORGANIQUE (CI) (33)

1	AGWARA ONDOH Moïse	Professeur	*Chef de Département*
2	DJOUFAC WOUMFO Emmanuel	Professeur	En poste
3	Florence UFI CHINJE épouse MELO	Professeur	*Recteur Univ.Ngaoundere*
4	GHOGOMU Paul MINGO	Professeur	*Ministre Chargé deMiss.PR*
5	NANSEU Njiki Charles Péguy	Professeur	En poste
6	NDIFON Peter TEKE	Professeur	*CT MINRESI*
7	NDIKONTAR Maurice KOR	Professeur	*Vice-Doyen Univ. Bamenda*
8	NENWA Justin	Professeur	En poste
9	NGAMENI Emmanuel	Professeur	*DOYEN FS UDs*
10	NGOMO Horace MANGA	Professeur	*Vice Chancelor/UB*

11	ACAYANKA Elie	Maître de Conférences	En poste
12	BABALE née DJAM DOUDOU	Maître de Conférences	*Chargée Mission P.R.*
13	EMADACK Alphonse	Maître de Conférences	En poste
14	KAMGANG YOUBI Georges	Maître de Conférences	En poste
15	KEMMEGNE MBOUGUEM Jean C.	Maître de Conférences	En poste
16	KONG SAKEO	Maître de Conférences	En poste
17	NDI NSAMI Julius	Maître de Conférences	En poste
18	NJIOMOU C. épse DJANGANG	Maître de Conférences	En poste
19	NJOYA Dayirou	Maître de Conférences	En poste
20	TCHAKOUTE KOUAMO Hervé	Maître de Conférences	En poste

21	BELIBI BELIBI Placide Désiré	Chargé de Cours	CS/ ENS Bertoua
22	CHEUMANI YONA Arnaud M.	Chargé de Cours	En poste
23	KENNE DEDZO GUSTAVE	Chargé de Cours	En poste
24	KOUOTOU DAOUDA	Chargé de Cours	En poste
25	MAKON Thomas Beauregard	Chargé de Cours	En poste
26	MBEY Jean Aime	Chargé de Cours	En poste
27	NCHIMI NONO KATIA	Chargé de Cours	En poste
28	NEBA nee NDOSIRI Bridget NDOYE	Chargée de Cours	CT/ MINFEM
29	NYAMEN Linda Dyorisse	Chargée de Cours	En poste
30	PABOUDAM GBAMBIE A.	Chargée de Cours	En poste

31	NJANKWA NJABONG N. Eric	Assistant	En poste
32	PATOUOSSA ISSOFA	Assistant	En poste
33	SIEWE Jean Mermoz	Assistant	En Poste

5- DÉPARTEMENT DE CHIMIE ORGANIQUE (CO) (34)			
1	DONGO Etienne	Professeur	Vice-Doyen/FSE/UYI
2	GHOGOMU TIH Robert Ralph	Professeur	Dir. IBAF/UDA
3	NGOUELA Silvère Augustin	Professeur	Chef de Département UDS
4	NYASSE Barthélemy	Professeur	En poste
5	PEGNYEMB Dieudonné Emmanuel	Professeur	*Directeur/ MINESUP/* Chef de Département
6	WANDJI Jean	Professeur	En poste

7	Alex de Théodore ATCHADE	Maître de Conférences	Vice-Doyen / DPSAA
8	AMBASSA Pantaléon	Maître de Conférences	En poste
9	EYONG Kenneth OBEN	Maître de Conférences	En poste
10	FOLEFOC Gabriel NGOSONG	Maître de Conférences	En poste
11	FOTSO WABO Ghislain	Maître de Conférences	En poste
12	KEUMEDJIO Félix	Maître de Conférences	En poste
13	KEUMOGNE Marguerite	Maître de Conférences	En poste
14	KOUAM Jacques	Maître de Conférences	En poste

15	MBAZOA née DJAMA Céline	Maître de Conférences	En poste
16	MKOUNGA Pierre	Maître de Conférences	En poste
17	MVOT AKAK CARINE	Maître de Conférences	En poste
18	NGO MBING Joséphine	Maître de Conférences	Sous/Direct. MINERESI
19	NGONO BIKOBO Dominique Serge	Maître de Conférences	**C.E**/ MINESUP
20	NOTE LOUGBOT Olivier Placide	Maître de Conférences	C.S/ MINESUP
21	NOUNGOUE TCHAMO Diderot	Maître de Conférences	En poste
22	TABOPDA KUATE Turibio	Maître de Conférences	En poste
23	TAGATSING FOTSING Maurice	Maître de Conférences	En poste
24	TCHOUANKEU Jean-Claude	Maître de Conférences	*Doyen /FS/ UYI*
25	TIH née NGO BILONG E. Anastasie	Maître de Conférences	En poste
26	YANKEP Emmanuel	Maître de Conférences	En poste
27	ZONDEGOUMBA Ernestine	Maître de Conférences	En poste

28	KAMTO Eutrophe Le Doux	Chargé de Cours	En poste
29	NGNINTEDO Dominique	Chargé de Cours	En poste
30	NGOMO Orléans	Chargée de Cours	En poste
31	OUAHOUO WACHE Blandine M.	Chargée de Cours	En poste
32	SIELINOU TEDJON Valérie	Chargé de Cours	En poste

33	MESSI Angélique Nicolas	Assistant	En poste
34	TSEMEUGNE Joseph	Assistant	En poste

6- DÉPARTEMENT D'INFORMATIQUE (IN) (25)

1	ATSA ETOUNDI Roger	Professeur	*Chef Div.MINESUP*
2	FOUDA NDJODO Marcel Laurent	Professeur	*Chef Dpt ENS/Chef IGA.MINESUP*

3	NDOUNDAM Réné	Maître de Conférences	En poste

4	ABESSOLO ALO'O Gislain	Chargé de Cours	En poste
5	AMINOU Halidou	Chargé de Cours	*Chef de Département*
6	DJAM Xaviera YOUH - KIMBI	Chargé de Cours	En Poste
7	DOMGA KOMGUEM Rodrigue	Chargé de Cours	En poste
8	EBELE Serge Alain	Chargé de Cours	En poste
9	KOUOKAM KOUOKAM E. A.	Chargé de Cours	En poste
10	MELATAGIA YONTA Paulin	Chargé de Cours	En poste
11	MONTHE DJIADEU Valery M.	Chargé de Cours	En poste
12	MOTO MPONG Serge Alain	Chargé de Cours	En poste
13	OLLE OLLE Daniel Claude Delort	Chargé de Cours	Directeur adjoint Enset. Ebolowa
14	TAPAMO Hyppolite	Chargé de Cours	En poste
15	TINDO Gilbert	Chargé de Cours	En poste
16	TSOPZE Norbert	Chargé de Cours	En poste
17	WAKU KOUAMOU Jules	Chargé de Cours	En poste

18	BAYEM Jacques Narcisse	Assistant	En poste
19	EKODECK Stéphane Gaël Raymond	Assistant	En poste
20	HAMZA Adamou	Assistant	En poste
21	JIOMEKONG AZANZI Fidel	Assistant	En poste
22	MAKEMBE. S . Oswald	Assistant	En poste
23	MESSI NGUELE Thomas	Assistant	En poste
24	MEYEMDOU Nadège Sylvianne	Assistante	En poste
25	NKONDOCK. MI. BAHANACK.N.	Assistant	En poste

7- DÉPARTEMENT DE MATHÉMATIQUES (MA) (30)

1	AYISSI Raoult Domingo	Professeur	Chef de Département
2	EMVUDU WONO Yves S.	Professeur	*Inspecteur MINESUP*

3	KIANPI Maurice	Maître de Conférences	En poste
4	MBANG Joseph	Maître de Conférences	En poste
5	MBEHOU Mohamed	Maître de Conférences	En poste
6	MBELE BIDIMA Martin Ledoux	Maître de Conférences	En poste
7	NKUIMI JUGNIA Célestin	Maître de Conférences	En poste

8	NOUNDJEU Pierre	Maître de Conférences	*Chef service des programmes & Diplômes/FS/UYI*
9	TCHAPNDA NJABO Sophonie B.	Maître de Conférences	Directeur/AIMS Rwanda
10	TCHOUNDJA Edgar Landry	Maître de Conférences	En poste

11	AGHOUKENG JIOFACK Jean Gérard	Chargé de Cours	Chef Cellule MINPLAMAT
12	CHENDJOU Gilbert	Chargé de Cours	En poste
13	DJIADEU NGAHA Michel	Chargé de Cours	En poste
14	DOUANLA YONTA Herman	Chargé de Cours	En poste
15	FOMEKONG Christophe	Chargé de Cours	En poste
16	KIKI Maxime Armand	Chargé de Cours	En poste
17	MBAKOP Guy Merlin	Chargé de Cours	En poste
18	MENGUE MENGUE David Joe	Chargé de Cours	En poste
19	NGUEFACK Bernard	Chargé de Cours	En poste
20	NIMPA PEFOUKEU Romain	Chargée de Cours	En poste
21	POLA DOUNDOU Emmanuel	Chargé de Cours	En poste
22	TAKAM SOH Patrice	Chargé de Cours	En poste
23	TCHANGANG Roger Duclos	Chargé de Cours	En poste
24	TETSADJIO TCHILEPECK M. E.	Chargé de Cours	En poste
25	TIAYA TSAGUE N. Anne-Marie	Chargée de Cours	En poste

26	BITYE MVONDO Esther Claudine	Assistante	En poste
27	MBATAKOU Salomon Joseph	Assistant	En poste
28	MBIAKOP Hilaire George	Assistant	En poste
29	MEFENZA NOUNTU Thiery	Assistant	En poste
30	TCHEUTIA Daniel Duviol	Assistant	En poste

8- DÉPARTEMENT DE MICROBIOLOGIE (MIB) (18)

1	ESSIA NGANG Jean Justin	Professeur	*Chef de Département*
2	NYEGUE Maximilienne Ascension	Professeur	*VICE-DOYEN / DSSE*
3	NWAGA Dieudonné M.	Professeur	En poste

4	ASSAM ASSAM Jean Paul	Maître de Conférences	En poste
5	BOYOMO ONANA	Maître de Conférences	En poste
6	RIWOM Sara Honorine	Maître de Conférences	En poste

7	SADO KAMDEM Sylvain Leroy	Maître de Conférences	En poste

8	BODA Maurice	Chargé de Cours	En poste
9	BOUGNOM Blaise Pascal	Chargé de Cours	En poste
10	ESSONO OBOUGOU Germain G.	Chargé de Cours	En poste
11	NJIKI BIKOÏ Jacky	Chargée de Cours	En poste
12	TCHIKOUA Roger	Chargé de Cours	En poste
13	ESSONO Damien Marie	Assistant	En poste
14	LAMYE Glory MOH	Assistant	En poste
15	MEYIN A EBONG Solange	Assistante	En poste
16	NKOUDOU ZE Nardis	Assistant	En poste
17	SAKE NGANE Carole Stéphanie	Assistante	En poste
18	TOBOLBAÏ Richard	Assistant	En poste

9. DEPARTEMENT DE PYSIQUE(PHY) (40)

1	BEN- BOLIE Germain Hubert	Professeur	En poste
2	DJUIDJE KENMOE épouse ALOYEM	Professeur	En poste
3	EKOBENA FOUDA Henri Paul	Professeur	*Vice-Recteur. UN*
4	ESSIMBI ZOBO Bernard	Professeur	En poste
5	KOFANE Timoléon Crépin	Professeur	En poste
6	NANA ENGO Serge Guy	Professeur	En poste
7	NANA NBENDJO Blaise	Professeur	En poste
8	NDJAKA Jean Marie Bienvenu	Professeur	Chef de Département
9	NJANDJOCK NOUCK Philippe	Professeur	En poste
10	NOUAYOU Robert	Professeur	En poste
11	PEMHA Elkana	Professeur	En poste
12	TABOD Charles TABOD	Professeur	Doyen FS Univ/Bda
13	TCHAWOUA Clément	Professeur	En poste
14	WOAFO Paul	Professeur	En poste
15	ZEKENG Serge Sylvain	Professeur	En poste

16	BIYA MOTTO Frédéric	Maître de Conférences	DG/HYDRO Mekin
17	BODO Bertrand	Maître de Conférences	En poste

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Répartition chiffrée des Enseignants de la Faculté des Sciences de l'Université de Yaoundé I

NOMBRE D'ENSEIGNANTS					
DÉPARTEMENT	**Professeurs**	**Maîtres de Conférences**	**Chargés de Cours**	**Assistants**	**Total**
BCH	8 (01)	14 (10)	13 (05)	3 (02)	**38 (18)**
BPA	15 (01)	8 (06)	18 (05)	05 (02)	**46 (14)**
BPV	07 (01)	10 (01)	9 (06)	07 (01)	**33 (9)**
CI	10 (01)	10 (02)	10 (02)	03 (0)	**33 (5)**
CO	6 (0)	21 (05)	05 (02)	02 (0)	**34(7)**
IN	2 (0)	1 (0)	14 (01)	08 (01)	**25 (2)**
MAT	2 (0)	8 (0)	15 (01)	05 (02)	**30 (3)**
MIB	3 (0)	4 (02)	05 (01)	06 (02)	**18 (5)**
PHY	15 (0)	14 (02)	09 (03)	02 (0)	**40 (5)**
ST	7 (1)	15 (01)	18 (05)	02 (0)	**42(7)**
Total	**75 (5)**	**105 (29)**	**116 (31)**	**43 (10)**	**339 (75)**

Soit un total de **339 (**75**)** dont :

- Professeurs **75 (5)**
- Maîtres de Conférences **105 (**29**)**
- Chargés de Cours **116 (31)**
- Assistants **43** (10**)**

( ) = Nombre de Femmes **75**

DEDICATION

To:

my wife and children.

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ABSTRACT

In most agriculturally based economies around the world, large scale industrial agriculture entails the use of modern agricultural machinery, herbicides and pesticides. Meanwhile there are recent debates around agroecology/organic agriculture in which less pesticides are used, the use of pesticide and herbicide in agriculture remain a dominant feature even among small scale farmers. Moreover, pesticides are used by public health experts for the control of vectors of infectious diseases. Therefore, pest control is largely dependent on pesticides some of which include: Imidacloprid, Chlorpyrifos, Lambda cyhalothrin and Oxamyl, belonging to the neonicotinoids, organophosphate, pyrethroids and carbamate classes respectively. In addition to their primary mode of action and their specific targets such as insects, recent studies show that they affect metabolic parameters in primates. The present study inscribes itself in these body of recent research and specifically sought to investigate the effects of gestational exposure to the above mentioned four pesticides on oxidative stress and metabolic parameters in female Wistar rats and their offspring.

This study was divided into 3 parts with a preliminary section carried out to determine the lethal dose 50 (LD_{50}) of the four studied pesticides. For LD_{50}, each pesticide was administered to female nulliparous Wistar rats (age: 10 - 12 weeks, weight: 180g - 200g) in increasing dose by oral gavage until death was recorded. The pesticide was diluted in distilled water with the volume being 10mL/kg rat. "The highest dose with no mortality and the lowest with mortality were used to calculate the LD_{50}". In the 1st part, thirty-female nulliparous Wistar rats (age: 12 - 14 weeks, weight: 200g - 220g) were divided into 5 equal groups matched by weight and fasting blood glucose levels. Each rat in group 2, group 3, group 4 and group 5 received 44 mg Imidacloprid, 13.5 mg Chlorpyrifos, 5.6 mg Imidacloprid + 5.6 mg Lambda cyhalothrin, and 0.4 mg Oxamyl per kg body weight/ day respectively. All pesticides were administered using a gastro-oesophageal probe throughout gestation. Each rat from the Control Group (group 1) received distilled water (10mL/kg rat). At birth, pesticide administration was discontinued, and the female rats were followed up for 4 weeks. They were fasted overnight and sacrificed upon local anaesthesia and blood samples, liver, kidney, pancreas and muscles collected. Part of the liver and kidney were used to quantify antioxidants and lipid peroxidation markers while serum was used to measure alanine transaminase and aspartate transaminase. In the 2nd part, all offspring were followed up by weekly measuring of body weight. At weaning (4 weeks), 8 offspring were randomly selected from each group, fasted overnight and sacrifice upon local anaesthesia. The remaining offspring were followed up until adult age (12 weeks), 8 randomly selected and sacrificed upon local anaesthesia after an overnight fast.

After sacrifice in each case (4 week and 12 week), blood samples, liver and kidney were collected. Part of the liver and kidney were used to quantify antioxidants and lipid peroxidation markers while serum was used to measure alanine transaminase and aspartate transaminase. In the 3rd part, samples were collected from parent rats, their offspring at weaning and at adult age. Blood samples were collected, and serum prepared for lipid profile and insulin quantification. Muscles samples were collected from the biceps femoris for glucose transporter-4 and nuclear factor kappa beta protein expression. Liver, kidney, and pancreas were collected for histological studies. Statistical analyses were performed using the GraphPad Prism software.

The LD_{50} values obtained were "440 mg/kg" for Imidacloprid, "135 mg/kg" for Chlorpyrifos, "56 mg/kg" for the formulation containing Lambda cyhalothrin+Imidacloprid and "4 mg/kg" for Oxamyl. Antioxidant enzyme levels (super oxide dismutase, catalase, glutathione peroxidase, glutathione reductase), reduced glutathione and malondialdehyde were altered in at-least one of the pesticide-exposed groups compared to the control group. These alterations observed in parents were also observed in offspring sacrificed at weaning as well as those sacrificed at adult age. Fasting Blood glucose, lipid profile (total cholesterol, triglycerides and low-density lipoprotein cholesterol), insulin and homeostasis model assessment of insulin resistance (HOMA-IR) values were significantly higher in the pesticide-exposed groups compared to the control group. Similar results were observed in offspring sacrificed at weaning and those sacrificed at adult age. Moreover, Imidacloprid and Chlorpyrifos significantly reduced glucose transporter-4 (GLUT4) and significantly increased nuclear factor kappa beta (NFkβ) expression in muscles of parents and offspring explored at adult age.

According to this study, pesticides induced oxidative stress, lipid peroxidation, insulin resistance and dyslipidemia in female Wistar rats during gestation and their offspring. The effect on offspring persisted until adult age, suggesting epigenetic mechanisms. These findings suggest that exposure to pesticides could contribute to an increase in the incidence of diabetes worldwide. Therefore, future studies are needed to elucidate the current findings in humans. Such studies might inform public health and agricultural policy makers on the need to regulate the use of pesticides especially in a country like Cameroon where the drive to a second-generation agriculture has paved the way for an increase in the use of pesticides sometimes by farmers not wearing any form of protection nor respecting prescribed doses.

Keywords: Foetal exposure, pesticides, Biochemical alterations, Offspring, Adult age

RESUME

Dans la plupart des économies agricoles du monde, l'agriculture industrielle à grande échelle implique l'utilisation de machines agricoles modernes, d'herbicides et de pesticides. Cependant, il y'a des débats récents sur l'agroécologie et l'agriculture biologique dans lesquels moins de pesticides sont utilisés, l'utilisation d'herbicides et de pesticides dans l'agriculture reste un problème majeur particulièrement parmi les petits agriculteurs. De plus, les pesticides sont utilisés par les experts en santé publique pour lutter contre les vecteurs de maladies infectieuses. Par conséquent, la lutte antiparasitaire dépend en grande partie des pesticides dont certains comprennent: l'imidaclopride, le chlorpyrifos, la lambda cyhalothrine et l'oxamyl, appartenant respectivement aux classes des néonicotinoïdes, des organophosphorés, des pyréthroïdes et des carbamates. En plus de leur mode d'action principal et de leurs cibles spécifiques comme les insectes, des études récentes montrent qu'ils affectent les paramètres métaboliques chez les primates. Cette étude visait donc à étudier les effets de l'exposition gestationnelle aux quatre pesticides mentionnés ci-dessus sur le stress oxydatif et les paramètres métaboliques chez les rats Wistar femelles et leur progéniture.

Cette étude a été divisée en 3 parties avec une section préliminaire réalisée pour déterminer la dose létale 50 (DL50) des quatre pesticides étudiés. Pour la dose lethaal-50 (DL50), chaque pesticide a été administré à des rats Wistar nullipares femelles (âge : 10 - 12 semaines, poids : 180 g - 200 g) à une dose croissante par gavage oral jusqu'à ce que la mort soit enregistrée. Le pesticide a été dilué dans de l'eau distillée avec un volume de 10 ml/kg de rat. " La plus petite dose qui a entrainé la mortalité ainsi que la dose la plus élevé sans mortalité associée ont été utilisées pour calculer la DL50". Dans la 1ère partie, trente rats Wistar nullipares femelles (âge : 12 - 14 semaines, poids : 200 g - 220 g) ont été divisés en 5 groupes égaux appariés en poids et en glycémie à jeun. Chaque rat du groupe 2, du groupe 3, du groupe 4 et du groupe 5 a reçu respectivement 44 mg d'imidaclopride, 13,5 mg de chlorpyrifos, un mélange contenant 5,6 mg d'imidaclopride + 5,6 mg de lambda cyhalothrine et 0,4 mg d'oxamyl par kg de poids corporel/jour. Tous les pesticides ont été administrés à l'aide d'une sonde gastro-œsophagienne tout au long de la gestation. Chaque rat du groupe témoin (groupe 1) a reçu de l'eau distillée (10 ml/kg de rat). À la naissance, l'administration de pesticides a été interrompue et les rats femelles ont été suivis pendant 4 semaines. À la fin du suivi, les rats ont été soumis à un jeun de 12 heures, sacrifiés sous anesthésie locale et les prélèvements sanguins, foie, et reins effectués. Une partie du foie et un rein ont été utilisées pour quantifier les antioxydants et les marqueurs de peroxydation lipidique tandis que le sérum a été utilisé pour quantifier l'alanine transaminase et l'aspartate transaminase. Dans la 2ème partie, tous les descendants ont été suivis par une mesure du poids corporel chaque semaine. Au sevrage (4 semaines), 8 descendants ont été sélectionnés dans chaque groupe. Après un jeun de 12 heure, ils ont été sacrifiés sous anesthésie locale. Les descendants restants ont été suivis jusqu'à l'âge adulte (12

semaines), et 8 sélectionnés et sacrifiés sous anesthésie locale après un jeûne de 12 heure. Après sacrifice dans chaque cas (4 semaines et 12 semaines), des échantillons de sang, de foie et de rein ont été prélevés. Une partie du foie et un rein ont été utilisées pour quantifier les antioxydants et les marqueurs de peroxydation lipidique tandis que le sérum a été utilisé pour quantifier l'alanine transaminase et l'aspartate transaminase. Dans la 3ème partie, des échantillons ont été prélevés sur des rats parents, leur progéniture au sevrage et à l'âge adulte. Des échantillons de sang ont été prélevés et du sérum préparé pour la quantification du profil lipidique, l'insuline et la glycémie. Des échantillons de muscles ont été prélevés dans le biceps fémoral des parents et descendants sacrifies à l'âge adulte pour l'expression du GLUT4 et NFKβ. Le foie, les reins et le pancréas ont été prélevés pour des études histologiques. Les analyses statistiques ont été réalisées à l'aide du logiciel GraphPad Prism.

Les DL50 obtenues étaient de "440 mg/kg" pour l'imidaclopride, "135 mg/kg" pour le chlorpyrifos, "56 mg/kg" pour la formulation contenant la Lambda cyhalothrine + Imidaclopride et "4 mg/kg" pour l'Oxamyl. Les taux d'enzymes antioxydantes (super oxyde dismutase, catalase, glutathion peroxydase, glutathion réductase), glutathion réduit et malondialdéhyde ont été modifiés dans au moins un des groupes exposés aux pesticides comparé au groupe témoin. Ces altérations observées chez les parents ont également été observées chez les descendants sacrifiés au sevrage ainsi que ceux sacrifiés à l'âge adulte. La glycémie à jeun, le profil lipidique (cholestérol total, triglycérides et HDL cholestérol), les taux HOMA-IR indiquant la sensibilité à l'insuline étaient significativement plus élevés dans les groupes exposés aux pesticides comparé au groupe témoin. Des résultats similaires ont été observés chez les descendants sacrifiés au sevrage et ceux sacrifiés à l'âge adulte. De plus, l'imidaclopride et le chlorpyrifos ont considérablement réduit l'expression du GLUT4 et NFkβ dans les muscles des parents et de la progéniture explorés à l'âge adulte.

Selon cette étude, les pesticides ont induit un stress oxydatif, une peroxydation lipidique, une hyperglycémie, une résistance à l'insuline et une dyslipidémie chez les rats femelles Wistar pendant la gestation et leur progéniture. L'effet sur la progéniture a persisté jusqu'à l'âge adulte, suggérant des mécanismes épigénétiques. Ces résultats suggèrent que l'exposition aux pesticides pourrait contribuer à une augmentation de l'incidence du diabète de type 2 dans le monde. Par conséquent, des études futures sont nécessaires pour élucider les résultats actuels chez l'homme. De telles études pourraient informer les décideurs de la santé publique et des politiques agricoles sur la nécessité de réglementer l'utilisation des pesticides, en particulier dans un pays comme le Cameroun où la tendance à une agriculture de deuxième génération a ouvert la voie à une augmentation d'utilisation des pesticides parfois par les agriculteurs qui n'utilise pas d'équipements protectifs ni respecte les doses prescrites.

Mots clés : exposition foetal, pesticides, altérations biochimiques, progéniture, âge adulte.

ABBREVIATIONS

AACE:	American Association of Clinical Endocrinologists
ACh:	Acetylcholine
AChE:	Acetylcholinesterase
ADA:	American Diabetes Association
AhR:	Aryl hydrocarbon Receptors
ALP:	Alkaline Phosphatase
APS:	Ammonium Persulphate
BPB:	Bromo-Phenol Blue
BSA:	Bovine Serum Albumin
CAD:	Coronary Artery Disease
CDNB:	1-Chloro-2,4-Dinitrobenzene
CVA:	Cerebrovascular events
DDT:	Dichloro-Diphenyl-Trichloroethane
DKA:	Diabetic Ketoacidosis
DMSO:	Dimethyl Sulfoxide
DNPH:	Di-Nitro-phenyl-hydrazine
DTNB:	5-5’ Dithiobis-2- nitro-benzoic acid
DTT:	Dithiothréitol
ED:	Erectile Dysfunction
EDP:	Endocrine Disruption Pesticides
EDTA:	Ethylene Diamine Tetra-acetic Acid
ELISA:	Enzyme Linked Immuno-Sorbent Assay
EPA:	Environmental Protection Agency
FBG:	Fasting Blood Glucose
FBS:	Fetal Bovine Serum
FCDP:	Fibrocalculous Pancreatic Diabetes Mellitus
FFA:	Free Fatty Acid
FTO:	Fat mass and obesity-associated protein
G6P:	Glucose-6-Phosphate
GABA:	Gamma Aminobutyric Acid
GAD:	Glutamic Acid Decarboxylase

GIP:	Gastric-Inhibitory Peptide
GLP:	Glucagon-Like Peptide
GLUT:	Glucose Transporter
GPx:	Glutathione Peroxidase
GR:	Glutathione Reductase
GSH:	Reduced Glutathione
GSSG:	Oxidised Glutathione
GST:	Glutathione S-Transferase
HDL:	High Density Lipoprotein
HEPES:	(4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid
HHEX:	Hematopoietically Expressed Homeobox Protein
HLA:	Human Leucocyte Antigen
HNC:	Hyperosmolar Non-ketotic Coma
IDDM:	Insulin Dependent Diabetes mellitus
IDF:	International Diabetes Federation
IGF2BP2:	Insulin-Like Growth Factor 2 mRNA Binding Protein 2
IGT:	Impaired Glucose Tolerance
IL-6:	Interleukin-6
IRS:	Insulin Receptor Substrate
KCNJ11:	Potassium Voltage-Gated Channel Subfamily J Member 11
LA:	Lactic Acidosis
LDL:	Low Density Lipoprotein
LNNB:	Laboratory for Nutrition and Nutritional Biochemistry
MDA:	Malondialdehyde,
MODY:	Maturity Onset Diabetes of the Young
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
nAChRs:	Nicotinic Acetylcholine Receptors
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
NDM:	Neonatal Diabetes mellitus
NFKβ:	Nuclear Factor Kappa Beta
NOTCH2:	Neurogenic Locus Notch Homolog Protein 2
OEDC:	Organisation for Economic Co-operation and Development
OGTT:	Oral Glucose Tolerance Test

OP:	Organophosphate
PAGE:	Poly-Acrylamide Gel Electrophoresis
PBS:	Phosphate Buffered Saline
PI-3K:	Phosphatidyl-Inositol 3-Kinase
PIP2:	Phosphatidyl-Inositol-3,4-Bisphosphate
PIP3:	Phosphatidyl-Inositol-3,4,5-Trisphosphate
PKB:	Protein Kinase B
PMSF:	Phenyl Methyl Sulfonyl Fluoride
PND:	Post Natal Day
POP:	Persistent Organic Pollutant
PPARγ:	Peroxisome Proliferator Activated Receptor Gamma
PVD:	Peripheral Vascular Disease
ROS:	Reactive Oxygen Species
SDS:	Sodium Dodecyl Sulphate
SLC30A8:	Solute Carrier Family 30 Member 8
SOD:	Superoxide Dismutase
SREBP:	Sterol Regulatory Element-Binding Proteins
SUR:	Sulphonylurea Receptor
TBARS:	Thiobarbituric Acid Reactive Substrate
TBS:	Tris Buffered Saline
TC:	Total Cholesterol
TCF7L2:	Transcription Factor 7-Like 2
TEMED:	N, N, N, N – Tetra-methyl Ethylene Di-amine
TG:	Triglycerides
TMB:	3,3′,5,5′-Tetramethylbenzidine
TNF:	Tumour Necrosis Factor
TZDs:	Thiazolidinediones
UCP:	Uncoupling Protein
WHO:	World Health Organisation
ZnT8:	Zinc Transporter 8

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INTRODUCTION

Diabetes mellitus (DM) is recognised as a global health problem that greatly affects the quality of life and increase mortality. The term "*Diabetes mellitus*" describes some metabolic disorders with multiple aetiologies, characterized by chronic hyperglycaemia with disturbances in carbohydrate, fat and protein metabolism resulting from defects in insulin secretion and/or insulin action **(WHO, 1999)**. The major forms of DM include Type 1 DM, Type 2 DM and gestational DM **(IDF, 2017**). Other forms include lipo-atrophic diabetes, fibrocalculous pancreatopathy, Maturity Onset Diabetes of the Young (MODY). The number of people aged 20 – 79 years with diabetes was estimated to be 537 million in 2021 and it is predicted to rise to 783 million in 2045 **(IDF, 2021).** Gestational DM is a carbohydrate intolerance resulting in hyperglycaemia of variable severity with onset or first recognition during pregnancy **(IDF, 2019**). Type 1 DM accounts for about 5% of all diabetes cases. It is an autoimmune disorder resulting from the destruction of pancreatic beta cells ending in little or no insulin production (**Atkinson and MacLaren, 1994**). It is common in children and adolescents. Type 2 DM is the most common form, accounting for 90% of all diabetic cases **(IDF, 2019).** It is characterized by "disorders in insulin action and secretion", either of which may be the predominant feature **(WHO, 1999)**. It results from the association of several factors classified as modifiable and non-modifiable. The modifiable factors are: decreased physical activity, a high calorie diet, alcohol consumption, cigarette smoking, environmental contaminants among others. The non-modifiable factors include: age, family history and associated genetic factors. One of the risk factors of diabetes that has not undergone a lot of investigation is environmental contaminants, that include pesticides.

A pesticide is defined as any substance or mixture of substances intended for preventing, destroying, or controlling any pests, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal foodstuff **(FAO, 2002).** They can be classified by several methods, the most useful being based on their mode of action wherein they are classified as contact and systemic pesticides. According to the classification based on chemical composition, there are: organochlorines, organophosphates, carbamates, pyrethroids and neonicotinoids. Organochlorine pesticides were banned in the 1970s due to their bioaccumulation in the food chain and their resistance to environmental degradation

which made them very toxic (**Aktar *et al.*, 2009).** The other classes are still commonly used for the control of household and agricultural pests and in public health disease control. These pesticides are either formulated in combination or as single components. Parastar®, a combination of Lambda cyhalothrin and Imidacloprid, confidor® containing the pesticide Imidacloprid and L-Drint-20® containing the pesticide Chlorpyrifos as well as oxamyl are amongst the 600 pesticides approved for use in Cameroon **(Pouokam *et al.*, 2017).**

The main mechanisms of action of these pesticides include phosphorylation and inhibition of the action of acetylcholinesterase by organophosphates **(Betancourt and Carr, 2004)** and carbamates **(Fukuto *et al.*, 1990)**. Pyrethroids act by blocking the open gate sodium channels **(Coats, 1990)**, while neonicotinoids act by binding to the nicotinic acetylcholine receptors (nAChRs) and acting as nAChR agonists **(Jeschke *et al.*, 2011).** The exposure of pests to these pesticides leads to paralysis and/or death. Despite the beneficial effects of these chemicals in agriculture and disease control, they are also known to induce adverse effects in humans and affecting the ecological system. According to the World Health Organization, 3 million cases of pesticides poisoning occur yearly, resulting to more than 250,000 deaths **(Lobin *et al.*, 2017).** Pesticide usage/hectare is less in Africa compared to Latin America and Asia **(Lobin *et al.*, 2017).** However, the concern in Africa is alarming due to farmers' and agricultural workers' poor knowledge of pesticides and their use. Moreover, little formal education and protection against exposure, minimal understanding of the health risks and, most importantly, inadequate safety warnings on the packages by the manufacturers increases the risk of contamination (**Bon *et al.*, 2014**). In Cameroon as many countries in sub–Saharan Africa, pesticide exposure mainly occurs in the agricultural sector. This happens mainly to local farmers (**Tambe *et al.*, 2019)** or those working in industrialized companies such as the Cameroon Development Corporation which cultivates rubber, oil palm and banana **(Kimengsi and Muluh, 2013).** Non respect of pesticide application measures, high run-off of pesticides into nearby water sources as a result of aircraft spraying and dumping of pesticide waste amongst others, result in pesticide poisoning **(Brice *et al.*, 2017)**. This has as consequence the dead of aquatic organisms and, the intoxication of the applicators as well as the local population which can result to dead.

In addition to poisoning caused by acute high dose exposure to pesticides, chronic exposure at low doses is associated with the development of several abnormalities and disease conditions. These include: "oxidative stress", lipid peroxidation, dyslipidemia, dysglycaemia and insulin resistance. The neonicotinoid insecticide (Imidacloprid) is one of the most used pesticides worldwide

(Pouokam *et al.*, 2017; Matthews *et al.*, 2003). Moreover, Parastar containing a mixture of Lambda cyhalothrin + Imidacloprid and Chlorpyrifos are widely used pesticides in Cameroon **(Pouokam *et al.*, 2017; Matthews *et al.*, 2003)**. Lambda cyhalothrin was observed to induce oxidative stress both *in-vivo* and *in-vitro* **(Abdallah *et al.*, 2012; Fetoui *et al.*, 2009; Fetoui *et al.*, 2008; El-Demerdash, 2007).** It significantly increased liver and kidney malondialdehyde (MDA) levels in male adult Wistar rats (**Fetoui *et al.*, 2010; Fetoui *et al.*, 2009).** Similarly, dysregulation in oxidative stress and lipid peroxidation biomarkers in rats exposed to Imidacloprid has been observed **(Mohany *et al.*, 2011; El-Gendy *et al.*, 2010).** Imidacloprid induced insulin resistance by affecting the insulin signalling cascade in cell lines **(Kim *et al.*, 2013)**, and modulated adipocyte differentiation and lipogenesis **(Park *et al.*, 2013)**. Chlorpyrifos induced oxidative stress and increased levels of lipid peroxides in rats **(Ahmed and Zaki, 2009; Tuzmen *et al.*, 2008**). Chronic oral administration of Chlorpyrifos either alone or in combination with Profenofos to male rats increased blood glucose levels while decreasing insulin levels **(Hamza *et al.*, 2014)**. Developmental exposure of male rats to Chlorpyrifos through subcutaneous injection caused hyperlipemia and hyper insulinaemia at adulthood **(Slotkin *et al.*, 2005).**

The effect of gestational exposure to maternal pesticides is understudied meanwhile there is increasing evidence on the role of various exposures during early life (pre-natal and post-natal) on adult metabolism and biochemical status (**Portha *et al.*, 2011).** In fact, the developmental origin of health and disease hypothesis stipulates that adverse foetal exposures may cause permanent foetal adaptations in structure, physiology and metabolism which might be beneficial for short term foetal survival, but may lead to foetal growth retardation, cardiovascular and metabolic diseases in adulthood **(Gluckman *et al.*, 2008)** as observed with several risk factors for Type 2 DM. Possible programming factors investigated include maternal diet, environmental factors such as exposure to Bispheol A, maternal hormonal levels and the metabolic situation during pregnancy **(Jiang *et al.*, 2013)**. However, studies investigating the effect of foetal exposure to commonly used agricultural pesticides such as Imidacloprid, Chlorpyrifos, Lambda cyhalothrin and Oxamyl are lacking. Therefore, whether the effect of foetal exposure to these pesticides could persist until adult age is unknown. We therefore undertook this study aiming at investigating whether biochemical alteration resulting from exposure to pesticides during pregnancy would persist until adult age in offspring. We studied some of the commonly used agricultural pesticides in Cameroon whose formulation contain a single or a combination of pesticides active principle

HYPOTHESIS

In utero exposure to pesticides has no effect on oxidative stress and metabolic markers in female Wistar rats, their offspring at weaning and at adult age.

MAIN OBJECTIVE

The main objective of the study was to investigate the effect of gestational exposure to certain pesticides (including Imidacloprid, Chlorpyrifos, Lambda cyhalothrin+imidacloprid and Oxamyl) on oxidative stress, lipid peroxidation and metabolic markers of glucose and lipid metabolism in female Wistar rats, their offspring at weaning and at adult age.

SPECIFIC OBJECTIVES

Our main objective was achieved through the following three specific objectives:

- To evaluate the effect of *in-vivo* oral exposure of pregnant rats to pesticides on biochemical markers of liver function and oxidative stress in parent rats.
- To evaluate the effect of *in-utero* exposure of offspring to pesticides on biochemical markers of liver function and oxidative stress at weaning and at adult age.
- To determine the metabolic pathways affected by oral and *in-utero* exposure of pregnant rats and their offspring to pesticides.

CHAPTER I:

LITERATURE REVIEW

I.1 DIABETES MELLITUS

I.1.1 Definition and epidemiology

Diabetes Mellitus (DM) is defined as a metabolic disorder of multiple aetiologies characterized by chronic hyperglycaemia with disturbances in carbohydrate, fat and protein metabolism resulting from defects in insulin secretion and/or insulin action **(WHO, 1999)**. The prevalence and incidence of DM are increasing both in developed and developing countries, and it is now one of the most common non-communicable diseases worldwide. It is also one of the fastest growing global health emergencies of the 21st century with over 4 million people aged 20–79 years estimated to die from diabetes and related causes in 2019 **(IDF, 2019).** The current prevalence of DM in adults aged from 20-79 years stands at 463 million in 2019 and this number is projected to reach 700 million by 2045 **(IDF, 2019).** It is estimated that 79.4% of individuals with DM live in low- and middle-income countries **(IDF, 2019).** Even though Africa had the smallest number of individuals with diabetes in 2019 (19 million), it is predicted that by 2045, this continent will be facing the highest increase in the percentage (143%) of people living with Type 2 DM (Table I).

Table I: Type 2 Diabetes Mellitus prevalence in 2019 and projections of 2045 (IDF, 2019).

IDF REGION	DM Prevalence 2019 (Million)	DM Prevalence 2045 (Million)	INCREASE (%)
Africa	19	47	143
The Middle East and North Africa	55	108	96
South-East Asia	88	153	74
South and Central America	32	49	55
North America and the Caribbean	48	63	33
Western Pacific	163	212	31
Europe	59	68	15
Total	463	700	51

Type 2 DM estimates for 2019 show a typically increasing prevalence by age. Similar trends are predicted for the years 2030 and 2045 (Figure 1).

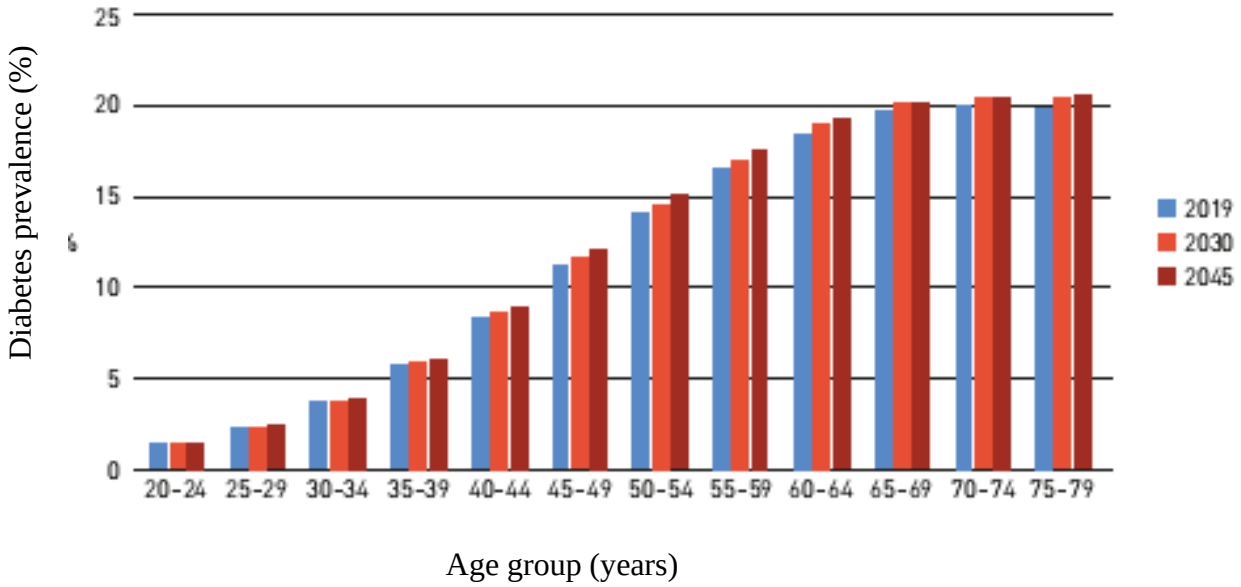


Figure 1: Prevalence of diabetes by age groups in adults (20–79 years) in 2019, 2030 and 2045 **(IDF, 2019)**

I.1.2 Classification

The first widely accepted classification of DM was published by the World Health Organisation (WHO) in 1980 and later modified in 1985 (**WHO, 1980; WHO, 1985**). The 1980 Expert Committee proposed two major classes of DM and named them, Insulin Dependent Diabetes Mellitus (IDDM) or Type 1 DM, and Non-Insulin Dependent Diabetes Mellitus (NIDDM) or Type 2 DM (**WHO, 1980)**. In the 1985 Study Group Report, the terms Type 1 and Type 2 were omitted. The classes IDDM and NIDDM were retained, and a class of Malnutrition–Related Diabetes Mellitus was introduced (**WHO, 1985**). Both the 1980 and the 1985 reports included two other classes of diabetes namely "other types" and Gestational Diabetes Mellitus (GDM). The classification of diabetes has been revised and includes the following.

I.1.2.1 Type 1 *Diabetes Mellitus*

Type 1 DM is an autoimmune disorder resulting from the destruction of pancreatic beta cells. It represents 5 to 10% of all cases of DM worldwide. Despite Type 1 DM occurring frequently in childhood, onset can occur in adults as 84% of people living with Type 1 DM are adults **(Center for Disease Control, 2017).** Between 70% and 90% of people with Type 1 DM at diagnosis have evidence of an immune-mediated process with β-cell autoantibodies against glutamic acid

decarboxylase (GAD)-65, islet antigen (IA)-2, Zinc Transporter 8 (ZnT8) or insulin, and associations with genes controlling immune responses (**Eisenbarth, 2007**). There is a genetic predisposition to autoimmune destruction of beta cells, and it is also related to environmental factors. In populations of European descent, most of the genetic associations are with the HLA-DQ8, and HLA-DQ2 **(WHO, 2019).**

I.1.2.2 Type 2 *Diabetes Mellitus*

Type 2 DM represents the major form accounting for about 90 to 95% of all diabetes cases. It is characterized by disorders in insulin action (insulin resistance) and insulin secretion (beta-cell dysfunction), either of which may be the predominant feature. Type 2 DM is most common in adults, but an increasing number of children and adolescents are also affected **(WHO, 2019).** Most patients with type 2 DM are obese, and obesity itself causes or aggravates insulin resistance (**Stumvoll *et al.*, 2005**). However, in Asians, β-cell dysfunction appears to be more prominent than in populations of European descent (**Ma and Chan, 2013**). This is also observed in thinner people from low- and middle-income countries such as India (**Narayan, 2016**), and among people of Indian descent living in high-income countries (**Gujral *et al.*, 2014; Kanaya *et al.*, 2014**). The risk of developing Type 2 DM increases with age, obesity, unhealthy lifestyle and prior GDM (**WHO, 2019**). It is also associated with strong familial, likely genetic or epigenetic predisposition (**Zimmet *et al.*, 2001**; **Stumvoll *et al.*, 2005**). However, the genetics of type 2 DM are complex and not clearly defined, though studies suggest that some common genetic variants of type 2 DM occur among many ethnic groups and populations **(Fuchsberger *et al.*, 2016).**

I.1.2.3 Hybrid form of diabetes

This form of DM has two classes which are slowly evolving immune-mediated diabetes and ketosis-prone type 2 DM

i) Slowly evolving immune-mediated diabetes:

This form has been described for many years and has often been referred to as “Latent Autoimmune Diabetes in Adults” (LADA). It is frequent in adults presenting clinical symptoms of type 2 DM but having evidence of pancreatic autoantibodies that can react with non-specific cytoplasmic antigens in islet cells, Glutamic Acid Decarboxylase (GAD), protein tyrosine phosphatase IA-2, insulin, or ZnT8 **(WHO, 2019).** This group of people are initially controlled with lifestyle modifications and oral agents, but progress to requiring insulin more rapidly than people with typical Type 2 DM

(Zimmet *et al.*, 1994). In some regions of the world this form of diabetes is more common than classic Type 1 DM **(Tuomi *et al.*, 2014).** A similar subtype has also been reported in children and adolescents with clinical Type 2 DM and pancreatic autoantibodies and has been referred to as latent autoimmune diabetes in youth **(Reinehr *et al.*, 2006; Klingensmith *et al.*, 2010).** Relative differences between slowly evolving immune-mediated diabetes and Type 1 DM include obesity, features of the metabolic syndrome, retaining greater β-cell function, expressing a single autoantibody (particularly GAD65), and carrying the transcription factor 7-like 2 (TCF7L2) gene polymorphism **(Cervin *et al.*, 2008).** There are no universally agreed criteria for identifying this subtype of diabetes, but three criteria are often used. They include: positivity for GAD autoantibodies, age older than 35 years at diagnosis, and no need for insulin therapy in the first 6–12 months after diagnosis.

ii) Ketosis-prone Type 2 diabetes

This subtype has been described as a variant of Type 1 DM or Type 2 DM. Ketosis-prone Type 2 diabetes is an unusual form of non-immune ketosis-prone diabetes first reported in young African Americans in Flatbush, New York, USA (**Winter *et al.*, 1987**; **Banerji *et al.*, 1994).** Subsequently similar phenotypes were described in populations in sub-Saharan Africa (**Mauvais-Jarvis *et al.*, 2004**). Typically, those affected present with ketosis and evidence of severe insulin deficiency but later go into remission and do not require insulin treatment. Reports suggest that further ketotic episodes occur in 90% of these people within 10 years. In high-income countries, obese males seem to be most susceptible to this form of diabetes, but a similar pattern of presentation has been observed in lean individuals of low-income countries **(WHO, 2019**). Ketosis-prone Type 2 diabetes is observed in all populations, but it is least common in populations of European origin (**WHO, 2019**). While it presents with diabetic ketoacidosis, the subsequent clinical course more closely resembles Type 2 DM **(Thomas and Philipson, 2015).** Though the underlying pathogenesis is unclear, there is a transient secretory defect of β-cells at the time of presentation with remarkable recovery of insulin-secretory capacity during the period of remission **(Mauvais-Jarvis *et al.*, 2004).** No genetic markers or evidence of autoimmunity has been identified. Ketosis-prone Type 2 DM can be differentiated from Type 1 DM and classical Type 2 DM by specific epidemiologic, clinical, and metabolic features of diabetes onset and by the natural history of impairment in insulin secretion and action. Glucose toxicity may play a role in the acute and phasic β-cell failure in ketosis-prone Type 2 diabetes. Restoration of normo-glycaemia after insulin therapy is accompanied by a dramatic and prolonged improvement in β-cell insulin secretory function **(Mauvais-Jarvis *et al.*, 2004).**

1.1.2.4 Other specific types of diabetes

i) Monogenic diabetes:

These are rare forms of DM resulting from mutations in a single gene. They account for about 1% to 5% of all cases of diabetes in young people. In most cases, the mutation is inherited while in the remaining cases the mutation develops spontaneously. Most of these mutations reduce the body's ability to produce insulin. Clinical manifestations of monogenic defects in β-cell function include: neonatal diabetes mellitus (NDM), maturity-onset diabetes of the young (MODY), and genetic syndromes where insulin-deficient diabetes is associated with specific clinical features (**Hattersley *et al.*, 2006).**

NDM is a monogenic form of diabetes that occurs in the first 6 months of life. It is a rare condition occurring in only one in 100,000 to 500,000 live births. Infants with NDM do not produce enough insulin, leading to an increase in blood glucose. Intrauterine growth retardation, fever, dehydration, hyperglycemia, acidosis with or without ketonuria are the clinical features of the disease **(Ferda *et al.*, 2006).** NDM can be mistaken for common Type 1 DM which usually occurs later than the first 6 months of life. In about half of those with NDM, the condition is lifelong and is called Permanent Neonatal Diabetes Mellitus. In the rest of the cases, the condition is transient and disappears during infancy but can reappear later in life **(Fosel, 1995).** This type of NDM is called Transient Neonatal Diabetes Mellitus.

MODY is a monogenic form of DM that usually first occurs during adolescence or early adulthood. However, it sometimes remain undiagnosed until later in life. Several gene mutations have been shown to cause MODY, all of which limit the ability of the pancreas to produce insulin. The commonest genetic subtypes are due to mutations in the glucokinase (GCK) gene (GCK MODY) and the hepato-nuclear factor (HNF) gene (HNF1A MODY and HNF4A MODY**) (Shields et al., 2010).**

ii) Diseases of the exocrine pancreas

This type of DM can be caused by any process that diffusely damages the pancreas. Acquired processes include: pancreatitis, trauma, infection, pancreatic cancer and pancreatectomy **(Ewald, 2012; Hart, 2016).** Apart from cancer, damage to the pancreas must be extensive for diabetes to occur. However, adenocarcinomas involving only a small portion of the pancreas have been associated with diabetes. This implies a mechanism other than simple reduction in β-cell mass

(Permert *et al.*, 1994). In cystic fibrosis, there is both exocrine pancreatic failure and reduced insulin secretion resulting in DM **(Dobson *et al.*, 2004)**. However, the relationship between these two defects is not clear **(Dobson *et al.*, 2004)**. DM following pancreatic disease (incidence 2.59 per 100 000 person-years) has been reported to be more common than Type 1 DM (incidence 1.64 per 100 000 person-years). Most of the diabetes following pancreatic disease is classified by clinicians as; Type 2 DM (87.8%), and uncommonly as, diabetes of the exocrine pancreas (2.7%) (**WHO, 2019**).

1.1.2.5 Hyperglycaemia first detected during pregnancy

This form of diabetes either exist as DM in pregnancy or GDM. DM in pregnancy is defined by the same criteria as in non-pregnant person while GDM is defined by newly recommended glucose cut-off points that are lower than those for diabetes **(WHO guideline, 2014**). Individuals at high risk include: older women, those with previous history of glucose intolerance, those with a history of large for gestational age babies, women from certain high–risk ethnic groups, and any pregnant woman who has elevated fasting or casual blood glucose levels.

1.1.2.6 Unclassified diabetes

Subtyping diabetes has become increasingly complex and it is not always possible to classify all newly diagnosed cases of diabetes as belonging to a specific category. Consequently, a category of "unclassified diabetes" has been introduced. For most individuals given this label at diagnosis, it is a temporary category as they can be classified into an appropriate type at some point after diagnosis.

I.1.3 Pathogenesis

Understanding the pathogenesis of Type 2 DM requires a prior understanding of insulin biosynthesis, secretion and action, as well as the insulin resistance and beta cell dysfunction processes.

I.1.3.1. Insulin biosynthesis

Insulin is a peptide hormone produced by the beta cells in the islets of Langerhans of the pancreas. It is made up of two polypeptide chains A and B comprising of twenty-one amino acids and thirty amino acids residues respectively (Figure 2). The two chains are linked to each other by two intermolecular disulphide bonds on cysteine residues. Moreover, chain A has an intra-molecular disulphide bond linking residues 6 and 11 as illustrated in figure 2 below.

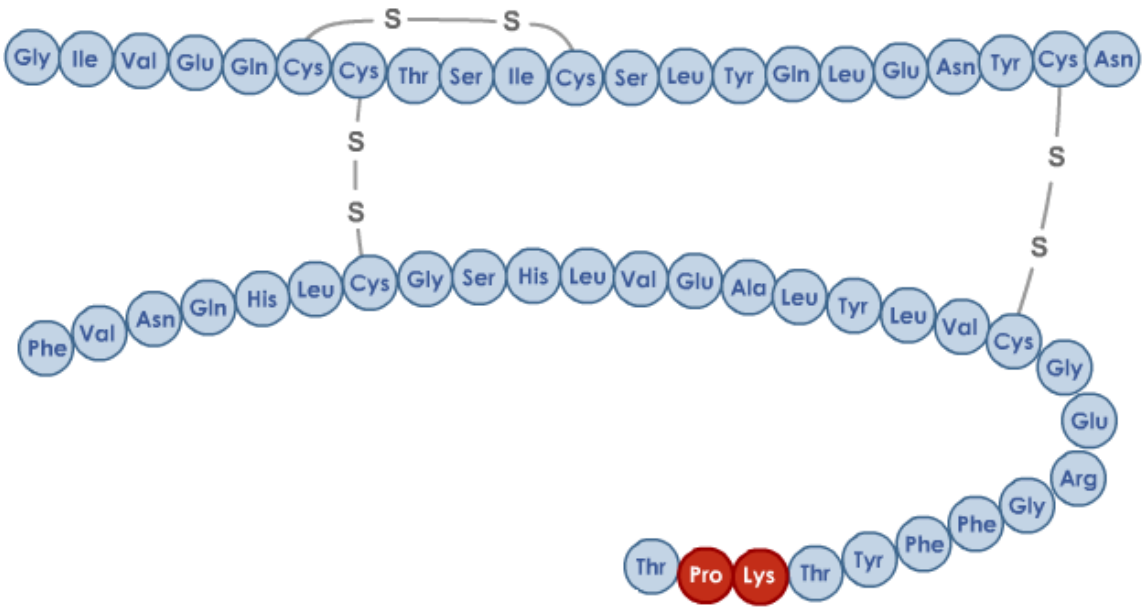


Figure 2: Insulin polypeptide structure[1]

Insulin is coded on the short arm of chromosome 11 and synthesised in the β cells of the pancreatic islets of Langerhans in the form of pre-proinsulin. After synthesis, pre-proinsulin is discharged into the cisternal space of the rough endoplasmic reticulum and cleaved by proteolytic enzymes into proinsulin by the removal of a signal peptide. Proinsulin is then transported by micro-vesicles to the Golgi apparatus whose aqueous zinc and calcium rich environment favours the formation of soluble zinc-containing proinsulin hexamers **(Shashank *et al.*, 2007)**. It is then converted to insulin and c-peptide in the maturing granules through the action of prohormone convertase 2 and 3 as well as carboxy-peptidase H. (Figure 3).

[1] https://www.pharmawiki.ch/wiki/index.php?wiki=Insulin%20lispro

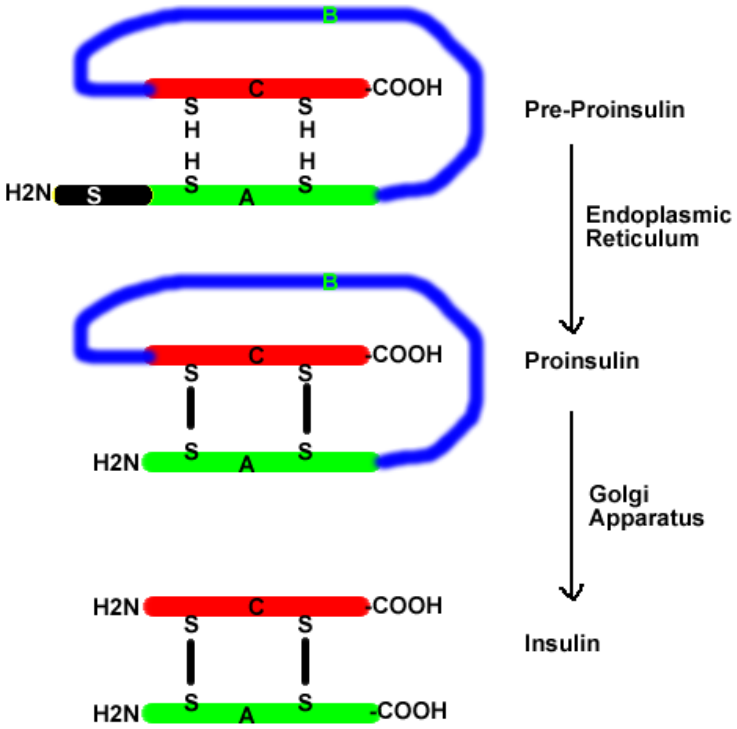


Figure 3: Biosynthesis of insulin[2]

I.1.3.2 Insulin secretion

Insulin is released from the pancreatic beta cells in a biphasic manner in response to a rapid increase in blood glucose concentration **(Straub and Sharp, 2004).** The first phase which is short-lasting is followed by a more slowly evolving second phase which lasts longer. Glucose is the principal stimulus for insulin secretion, though other macronutrients, hormones, humoral factors and neural input may modify this response. The process is initiated by the transport of glucose into beta-cells through diffusion facilitated by glucose transporter (GLUT)-2 in rodents **(Chen *et al.*, 1990)** or GLUT-1 in humans **(De Vos *et al.*, 1995).** Glucose entry into the β cell is sensed by glucokinase, which phosphorylates glucose to glucose-6-phosphate (G6P), generating ATP and consequently increasing the ATP/ADP ratio. This increase in ATP/ADP ratio induces the closure of cell-surface ATP-sensitive potassium-ion (K^+) channels leading to the depolarization of the cell membrane. Next, trans-membrane voltage-dependent calcium-ion (Ca^{2+}) channels are opened due to depolarisation, facilitating the influx of extracellular Ca^{2+} into the beta-cell **(Ashcroft *et al.*, 1984**).

[2] http://biochem.co/2008/08/proteins-quaternary-structure-overview/

Finally, a rise in free cytosolic Ca^{2+} triggers the exocytosis of insulin (Figure 4 below). On the other hand, a slow increase in plasma glucose level induces a gradually larger secretion without the first phase **(Henquin *et al.*, 2002).**

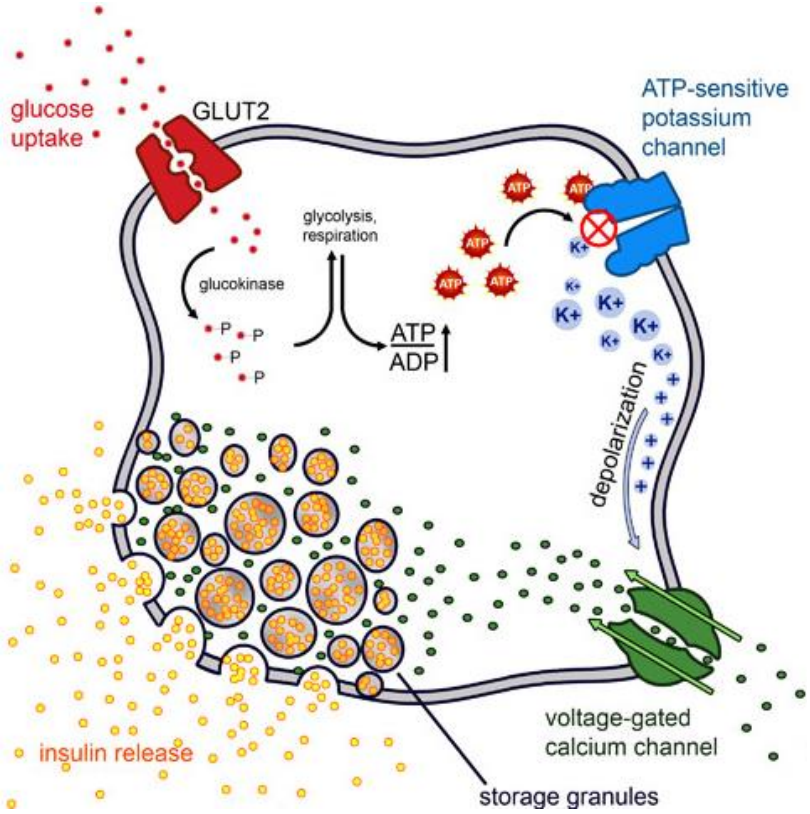


Figure 4: Insulin secretion mechanism[3]

Other mediators of insulin secretion include: activation of phospholipases and protein kinase C (e.g. by acetylcholine), stimulation of adenylyl cyclase activity and activation of β cell protein kinase A, which potentiates insulin secretion. This latter mechanism may be activated by hormones such as Vasoactive Intestinal Peptide, Glucagon-Like Peptide (GLP) 1, and Gastric-Inhibitory Peptide (GIP).

I.1.3.3 Insulin action

In the fed state, excess fuel is stored as glycogen and as triglycerides. During fasting or exercise, this stored fuel is broken down to glucose to be used as a source of energy. These two

[3] http://health4happy.blogspot.com/2011/07/diabetes-what-type-of-insulin-is-right.html

processes are coordinated by insulin and glucagon respectively. Insulin exerts its action by promoting glucose uptake from blood, glycogen synthesis in the liver and muscle, lipid formation to be stored in the adipose tissue, and protein synthesis in most cells. Insulin starts its action by binding to the insulin receptor which was first characterised in 1971. This receptor is a hetero-tetramer consisting of two α and two β glycoprotein subunits that are linked by disulphide bonds (Figure 5) and located in the cell membrane.

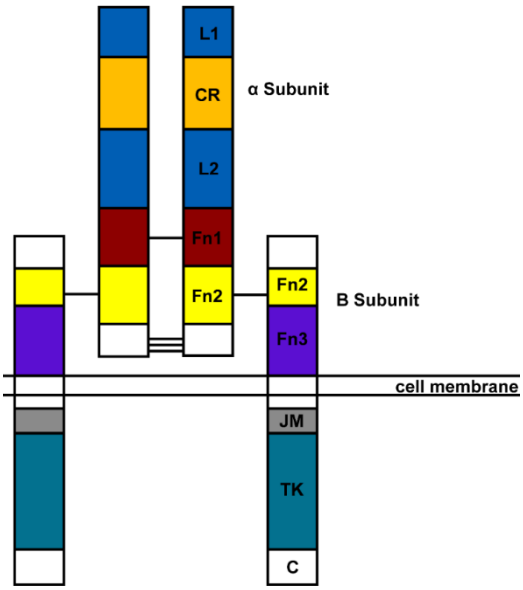


Figure 5: Structure of the insulin receptor[4]

Legend: L1 and L2 denote leucine-rich repeat domains, CR denotes cysteine-rich regions, Fn represents the fibronectin domain

Insulin binds to the extracellular α subunits of the insulin receptor and activates the tyrosine kinase in the β subunits (Figure 6). Once activated, the tyrosine kinase promotes auto-phosphorylation of the β subunits, with phosphorylation of three tyrosine residues (Tyr-1158, Tyr-1162, and Tyr-1163). This leads to the phosphorylation of the Insulin Receptor Substrate (IRS) proteins, and the activation of Phosphatidylinositol 3-Kinase (PI-3K) and Protein Kinase B (PKB) also called Akt. Activated PI-3K generates 3′-phospho-inositides [Phosphatidyl-Inositol-3,4-bisphosphate (PIP2) and phosphatidyl-inositol-3,4,5-trisphosphate (PIP3)], which binds to Phosphoinositide-Dependent Kinase 1 (PDK1). Downstream from PI-3K, the serine/threonine

[4] https://www.pancreapedia.org/sites/default/files/ir_receptor _domains_w-o_title.png

kinase (Akt) triggers insulin effects on the liver, such as glycogen synthesis and the suppression of hepatic glucose production. Akt plays an important role in linking the insulin-dependent glucose transporter protein (GLUT4) to the insulin signalling pathway. It activates GLUT4 which moves to the cell surface to transport glucose into the cell **(Burks and White, 2001; Kido *et al.*, 2001)**

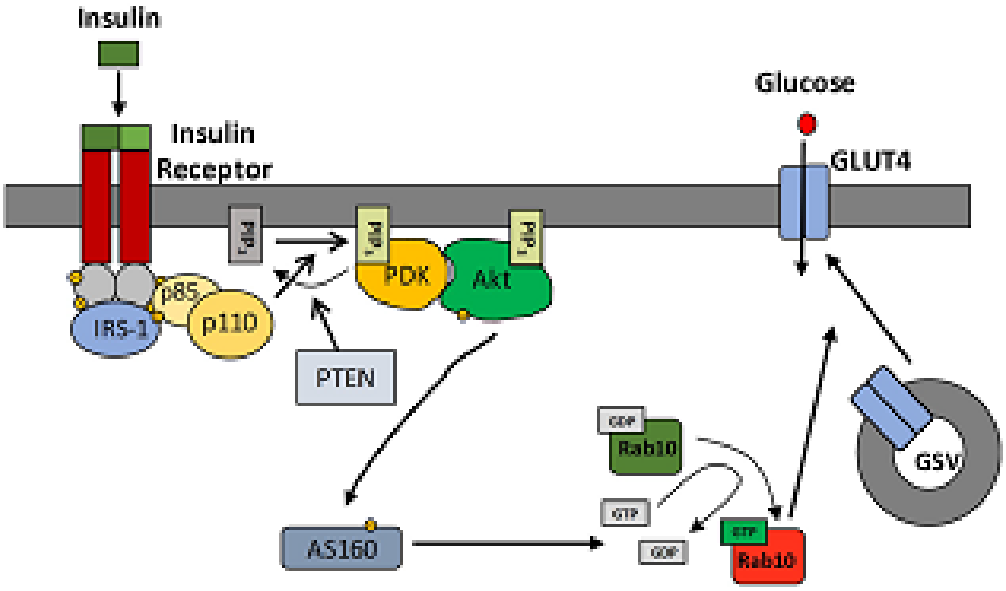


Figure 6: Insulin signalling mechanism (Carmichael et al., 2019)

I.1.3.4 Insulin resistance

Insulin resistance can be defined as the inability of insulin-sensitive tissues to respond properly to normal circulating concentrations of insulin. The main insulin-sensitive tissues are the skeletal muscle, liver and adipose tissue, accounting for 60 – 70%, 30% and 10% of whole-body glucose uptake respectively. Multiple abnormalities in the insulin signalling pathway have been identified to be responsible for insulin resistance. The most important sites are IRS-1, IRS-2, PI-3K and PKB. Rare mutations in the IRS-1 in humans and disruption of the IRS-1 gene in mice have been shown to be associated with insulin resistance **(Yamauchi *et al.*, 1996; Whitehead *et al.*, 1998).** Moreover, serine phosphorylation of IRS proteins can reduce their ability to attract PI-3K, thereby minimizing their activation **(Aguirre *et al.*, 2002; Qiao *et al.*, 2002).** This phosphorylation can also lead to an accelerated degradation of the IRS-1 protein **(Shah *et al.*, 2004)**. This serine phosphorylation in turn decreases IRS-1 tyrosine phosphorylation, impairing downstream effectors **(Yu *et al.*, 2002)**. Circulating free fatty acids (FFA) and the adipokine tumour necrosis factor (TNF) have been shown

to increase serine phosphorylation of IRS-1, thereby causing impaired insulin signal transduction **(White, 2002).** Some studies have demonstrated hyper serine phosphorylation of IRS-1 on Ser-302 **(Um *et al.*, 2004)**, Ser-307, Ser-612 and Ser-632 **(Yu *et al.*, 2002; Kim *et al.*, 2004; Furukawa *et al.*, 2005)** in several insulin-resistant rodent models as well as in young lean insulin-resistant offspring of parents with type 2 diabetes **(Morino *et al.*, 2005).** Based on *in vitro* studies, serine phosphorylation may lead to dissociation between the insulin receptor/IRS-1 and/or the IRS-1/PI-3K complex, preventing PI-3K activation **(Li *et al.*, 1999; Moeschel *et al.*, 2004**) or increasing degradation of IRS-1 **(Egawa *et al.*, 2000**). Insulin resistance can also be established as a result of disruption in the balance between the amounts of the PI-3K subunits. The PI-3K family is divided into four different classes which are Class I, Class II, Class III and Class IV, with Class I further divided into Class IA and Class IB. Class 1A **(Shepherd, 1998)** exist as heterodimers, consisting of a regulatory subunit (p85) which is tightly associated with a catalytic subunit (p110). The regulatory subunit normally exists in stoichiometric excess to the catalytic one, resulting in a pool of free p85 monomers not associated with the p110 catalytic subunit. However, there exists a balance between the free p85 monomer and the p85/p110 heterodimer, with the latter being responsible for the PI-3K activity **(Mauvais-Jarvis *et al.*, 2000; Ueki *et al.*, 2003)**. Because the p85 monomer and the p85/p110 heterodimer compete for the same binding sites on the tyrosine-phosphorylated IRS proteins, an imbalance could cause either increased or decreased PI-3K activity **(Giorgino *et al.*, 1997).** Overexpression of the P85 monomer is responsible for a decrease in the PI-3K activity.

I.1.3.5 Beta cell dysfunction.

Beta-cell dysfunction is characterised by a defect in insulin biosynthesis, a defect in the secretion pathway and a failure to maintain functional beta cell mass. This defect might stem from the impaired capacity for regeneration and/or an increased rate of cell death. The plausible pathways include: reversible metabolic abnormalities (glucotoxicity and lipotoxicity), hormonal changes (inadequate incretin secretion and action), reduction of beta-cell mass due to apoptosis, increased FFA, cytokine dysregulation, mitochondrial dysfunction, metabolic stress and genetic abnormalities. A progressive decrease in beta cell mass arises from a marked increase in beta cell apoptosis which prevails over beta cell replication and neogenesis **(Rhodes, 2005).** Hyperglycemia decreases insulin secretion and is implicated in the damage of beta cells **(Robertson *et al.*, 2003).** Chronic hyperglycemia impairs insulin gene expression of two major beta cell transcription factors which are pancreatic-duodenum homeobox-1 and the activator of the rat insulin promoter element 3b1**.**

Proinflammatory cytokines cause beta cell death via the induction of mitochondrial stress and other responses (**Gurgul-Convey *et al.*, 2011**). Cytokines secreted by immune cells that have infiltrated the pancreas are reported to be crucial mediators of beta cell destruction (**Lin *et al.*, 2012**). A rise in the level of FFAs has been shown to promote pro-apoptotic effects on beta cells **(Unger *et al.*, 1999),** possibly as a result of endoplasmic reticulum stress **(Cnop *et al.*, 2005)** caused by lipotoxicity. Moreover, high levels of FFAs can also contribute to beta cell dysfunction through the intracellular accumulation of triglycerides in response to the activation of the Sterol Regulatory Element-Binding Proteins **(Yamashita *et al.*, 2004)** or by the increased expression of Uncoupling Protein (UCP) 2 which regulates cellular ATP production **(Joseph *et al.*, 2004).** The deleterious effects of FFAs are observed predominantly in the presence of high glucose. In a similar manner, gene variants may affect insulin secretion in type 2 DM through their effects on glucose-stimulated insulin release, incretin sensitivity or secretion, pro-insulin conversion, beta cell proliferation and apoptosis.

The onset of type 2 DM result from impairment of the insulin secretion and insulin action pathways. Even though scientists/scholars are not very certain on which factor precedes the other, it is presently accepted that insulin secretion defects precede insulin resistance, conferring insulin resistance as an early abnormality in the disease. At the pre-onset of type 2 DM, there is resistance to the glucose-lowering action of insulin. This slightly increases blood glucose concentration, stimulating excess insulin secretion and causing hyperinsulinemia. Initially, hyperinsulinemia can overcome insulin resistance and prevents hyperglycaemia. When insulin secretion can no longer be sustained to compensate insulin resistance because of acquired beta cell secretory dysfunction and/or decreased beta cell mass, hyperglycemia occurs. This hyperglycemia can lead to glucose toxicity **(Yki-Jarvinen, 1992**) which then progresses through impaired fasting glycaemia and impaired glucose tolerance to type 2 DM.

I.1.3.6 Signs and symptoms

In 2019, 50.1% of the 463 million adults living with diabetes between the ages 20–79 were undiagnosed **(IDF, 2019**). This is because the disease can go undetected for a long period of time. Moreover, the preliminary symptoms are usually similar to those of other diseases. The common symptoms are excessive thirst (polydipsia), excessive urination (polyuria) and dehydration, excessive hunger or appetite (polyphagia), unexplained weight loss, blurred vision, near-sightedness or other vision problems, slow healing of sores, skin problems such as itchiness, fatigue, lethargy or

drowsiness, shakiness or trembling, mood swings or irritability, dizziness or fainting, numbness, tingling or pain in the feet, legs or hands **(Ramachandran, 2014).**

I.1.3.7 Diagnosis

Diagnostic criteria for Type 2 DM is based on measurement of Fasting Blood Glucose (FBG), casual plasma glucose, glycated haemoglobin (HbA1c) as well as an oral glucose tolerance test (OGTT). People with FBG values of ≥ 7.0 mmol/L (126 mg/dL), 2-h post-load plasma glucose ≥ 11.1 mmol/L (200 mg/dL) **(WHO, 2006**), HbA1c ≥ 6.5% (48 mmol/mol); or a random blood glucose ≥ 11.1 mmol/L (200 mg/dL) in the presence of signs and symptoms are considered to have diabetes (**WHO consultation report, 2011).**

I.1.4 Risk factors

Many studies have elaborated a causal relationship between several factors and the occurrence of type 2 DM. They can be classified as modifiable and non-modifiable risk factors. The non-modifiable risk factors include: age, gender, family history, race/ethnicity and genetic predisposition while the modifiable risk factors include; a sedentary lifestyle, overweight/obesity, high blood pressure, an unhealthy diet and environmental contaminants.

I.1.4.1 Modifiable risk factors

A poor or unhealthy diet and a sedentary lifestyle are important factors associated with the development of Type 2 DM. Positive associations have been reported between different patterns of food intake and the risk of Type 2 DM (**Van-Dam *et al.,* 2002; Liese *et al.,* 2009; Sun *et al.,* 2010**). Some cohort studies have reported an association between a high dietary glycaemic index and the risk of Type 2 DM (**Schulze *et al.,* 2004; Villegas *et al.,* 2007**). A prospective study also found that regular consumption of white rice was associated with an increased risk of Type 2 DM whereas its replacement with brown rice or other whole grains was associated with a lower risk (**Sun *et al.,* 2010**). A higher consumption of butter, potatoes and whole milk was associated with an increased risk (**Hu *et al.,* 2001**) while a higher consumption of fruits and vegetables was associated with a reduced risk of the condition (**Montonen *et al.,* 2005**). The possible mechanism suggested for the latter was that insoluble fibre intake was consistently associated with improved insulin sensitivity thereby decreasing the risk of Type 2 DM (**Salmeron *et al.,* 1997; Meyer *et al.,* 2000**). Moreover, large observational studies have suggested an association between low vitamin D status or low

vitamin D intake and increased incidence of Type 2 DM (**Pittas *et al.*, 2006**; **Knekt *et al.*, 2008**). The suggested mechanism is that vitamin D deficiency may contribute to beta cell dysfunction, insulin resistance and inflammation that may result in Type 2 DM. Dietary factors act in combination with sedentary lifestyle. Longitudinal studies have found physical inactivity to be a strong risk factor for Type 2 DM (**Villegas *et al.*, 2006; Jeon *et al.*, 2007; Fretts *et al.*, 2009**). Prolonged television watching, a surrogate marker for sedentary lifestyle, was reported to be positively associated with DM risk in both men and women (**Hu *et al.*, 2003; Krishnan *et al.*, 2009**). On the other hand, moderate and vigorous physical activities were associated with a lower risk (**Weinstein *et al.*, 2004; Fretts *et al.*, 2009**). Physical activity plays an important role in delaying or preventing the development of Type 2 DM in those at risk both directly by improving insulin sensitivity and reducing insulin resistance, and indirectly by beneficial changes in body mass and body composition **(Hamman *et al.*, 2006).** Moreover, physical activity and a healthy diet prevent the occurrence of two other important risk factors of diabetes which are overweight and high blood pressure. Some habits predispose individuals to Type 2 DM. Alcohol consumption and smoking are two lifestyle factors that contribute in this direction. Several prospective studies have reported that smoking is a risk factor for developing Type 2 DM (**Manson *et al.*, 2000; Sairenchi *et al.*, 2004; Yeh *et al.*, 2010**). Other studies found this increased risk of Type 2 DM even after smoking was stopped for 2 to 3 years. Smoking induces insulin resistance and inadequate compensatory insulin secretion response (**Facchini *et al.*, 1992**). The relationship between alcohol intake and the risk of Type 2 DM is bi-directional whereby light to moderate alcohol intake protects against the development of diabetes while heavy drinking is a risk factor **(Dolly *et al.*, 2009).** The possible mechanisms that explain the relationship include; increase in insulin sensitivity after moderate alcohol consumption **(Hendriks, 2007)**, changes in levels of alcohol metabolites **(Sarkola *et al.*, 2002)**, increase in HDL cholesterol concentration **(Rimm *et al.*, 1999)**, or the anti-inflammatory effect of alcohol **(Imhof *et al.*, 2001).** On the other hand, excess alcohol has a direct toxic effect on the pancreatic islet cells and consequently, increasing the risk in heavy drinkers. Exposure to environmental contaminants principally pesticides is a well characterised Type 2 DM risk factor **(Acker and Nogueira, 2012; Kim *et al.*, 2013; Hamza *et al.*, 2014).**

I.1.4.2 Non-modifiable risk factors

Environmental factors alone cannot explain the rise in the prevalence and incidence of Type 2 DM. They act along with genetic and other non-modifiable factors. Several studies, both prospective and

epidemiological, have reported that first-degree relatives of persons with Type 2 DM are at a higher risk of developing the disease compared to others (**Meigs *et al.*, 2000; Amini and Janghorban, 2007**). This risk is shown to be greater when both parents are affected (**Bjornholt *et al.*, 2000; Ma *et al.*, 2008**). Moreover, twin studies also demonstrate that the concordance estimate for Type 2 DM is higher in monozygotic compared to dizygotic twins and the rate of the disease increases with the duration of follow-up (**Medici *et al.*, 1999**). Also, the prevalence of this disease varies substantially amongst different ethnic groups even when they share a similar environment. This supports the idea that some genes predispose for Type 2 DM. Studies have identified variants in many genes to be associated with the risk of Type 2 DM – amongst which are: Transcription Factor 7-Like 2 (TCF7L2), Peroxisome Proliferator Activated Receptor gamma (PPARγ), Fat mass and obesity-associated protein, Potassium Voltage-Gated Channel Subfamily J Member 11, Neurogenic locus notch homolog protein, Insulin-Like Growth Factor 2 mRNA Binding Protein 2, Solute Carrier Family 30 Member 8, Haematopoietically expressed homeobox protein **(Singh S, 2011).** Of all these, TCF7L2 is the locus with the highest risk of Type 2 DM (**Cauchi and Froguel, 2008; Lyssenko, 2008).** The possible modifications include: single nucleotide polymorphisms, additions, deletions which modify the expression of these genes, leading to the development of the condition. However, the expression of genes can also be modified without a change in the DNA sequence. This phenomenon, described as epigenetics, principally involves DNA methylation and histone modification on specific portions of the gene **(Bramswig and Kaestner, 2012)**. DNA methylation represses gene expression and can result from direct or *in utero* exposure to environmental factors, some of which are risk factors for type 2 diabetes. Several studies have confirmed this positive association. Age is another non-modifiable risk factor. Generally, Type 2 DM individuals are in the 40-and-above age range. In developing countries, the majority are younger, between 45 and 64 years whereas in developed countries they are aged above 65 years.

I.1.5 Associated disorders (Metabolic syndrome)

I.1.5.1 Obesity

Obesity is a metabolic disease that is associated with Type 2 DM. It is estimated that the risk of diabetes in obese people is seven times higher compared to those of healthy weight, with a threefold increase in risk for overweight people **(Abdullah *et al.*, 2010).** Several mechanisms have been proposed to link obesity to Type 2 DM.

Increased upper body fat, also called abdominal fat, favours the production of the pro-inflammatory cytokines – tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and the adipocytokine - leptin, while decreasing the production of adiponectin. TNF-α, IL-6 and leptin are suspected to favour insulin resistance while adiponectin is known to promote insulin sensitivity. Moreover, there is an alteration in the profile of these hormones in the obese state whereby the adipose tissue secretes proportionally more adipocytokines that cause insulin resistance and fewer that promote insulin sensitivity. It has also been shown that obesity is associated with an increase in the adipocyte secretion of chemokines which promote macrophage infiltration as well as increased macrophage activation. Activated macrophages produces cytokines that can negatively impact insulin sensitivity **(Weisberg *et al.*, 2003).** Central obesity resulting from fat accumulation in the subcutaneous, abdominal and visceral depots is most strongly associated with the risk of metabolic complications **(Kissebah and Krakower, 1994)**. Upper body obesity results in an increased mass effect in the visceral region coupled with increased mobilization of FFAs from the individual fat cells in the visceral depots into the portal vein **(Arner, 1997).** The combination of these factors produces markedly elevated FFA levels in obese subjects, resulting in hyperglycaemia, hyperinsulinemia, and hepatic insulin resistance. In addition, the increase in upper-body subcutaneous fat in obese subjects generates an excess of FFA in the peripheral circulation. This is likely to inhibit insulin-stimulated glucose uptake in muscle and impair insulin secretion by the pancreas **(Arner, 1997).** Another mechanism is mitochondrial dysfunction which is evident by decreased mitochondrial mass and/or function. This could be one of the many important underlying defects linking obesity to diabetes, both by decreasing insulin sensitivity and compromising β cell function.

I.1.5.2 Hypertension

The association between Type 2 DM and hypertension has been extensively studied and reviewed **(Epstein and Sowers, 1992**), weighing in the direction of diabetes causing hypertension. The prevalence of hypertension in individuals with diabetes is higher than that of age- and sex-matched patients without diabetes, ranging between 32% and 82% **(Baskar *et al.*, 2006).** Insulin plays an important role in the regulation of the Ca^{2+}-ATPase **(Goewert *et al.*, 1983)** and the Na^{+}/K^{+}-ATPase **(Lytton *et al.*, 1985)** membrane pumps. Hence, defects in insulin secretion and action could result in decreased activity of these pumps. Moreover, decreased activity of either of the pumps results in increased concentration of Ca^{2+} which is associated with systemic hypertension and increased

peripheral vascular resistance **(Weidmann *et al.*, 1972).** High levels of intracellular calcium increase vascular smooth muscle tone, peripheral vascular resistance, and responsiveness to the sympathetic systems - actions that elevate blood pressure. Increased cell levels of Ca^{2+} have been observed in skeletal muscle cells, bone cells and erythrocytes in association with reduced membrane Ca^{2+}-ATPase activity in insulin-resistant rats (**Levy *et al.*, 1989)**. Therefore, abnormalities in vascular smooth muscle cation metabolism and a state of decreased cellular insulin action may play an important role in the increased peripheral vascular resistance that characterizes hypertension in patients with diabetes. Moreover, chronic hyperglycaemia can contribute to the genesis of hypertension **(Epstein and Sowers, 1992)**. One such hypertensive effect engendered by hyperglycaemia is that of sodium retention and the increase in exchangeable body sodium that has been observed in diabetic hypertensive individuals **(Weidmann and Ferrari, 1991**) Hyperglycemia results in glomerular hyper-filtration of glucose, which in turn stimulates the proximal tubular glucose-Na^+ cotransporter. This mechanism is insulin-independent and is rapidly operative, as evidenced by elevated proximal tubular cell Na^+ concentration and Na^+/K^+-ATPase activity within 4 days of streptozotocin-induced hyperglycaemia in rats **(Kumar *et al.*, 1988).** Thus, sodium retention occurs in association with mild-to-moderate hyperglycaemia and likely contributes to increased total exchangeable Na^+ and blood pressure elevations in diabetic hypertensive patients. Chronic hyperglycaemia may also contribute to increased vascular rigidity by promoting vascular structural changes **(Epstein and Sowers, 1992)**. At high concentrations, glucose appears to have a direct toxic effect on endothelial cells, which may result in decreased endothelial-mediated vascular relaxation, increased constriction, promotion of vascular smooth muscle cell hyperplasia, and vascular remodelling. High glucose levels mimicking the diabetic hyper-glycaemic state have also been shown to induce fibronectin and collagen IV overexpression in cultured human vascular endothelial cells **(Roy *et al.*, 1990).** Enhanced expression of fibronectin and collagen IV may further contribute to endothelial dysfunction which is a predictor of hypertension **(Epstein and Sowers, 1992)**. Moreover, hyperglycaemia accelerates the formation of non-enzymatic advanced glycosylation products, which accumulates in blood vessel walls at a rate proportional to time. A highly significant correlation has been noted between increased levels of advanced glycosylation end products and vascular complications **(Monnier *et al.*, 1986).**

I.1.5.3 Oxidative stress and lipid peroxidation

Oxidative stress is defined as a state in which Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) reach excessive levels due to excess production or insufficient removal. ROS are chemically reactive species containing oxygen. They are formed as natural by-products of the normal metabolism of oxygen and have important roles in cell signalling and homeostasis (**Devasagayam *et al.*, 2004**). However, exogenous ROS can also be produced especially during times of environmental stress caused by ultraviolet light, heat exposure or exposure to some environmental contaminants such as pesticides. Consequently, a dramatic increase in the ROS levels is observed which will be responsible for damage to proteins, lipids and DNA, altering the functioning of the cell and leading to cell death (**Johansen *et al.*, 2005**). The association between oxidative stress and Type 2 DM is bidirectional. Oxidative stress is responsible for the development of insulin resistant and beta cell dysfunction. ROS increase the stress signalling pathways in the beta cells, such as nuclear factor kappa beta (NFκβ) activity. This potentially leads to beta cell apoptosis **(Rhodes, 2005)**, suppression of insulin gene expression, contributing to beta cell dysfunction **(Kaneto *et al.*, 2001).** Moreover, over production of ROS as a result of oxidative stress disrupts the insulin signalling pathway at different stages resulting in insulin resistance **(Erejuwa, 2012)**. One of these is increased Ser/Thr phosphorylation of insulin receptor substrate (IRS) molecules when stress-responsive signalling cascades, such as the MAP kinase are activated **(Pitocco *et al.*, 2013).** Moreover, mitochondrial production of ROS induces Apoptosis Signal-regulating Kinase-1 (ASK-1) and activates JNK, thereby increasing Ser-307 phosphorylation of IRS-1 and decreasing insulin-stimulated tyrosine phosphorylation **(Imoto *et al.*, 2006)** which is detrimental for insulin signalling. Lipid peroxidation resulting from lipid damage by ROS species has also been shown to play a central role in the development of Type 2 DM (**Januszewski *et al.*, 2003)**. On the other hand, hyperglycemia and excess FFA levels are responsible for the development of oxidative stress as persistent hyperglycemia causes increased production of free radicals especially ROS, from all tissues as a result of glucose auto-oxidation and protein glycosylation (**Evans *et al.*, 2002; Robertson, 2004).** Moreover, glucose and its metabolites have been reported to react with hydrogen peroxide in the presence of iron and copper ions to form hydroxyl radical during auto-oxidation in diabetes thereby promoting the generation of ROS and development of diabetic complications **(Harani *et al.*, 2012).**

I.1.5.4 Dyslipidemia

Several studies have reported aa possible association between dyslipidemia and type 2 diabetes, recognising dyslipidemia as a risk factor of T2D in various regions of the world. A study carried out in middle-aged adults in the USA showed low levels of high-density lipoprotein (HDL) cholesterol and elevated triglycerides (TG) being significantly related to the development of diabetes (4). In a Korean population, elevated concentrations of total cholesterol, low density lipoprotein (LDL) cholesterol and TG were observed to be independent risk factors for the development of new onset T2DM (5). Moreover, increasing number of studies demonstrated that combined lipid parameters such as TG/HDL-C, LDL-C/HDL-C, TC/HDL-C and non-HDL-C were associated with T2DM (6-7). Other lipid abnormalities include: elevation of very low-density lipoprotein (VLDL) cholesterol, exaggerated postprandial lipidemia and a predominance of small dense LDL cholesterol **(Joshua *et al.*, 2002)** with insulin resistance often involved in the process **(Frank *et al.*, 2002).** However, the most characterised lipid abnormality in patients with diabetes is hypertriglyceridemia and low HDL cholesterol with or without associated increased in serum total and LDL cholesterol **(Taskinen, 1990; Packard *et al.*, 2002).**

I.2 PESTICIDES

I.2.1 Definition and historical background

A pesticide can be defined as a natural or synthetic substance that is used to repel or kill a pest (unwanted plant or animal). The historical background of pesticides dates to the beginning of agriculture and became more pronounced with time due to increased pest population paralleled with decreasing soil fertility **(Tano, 2011)**. However, the use of modern pesticides in agriculture and its public health impact dates to the 19th century. The first generation of pesticides involved the use of highly toxic compounds such as arsenic (calcium arsenate and lead arsenate) and a fumigant called hydrogen cyanide in the 1860's for the control of pests like fungi, insects and bacteria. Other compounds included bordeaux mixture (copper sulphate, lime and water) and sulphur. Their use was abandoned because of their toxicity and ineffectiveness **(Tano, 2011)**. The second generation involved the use of synthetic organic compounds. The first important synthetic organic pesticide was an organochlorine insecticide called Dichloro Diphenyl Trichloroethane (DDT) which was first synthesized by a German scientist in 1873 **(Othmer, 1996).** Its insecticidal effect was discovered by

a Swiss chemist in 1939. In its early days, DDT was hailed as a miracle because of its broad-spectrum activity, persistence, insolubility, low cost and ease of application **(Keneth, 1992).**

I.2.2 Classification

Pesticides are classified based on their mode of action, the targeted pest species and their chemical composition **(Drum, 1980).** Based on the mode of action, pesticides are classified according to the way in which they act to bring about the desired effect. The classes include contact (non-systemic) and systemic pesticides. The non-systemic pesticides are those that do not appreciably penetrate plant tissues and are consequently not transported within the plant vascular system. They only bring about the desired effect when they encounter the targeted pest, hence the name "contact" pesticides. On the other hand, the systemic pesticides are those which effectively penetrate the plant tissues and move through the plant's vascular system in order to bring about the desired effect. The second classification depends on the target pest and includes insecticides, herbicides, fungicides, bactericides, rodenticides, acaricides, nematicides and many others. The third classification is based on the chemical composition and is the most useful for farmers and other pesticide applicators. This is because it gives a clue on the efficacy, physical and chemical properties of the respective pesticides. This knowledge is very useful and provides information on the mode of application, precautions that need to be taken during application and the application rates. On this basis, pesticides are classified into five main groups, namely organochlorines, organophosphorus (organophosphates), carbamates, pyrethroids and neonicotinoids.

I.2.2.1 Organochlorine

I.2.2.1.1 Background

These are synthetically produced chemical compounds that were used mostly for the control of agricultural insects and weeds. They are compounds with at least five chlorine atoms, which are classified as organic because of their carbon ring molecular structures. They are resilient to environmental degradation through chemical, biological, and photolytic processes and have relatively long half-lives. Because of their long half-lives, their lipophilicity and resilience to degradation, they have been observed to persist in the environment, to be capable of long-range transport, to bioaccumulate in human and animal tissue, and to get biomagnified in food chains **(Swackhamer and Hites, 1998).** The members of this class include: hexachlorobenzene, aldrin,

dieldrin, DDT and its derivatives, lindane, heptachlor, endrine, hexachlorocyclohexane and many others **(Bharti *et al.*, 2019)** (Figure 7). These pesticides were banned in the 1970s because of their long-life persistence in the atmosphere and their lipophilicity which made them very toxic **(Aktar *et al.*, 2009).**

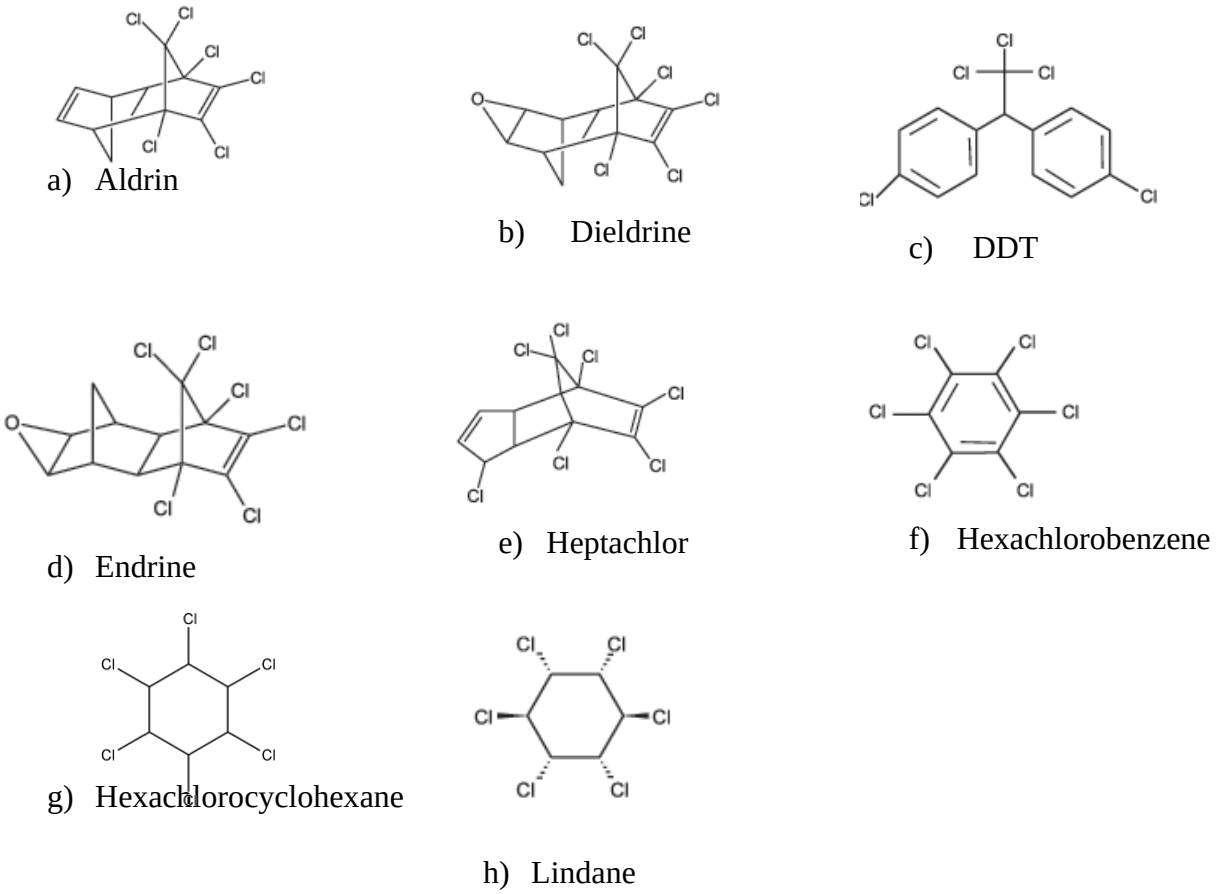


Figure 7 (a – h): Chemical structures of organochlorine pesticides

I.2.2.1.2 Mode of action

The modes of action differ between DDT type and chlorinated alicyclic compounds. DDT type compounds exert their toxic potential by acting on the peripheral nervous system. At the sodium gates of the axon, they prevent the deactivation or closing of the gate after activation and membrane depolarization. The result is leakage of Na^+ through the nerve membrane, creating a destabilizing negative after-potential **(Coats, 1990)**. Hyperexcitability of the nerve results in trains of repetitive discharges in the neuron after a single stimulus. This uncoordinated discharge can lead to central nervous system alterations including seizures, paralysis and death. The mechanism of action of

chlorinated alicyclic compounds involves binding at the picrotoxinin site in the Gamma Amino Butyric Acid (GABA) chloride ionophore complex **(Lawrence and Casida, 1983)**. This binding inhibits Cl^- flux into the nerve. With the function of the GABA-ergic inhibitory neurons impaired, hyperexcitation results, followed by paralysis of the organism. Early manifestations of poisoning by organochlorine pesticides, particularly DDT, are sensory disturbances, hyperesthesia and paraesthesia of the face and extremities. Headache, dizziness, pulmonary cough, nausea, vomiting, diarrhoea, dermatologic rash, incoordination, tremor and mental confusion are also reported. More severe poisoning results in myoclonic jerking movements, often followed by generalized convulsions. Coma and respiratory depression may follow the seizures. Poisoning by the cyclodienes and toxaphene is more likely to begin with the sudden onset of convulsions, often not preceded by the premonitory manifestations mentioned above.

I.2.2.1.3 Pharmacodynamics and metabolism

Exposure to organochlorine pesticide generally occur via ingestion of food and water and skin absorption (**Nicolopoulou-Stamati and Pitsos, 2001**), with the main route being via food. Moreover, application of cosmetic products with estrogenic activities may lead to continuous direct dermal exposure with subsequent absorption and accumulation in underlying tissues **(Harvey and Darbre, 2004).** The levels of organochlorine in human tissues have been shown to be positively correlated with age **(Cocco, 2002**). Human exposure has also been shown to occur during neonatal and prenatal periods, through placenta transfer **(Ando *et al.*, 1986**) and continue postnatally during lactation.

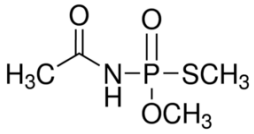
The metabolism of organochlorine occurs through the phase I reactions of biotransformation. These include: reductive dichlorination, dehydrochlorination and oxidation of the parent molecule. Reductive dichlorination of organochlorine insecticides is an important microbial reaction. The reaction proceeds by replacing a chlorine atom on a nonaromatic carbon with a hydrogen atom. This can be seen during the conversion of DDT to DDD and has been shown to occur in yeast **(Kallman and Andrews, 1963).** Dehydrochlorination involves the simultaneous removal of hydrogen and chlorine from organochlorine insecticides. Typically, the reaction takes place between the saturated chlorinated carbon and the adjacent hydrogen on the neighbouring carbon. The formation of DDE from DDT and the formation of -yPCCH from y-BHC are the most familiar examples of this reaction. Oxidation reactions convert the parent compounds such as aldrin, dieldrine and heptachlor

to their respective epoxides **(Korte *et al.*, 1962; Lichtenstein and Schulz, 1960).** After biotransformation, organochlorine pesticides are predominantly eliminated via sweat and urine **(Genius *et al.*, 2016).**

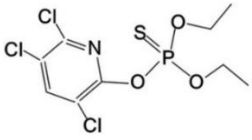
I.2.2.2 Organophosphate

I.2.2.2.1 Background

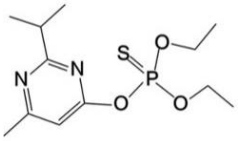
Organophosphate (OP) compounds were first developed by Schrader shortly before and during the Second World War and later synthesized in significant quantities in the 1940s **(Costa, 2018).** They have been used increasingly since the 1970s when organochlorine pesticides were banned. These synthetic organic compounds are all esters of phosphorus having varying combinations of oxygen, carbon, sulphur and nitrogen. Typically, OPs contain a phosphorus atom that is linked by a double bond to a sulphur or oxygen atom. Moreover, two alkyl chains are also linked to the phosphorus with an oxygen bridge to a methyl, ethyl or isopropyl moiety. The remaining bond of the pentavalent phosphorus atom is reserved for the leaving group which is removed from the molecule upon its reaction with the target molecule. These OPs are generally divided into three groups, namely aliphatic, phenyl and heterocyclic derivatives. Some commonly used OPs are ethyl-parathion, diazinon, chlorpyrifos, malathion, methamidophos and acephate **(Rani and Shanker, 2017)** (Figure 8).



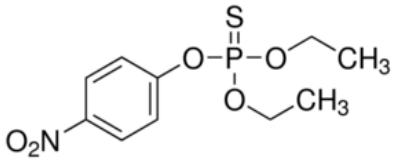
a) Acephate



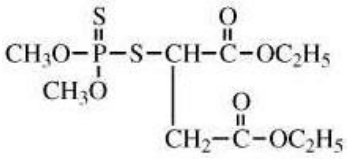
b) Chlorpyrifos



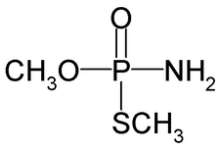
c) Diazinon



d) Ethyl parathion



e) Malathion



f) Methamidophos

Figure 8 (a – f): Chemical structures of organophosphate pesticides

I.2.2.2.2 Mode of action

The key target of OP insecticides is the enzyme acetylcholinesterase (AChE) which is found in synaptic membranes. Acetylcholinesterase hydrolyses the neurotransmitter acetylcholine (ACh), producing choline and acetate. This reaction is important for the regulation of synaptic activity in the central and peripheral neural systems. Choline is removed from the synaptic cleft by reuptake via a high-affinity transport system back into the presynaptic nerve terminal and used in the synthesis of new acetylcholine molecules. OP molecules, when present, inhibit the action of AChE by an irreversible phosphorylation of the molecule. This leads to a dramatic accumulation of ACh in the brain, sympathetic and parasympathetic ganglia of the autonomic nervous system, as well as in the neuromuscular end plate of striated or voluntary muscles (**Yang and Deng, 2007)**. ACh accumulation leads to excessive stimulation of cholinergic receptors, both nicotinic and muscarinic. This stimulation is associated with a dramatic increase in the influx of sodium into the target cells which in turn leads to neuronal depolarization and cellular activation with consequences paralysis and death of the organism. Activation of muscarinic receptors leads to activation through a G-protein linked to the enzyme phospholipase C, which cleaves a membrane phospholipid (phosphatidyl inositol-4,5-bisphosphate) into diacylglycerol and inositol-1,4,5-triphosphate. These two second messengers cause elevation of free intracellular calcium and subsequent cellular excitation. Non-neuronal cholinergic activation, on the other hand, leads to the increased formation of ROS, changes in gene expression and activation of apoptotic events (**Yu *et al.*, 2008)**. Symptoms of acute OP poisoning develop within minutes to hours during or after exposure, depending on the method of application. Inhalation exposure results in the fastest appearance of toxic symptoms, followed by the gastrointestinal route and finally the dermal route. Some of the most reported early symptoms include headache, nausea, dizziness, hypersecretion (sweating and salivation), muscle twitching, weakness, tremors, incoordination, paralysis and starvation **(Petras, 1981; Rickett *et al.*, 1986).**

I.2.2.2.3 Pharmacodynamics and metabolism

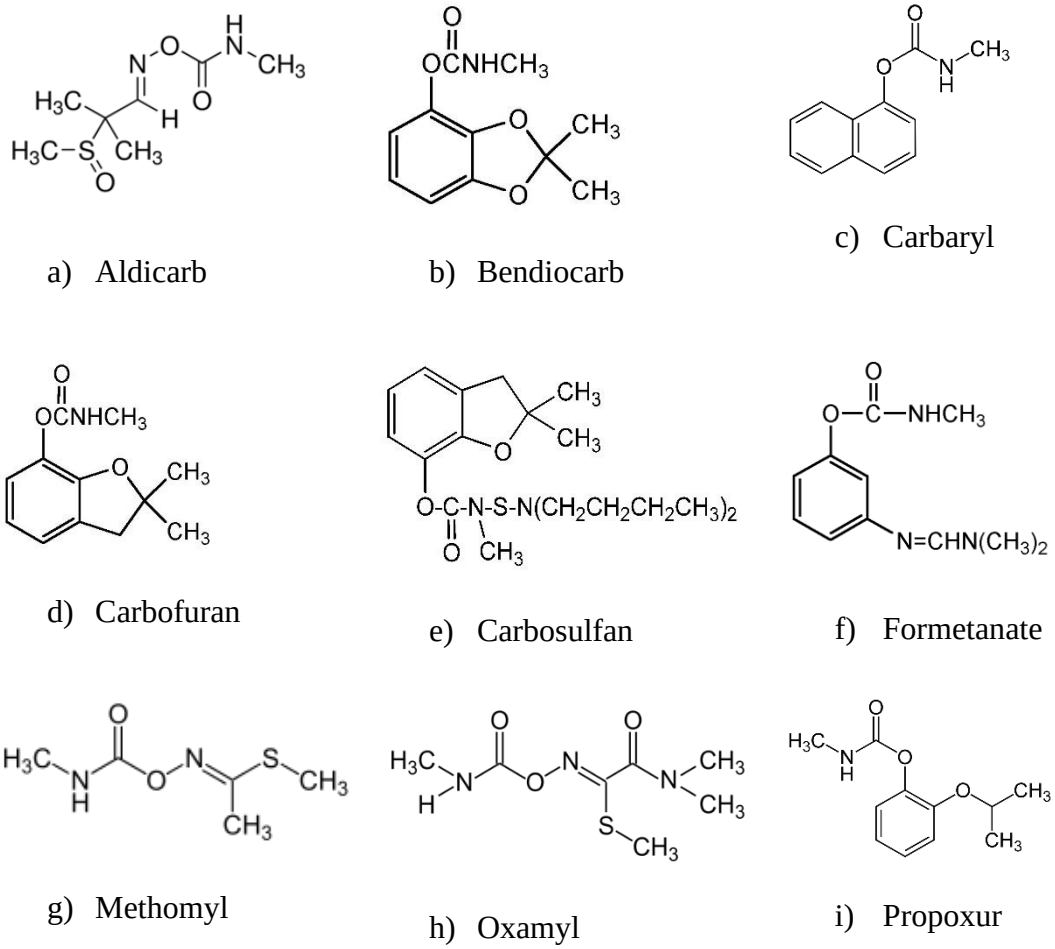
Organophosphate exposure occurs through ingestion, inhalation, and dermal absorption, with uptake through the skin being significant because of the lipophilic nature of these compounds. Moreover, they readily cross the placenta and target the cholinergic components of the developing nervous system and other vital organs **(Pelkonen *et al.*, 2006).**

Organophosphate insecticides and their metabolites are subject to both the phase I (oxidation, reduction and hydrolysis) and phase II (conjugation) biotransformation reactions. During the oxidation reaction, cytochrome P450 favors the addition of molecular oxygen to organophosphate insecticides to produce a more highly oxidized product which are more biologically reactive and therefore more toxic than the parent compound. As such, most oxidation reactions are responsible for activating the pesticide (**Forsyth and Chambers, 1989**). An example is chlorpyrifos which upon entry into the body, undergoes cytochrome P-450 (CYP) mediated metabolism to its active metabolite, chlorpyrifos-oxon **(Ma and Chambers 1994)** which is the metabolite primarily responsible for the inhibition of AChE. Reduction and hydrolysis reactions produces metabolites which are less toxic than the parent compound and therefore considered more important than oxidation reactions. Conjugation reactions frequently occur with the leaving groups produced by organophosphate hydrolysis, such as some of the phenols and heterocyclic alcohols or amines. This second phase render the metabolites more water soluble than the parent compounds or intermediate metabolites for easy excretion. Some of the conjugation reactions are ensured by glutathione and glutathione s-transferase.

I.2.2.3 Carbamate

I.2.2.3.1 Background

These are a group of pesticides which are derivatives of carbamic acid (NH_2COOH). Carbamate pesticides are derived from the substitution of the hydrogen atoms of carbamic acid by other groups of atoms. The carboxylic hydrogen is substituted either by a heterocyclic ring or a nitrogen-containing group, while one of the hydrogen atoms in the amino group is substituted by a methyl group and the other by a sulphur-containing group. The first successful carbamate pesticide (carbaryl) was introduced in 1956 **(Thacker, 2002**). Carbamate pesticides are used worldwide to protect crops against a wide range of insects due to their broad spectrum of insecticidal activity, effectiveness, and their non-persistence in the environment (**Thacker, 2002**). Some common members of this group are aldicarb, carbaryl, carbofuran, propoxur, methomyl, oxamyl, bendiocarb, carbosulfan and formetanate **(Gupta *et al.*, 2018)** (Figure 9).



a) Aldicarb b) Bendiocarb c) Carbaryl

d) Carbofuran e) Carbosulfan f) Formetanate

g) Methomyl h) Oxamyl i) Propoxur

Figure 9: (a – i): Chemical structures of carbamate pesticides

I.2.2.3.2 Mode of action

Carbamates act by inhibiting the AChE enzyme. This occurs by a mechanism virtually identical to that of organophosphate insecticides. However, this inhibition is reversible contrary to the irreversible inhibition by organophosphate compounds. The first step in the process involves the formation of the enzyme-inhibitor complex with subsequent carbamylation of the serine hydroxyl in the active site, resulting in the inhibition of the enzyme. This binding therefore leads to accumulation of ACh and subsequent over-activation of cholinergic receptors at the neuromuscular junctions and in the autonomic and central nervous systems **(Paudyal, 2008)** causing rapid twitching of voluntary muscles and finally paralysis as seen with organophosphates. It is evident that apart from the anticholinesterase activity, carbamates may also influence the functioning of the haemopoietic system especially at high doses, leading to the degeneration of the liver, kidneys and testes. The

commonly reported early symptoms are malaise, muscle weakness, dizziness and sweating. Moreover, blurred vision, incoordination, muscle twitching and slurred speech are also reported. Headache, salivation, nausea, vomiting, abdominal pain and diarrhoea are often prominent. However, the most severe manifestations of carbamate poisoning include coma, seizures and hypotonicity. Dyspnoea, bronchospasm and bronchorhea with eventual pulmonary oedema are other serious signs.

I.2.2.3.3 Pharmacodynamics and metabolism

The metabolic fate of carbamates is basically the same in plants, insects, and mammals. Carbamates are usually easily absorbed through the skin, mucous membranes, respiratory and gastrointestinal tracts. However, they do not accumulate in the body but are rapidly excreted, mainly via the urine. Generally, the metabolites are less toxic than the parent compounds. However, in very few cases, the metabolites are just as toxic or even more toxic than the parent carbamate as is the case of the oxon metabolites. Like organophosphates, carbamates also cross the placenta with little or no restriction and have been detected in the placenta, fetal cord and in the fetus, with target the cholinergic components of the developing nervous system and other vital organs **(Pelkonen *et al.*, 2006).**

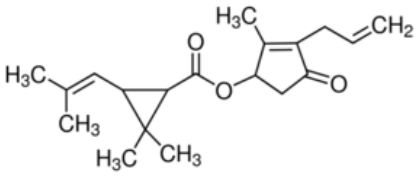
The first step in the metabolism of carbamates is hydrolysis to carbamic acid, which decomposes to carbon dioxide and the corresponding amine. Apart from hydrolysis, oxidation also takes place including: hydroxylation of the aromatic ring, O -dealkylation, N -methyl hydroxylation, N -dealkylation, oxidation of aliphatic side chains, and sulfoxidation to the corresponding sulfone. Oxidation is associated with the mixed-function oxidase (MFO) enzymes. Conjugation leads to the formation of O - and N -glucuronides, sulfates, and mercapturic acid derivatives in mammals.

I.2.2.4 Pyrethroid

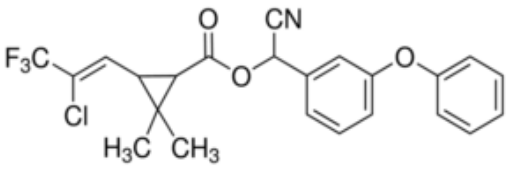
I.2.2.4.1 Background

Pyrethroids were initially extracted from the dried and powdered flower heads of *Chrysanthemum cinerariaefolium*. The extracts contain chrysanthemic and pyrethric acid esters in about equal amounts. Pyrethroids fall into two distinctive categories described as type I and type II. Type I pyrethroids do not have an alpha-cyano group while type II pyrethroids contain an alpha-cyano group, making them more potent pesticides than their type I counterparts. They are rapidly

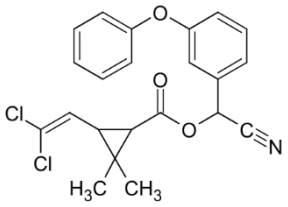
metabolised in mammals. Although lipophilic, no bioaccumulation has been observed after sub-acute administration to mammals **(Miyamoto *et al.*, 1995).** The first molecule synthesized in 1949 was called Allethrin **(Bradberry *et al.*, 2005)** but the first commercialization came about in 1978 with Fenvalerate **(WHO, 2005).** Other compounds include cyhalothrin, cypermethrin, permethrin and tefluthrin **(Wu *et al.*, 2011)** (Figure 10). They are used to eradicate domestic pests such as cockroaches, agricultural and animal pests.



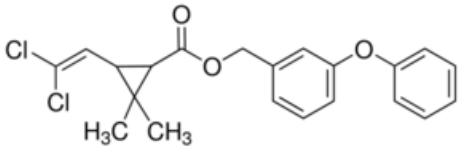
a) Allethrin



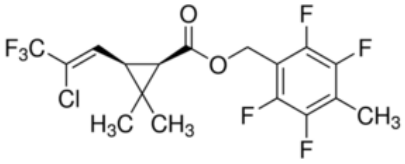
b) Cyhalothrin



c) Cypermethrin



d) Permethrin



e) Tefluthrin

Figure 10: (a – e): Chemical structures of pyrethroid pesticides

I.2.2.4.2 Mode of action

Pyrethroid pesticides interfere with signal propagation in neurons. Specifically, they act on the sodium channels located along the cell membrane on the neuron tail (axon). By blocking open gates, they may create repetitive firing and depolarization that leads to symptoms like tremors, involuntary movements and salivation. Other proposed mechanisms likely representing secondary modes of action include: antagonism with GABA receptors, modulation of nicotinic cholinergic transmission, increase in noradrenaline release and action on calcium channels **(WHO, 2005).** Short-term skin exposure to pyrethroids may lead to abnormal facial sensations. Ingestion may cause sore throat, nausea, vomiting and abdominal pain, with or without mouth ulcers, increased secretions and swallowing difficulty.

I.2.2.4.3 Pharmacodynamics and metabolism

Exposure to pyrethroids occurs primarily using pyrethroid containing household insecticides and pet sprays, and through the ingestion of food and drinking water contaminated with pyrethroid residues (**ATSDR, 2003**). Like many other classes of pesticides, pyrethroids compound cross the placental barrier and are known to interfere with hormonal and neurological development, the immune system and other physiological functions (**Doucet *et al.,* 2009**). Following oral ingestion, inhalation, or dermal intake, pyrethroids are metabolized into carboxylic and phenoxy-benzoic acids and then excreted in the urine.

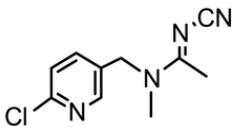
The main reactions of biotransformation are oxidation, cleavage of ester bonds and conjugation reactions **(Kaneko, 2010).** Oxidation and ester cleavage metabolizes pyrethroids mediated by CYP isoforms and carboxylesterases respectively. Oxidation reactions occur on several sites of the acid and alcohol moieties, depending on the chemical structures. Ester hydrolysis occurs to a larger extent with the trans and primary alcohol derivatives as compared with the corresponding cis and secondary alcohol derivatives respectively. Conjugation reactions including glucuronides, sulfates and amino acid conjugates leads to the formation of highly hydrophilic metabolite which is rapidly excreted and eliminated into urine **(Kaneko, 2011).** These metabolites show less acute oral toxicity than their corresponding parent compounds with rapid metabolism leading to low mammalian acute toxicity **(Soderlund *et al.,* 2002).**

I.2.2.5 Neonicotinoid

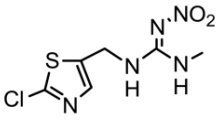
I.2.2.5.1 Background

Neonicotinoids are a class of highly selective neuro-active pesticides derived from nicotine **(Cresswell *et al.,* 2012).** They are systemic chemicals that are absorbed by the plant and transferred through the vascular system, making it toxic to sap-sucking and chewing insects. These chemicals were first registered for use in mid-1990's and have become widely adopted for use as seed treatment and spray on farm crops in agricultural and horticultural landscapes (**Jeschke *et al.,* 2011**). They have also been widely used in veterinary applications as effective flea treatment on cats and dogs (**Dryden *et al.,* 2000).** Neonicotinoids act primarily as water-soluble plant systemic pesticides. When used as soil or seed treatment or applied topically, they are taken up by the plant roots or tissues and translocated through the entire plant. This protects the plant from piercing/sucking insects such as aphids, leafhoppers, and whiteflies **(Tomizawa and Casida, 2005)**. Although these properties play

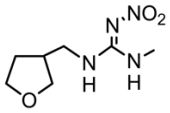
a valuable role in crop protection, these same features increase the potential for unintentional exposure to non-target insect species, animals and humans. Moreover, the persistence of neonicotinoids in the environment is attributed to their chemical and physical properties as shown in studies demonstrating residual concentrations in the environment following application. Cold soil temperatures have been associated with higher soil half-lives **(Main *et al.*, 2014).** Based on the soil half-lives, it is anticipated that their repeated applications would lead to an accumulation in the soil. Due to their high-water solubility, they have been detected in both groundwater and surface water. In 2005, neonicotinoids accounted for approximately 11%–15% of the total insecticide market **(Tomizawa and Casida, 2005)**. With trends of increasing detection of residue levels in food from 4.7% in 2007 to 12.6% in 2010, these results points to an increase in their overall usage worldwide **(Chen *et al.*, 2014)**. Individual neonicotinoids include imidacloprid, thiamethoxam, thiacloprid, clothianidin, acetamiprid, dinotefuran and nitenpyram **(Kasiotis and Machera, 2015**) (Figure 11).



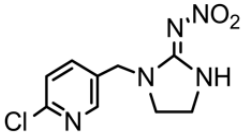
a) Acetamiprid



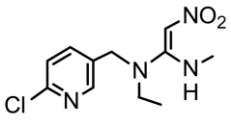
b) Clothianidin



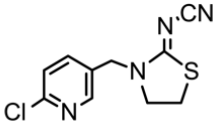
c) Dinotefuran



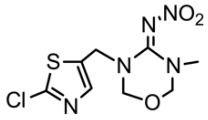
d) Imidacloprid



e) Nitenpyram



f) Thiacloprid



g) Thiamethoxam

Figure 11: (a –g): Chemical structure of neonicotinoid pesticides

I.2.2.5.2 Mode of action

Neonicotinoids exert their insecticidal action by binding to the nicotinic acetylcholine receptors (nAChRs) and acting as nAChR agonists. These receptors are in the post-synaptic dendrites of all neurons in the brain, spinal cord, ganglia, and muscular junctions. Normally, they are activated by binding on ACh and opening the ion channels which allow the entry of cations including sodium and calcium. They are then inactivated when ACh is broken down by AChE. Neonicotinoids mimic the action of ACh by opening the ion channels in a similar manner. However, they cannot be inactivated by AChE, leading to overstimulation of the nervous system. This finally ends with hyperactivity and death of the organism.

I.2.2.5.3 Pharmacodynamics and metabolism

Neonicotinoids are rapidly absorbed in the intestine, rapidly distributed, and excreted after oral exposure. An example is observed with imidacloprid which is quickly absorbed in the intestinal lumen followed by distribution from the plasma into the body (**Tomlin, 2003).** Neonicotinoids have been shown to cross the placenta membrane to affect the growth and development of offspring **(Tanaka, 2012).**

Neonicotinoids undergo phase I and phase II biotransformation in insects, mammals and plants. The phase I reactions include oxidation and reduction reactions catalyzed by the mixed function oxidase cytochrome P450s. IMI is oxidized to the 5-hydroxy (IMI-5-OH) and olefin (IMI-ole) metabolites and reduced to the nitrosoguanidine (IMI-NNO), aminoguanidine (IMI-NNH2), desnitro (IMI-NH) and urea (IMI-urea) metabolites by a variety of human CYP isozymes **(Schulz-Jander and Casida, 2002; Schulz-Jander *et al.*, 2002).** Another phase I reaction is hydroxylation of the imidazolidine ring at the 4 or 5 position to yield IMI-4-OH and IMI-5-OH (16% of the radiolabel) and then loss of water to produce IMI-ole. The phase 2 reaction involves conjugation with glycine and dichlorination of the chloropyridinyl ring producing 6-hydroxynicotinic acid and its methyl-mercapturic acid derivative likely via a glutathione conjugate.

I.2.3 Global use of pesticides

Pesticides are used for the control of pests such as insects, herbs and others. Globally, there are approximately 9000 species of insects and mites, 50000 species of plant pathogens, and 8000 species of weeds that injure crops **(Zhang *et al.*, 2011**). In 2009, insect pests were responsible for an

estimated 14% of loss, plant pathogens accounted for 13% of loss and weeds caused 13% of loss in crops worldwide. (**Pimentel, 2009a; Pimentel, 2009b).** With the continuous increase in the world's population, pesticides use is imperative to increase crops yield and meet the ever-demanding population. It was estimated that nearly one-third of the agricultural products are produced with the help of pesticides **(Liu *et al.*, 2002; Zhang *et al.*, 2011**). Without pesticide use, the loss of fruits, vegetables and cereals from pest injury may reach 78%, 54% and 32% respectively (**Cai, 2008**). Crop loss from pest injury declined by 35% to 42% when pesticides were used (**Pimentel, 1997**). Fungicides were used on 80% fruit and vegetable crops in USA. The economic value of the apple was estimated to increase by 1223 million US dollars using fungicides (**Guo *et al.*, 2007**). Without pesticide use, the export of cotton, wheat and soybean in USA would decline by 27% (**Zhang *et al.*, 2011**).

Through-out the globe, approximately 2 million tonnes of pesticides were utilized in 2018 with the amount projected to reach 3.5 million tonnes by 2020 (**Zhang, 2018**). Insecticides were the second most used pesticides, accounting for 29.5% after herbicides which accounted for 47.5% **(De *et al.*, 2014**). The top ten pesticide consuming countries in the world are China, the USA, Argentina, Thailand, Brazil, Italy, France, Canada, Japan and India[5], while Africa is contributing between 2 – 4% of the global use of pesticides. Average pesticide usage per hectare in Africa is 1.23 kg/hectare. Morocco was the 1st African country with the highest amount of pesticides used (13,697 tons) and the 32nd in the world in 2017 while Cameroon occupied the 80th position worldwide with 1372 tonnes[6] during the same period. Highly used pesticides in Cameroon in the early 2000s include Paraquat and Glyphosate (herbicides), Cypermethrin and Chlorpyrifos (insecticides), and Metalaxyl and Maneb copper (**Matthews *et al.*, 2003**). A study on the misuse of pesticides in the North Region of Cameroon by farmers revealed that organochlorine residues (lindane, alpha-endosulfan and beta-endosulfan), organophosphorus residues (malathion, pirimiphomethyl), synthetic pyrethroids (permethrin) and carbamates (carbofuran) are found in maize and millet in certain localities of that region at levels higher than the recommended Maximum Residue Limits MRLs (**Sonchieu *et al.*, 2010**). Nine pesticide residues were detected in cooked foods including atrazine in spices, chlorothalonil in vegetables, cypermethrin in tomatoes, deltamethrin in bread, endosulfan in tomatoes and vegetables, malathion in wheat doughnut, pirimiphos-methyl in spaghetti,

[5] https ://www.world atlas .com/artic les/top-pesticide-consuming-countries-of-the-world .html
[6] https://www.worldometers.info/food-agriculture/pesticides-by-country/

dithiocarbamates in papaya, pineapple and spices and chlordecone in tomatoes **(Gimou *et al.*, 2008).** A recent study revealed that pesticides including imidacloprid, lambda cyhalothrin and chlorpyrifos are widely used in five agro-ecological zones in Cameroon **(Pouokam *et al.*, 2017).**

However, the current use of pesticide is observed to be more than the amount required leading to environmental hazards and harmful effects on non-target organisms. In 2013, Greenpeace reported that 70% of pesticide used in China was not absorbed by plants, but instead seeped into the soil and groundwater[7]. Overuse of pesticides generally results in the development of resistance by the target pest and exposure of non-target organisms. In addition to the overuse of pesticides, the negative impact is also attributed to the misuse of pesticides. A lot of misuses have been reported in field farms because of absence of clear instructions, low literacy level of pesticide applicators, lack of knowledge on risks from bad uses, uses of pesticides on crops for which the product was not homologated, difficulties to properly prepare the solution to be used, and poor respect of dosage. (**Bon *et al.*, 2014**). This has led to devastating effects on the environment and humans (**Bon *et al.*, 2014**). Among all the categories of pesticides, insecticides are most toxic whereas fungicides and herbicides are second and third on the toxicity list respectively (**Mahmood *et al.*, 2016**). Pesticides enter the natural ecosystems by two different means depending upon their solubility. Water soluble pesticides get dissolve in water and enter ground water, streams, rivers and lakes. Pesticide-contaminated water poses a great threat to aquatic forms of life. It can affect aquatic plants, decrease dissolved oxygen in the water and cause physiological and behavioural changes in fish and other amphibian populations. On the other hand, fat soluble pesticides enter the bodies of animals by the process of bio-amplification. They get absorbed in the fatty tissues of animals hence resulting in persistence of pesticide in food chains for extended periods of time. In addition, pesticides such as carbamates, organophosphates and pyrethroids also affect beneficial insects such as bees and beetles. Neonicotinoid insecticides such as clothianidin and imidacloprid are toxic to bees such that low doses in addition to reducing learning capacity **(Decourtye *et al.*, 2003**) negatively affects bee foraging behaviour (**Yang *et al*., 2008**).

According to World Health Organization, about 3 million cases of pesticide poisoning and 220,000 deaths are recorded annually across the globe, the majority of which are reported from developing countries **(Lobin *et al.*, 2017**). Moreover, about 2.2 million people, mainly belonging to developing

[7] https://chinadialogue.net/en/pollution/10148-china-founds-pesticide-office-to-combat-pollution-overuse/

countries are at increased risk of exposure to pesticides **(Mahmood *et al.*, 2016**). Those most susceptible to the toxic effects are infants, young children, agricultural farm workers and pesticide applicators (**Pesticides and Human Health, 2020).** Pesticides enter the human body through ingestion, inhalation or penetration via the skin (**Spear, 1991),** and most people get affected via the intake of pesticide contaminated food. After crossing several barriers, they ultimately reach human tissues or storage compartments **(Hayo and Werf, 1996**). The toxic effects are produced when the pesticide is not metabolized an eliminated rapidly and its concentration in the body increases far more than its initial concentration in the environment **(Hayo and Werf, 1996).** The effects of pesticides on human health are highly variable with some being acute and others chronic. The immediate effects of acute exposure to pesticides result from the toxicity of the chemical. Acute exposure to high dose immediately results to toxic effect. Therefore, to determine the mechanism involved, biomarkers of toxicity are identified. A common biomarker of pesticide toxicity is oxidative stress characterized by an increase or a decrease in antioxidant enzymes as ROS levels increases. The increase in the concentration of ROS can result from by-products of the operation of the detoxification pathways, alteration of the mitochondrial and endoplasmic reticulum electron transport chains **(Lambert and Brand, 2009).** Common antioxidant enzymes measured to access toxicity are, Superoxide Dismutase (SOD), catalase, reduced glutathione (GSH), Glutathione Peroxidase (GPx), Glutathione S-Transferase (GST) and Glutathione Reductase (GR). SOD acts at the primary level where it converts superoxide radicals to hydrogen peroxide which is further converted by catalase to water and oxygen which are non-toxic **(Birben *et al.*, 2012).** GPx and GST are two important enzymes in the phase II reactions of detoxification. They catalyse the conjugation of several xenobiotics with GSH thereby eliminating these toxic substances. Glutathione reductase on the other hand serves in the regeneration of GSH which is an antioxidant reacting with free radicals and organic peroxides (**Birben *et al.*, 2012)**. Moreover, blood glucose concentration and activities of serum enzymes can also be used as biomarkers of pesticide toxicity. Serum enzymes such as alanine transaminase and aspartate transaminase are markers of hepatotoxicity which are associated with oxidative stress (**Cheraghi *et al.*, 2019).** Increased levels of these enzymes in serum or plasma is an indication of cellular damage. Therefore, the levels of these biomarkers can serve to determine pesticide toxicity. Common acute toxic effects include headache, stinging of the eyes and skin, irritation of the nose and throat, skin itching, appearance of rashes and blisters on the skin, dizziness, diarrhoea, abdominal pain, nausea and vomiting, blurred vision, blindness and very rarely

death (**Mahmood *et al.*, 2016).** Chronic toxic effects may take months and even years to manifest causing several diseases and disorders in the human system.

I.3 PESTICIDES AND DIABETES

Many chemicals that have been identified as endocrine disruptors are pesticides. About 105 pesticides have been identified as endocrine disruptors amongst which 46% are insecticides, 21% herbicides and 31% fungicides **(Wissem *et al.*, 2011).** An Endocrine Disruptor Pesticide (EDP) is an exogenous substance or mixture that alters the function of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny or sub-populations. These pesticides act by interfering with the endocrine network of vertebrates, provoking adverse dysregulation of the hormonally controlled physiological parameters or functions. The mechanisms of interference involve direct binding of the EDP to the hormone receptor leading to stimulation or inhibition of downstream cellular pathways in target cells or indirectly by increasing or decreasing the concentration of endogenous hormones. Examples could be seen with organophosphates and carbamates (acetylcholinesterase inhibitors), pyrethroids (sodium channel agonists) acting on insect's nervous systems **(Garcia-Reynaga *et al.*, 2013; Cordova *et al.*, 2016)** and neonicotinoid insecticides which act through their preferential affinity for the nAChR subtypes (**Casida, 2009).** EDPs mainly affect the endocrine system via the Nuclear Receptors (NR) family (oestrogen receptors, androgen receptors and thyroid hormone receptors) and the non-nuclear receptor aryl hydrocarbon receptor (AhR). Moreover, they may also affect hormone metabolism and synthesis by regulating their related enzymes including cytochrome P450, UDP-glucuronosyl transferase (UGT) or sulfotransferase as activators of the constitutive androstane receptor (CAR) and the peroxisome proliferator-activated receptors (PPARs) **(Lovekamp-Swan *et al.*, 2003; Tabb and Bumberg, 2006).**

Disruption of the endocrine system by pesticides leads to a plethora of dysfunctions and diseases. The common diseases include reproductive disorders and cancer. In males, pesticide exposure has been shown to affect the reproductive organ development as well as the semen quality. EDPs may also act in the brain by stimulating hypothalamic neurons, thereby releasing kisspeptin and promoting the maturation of the hypothalamus, causing earlier onset of puberty, or even precocious puberty **(Patisaul, 2013)**. Moreover, EDPs interfere in the hormonal regulation of the menstrual cycle leading to menstrual irregularity and fecundability. Endocrine disruptors may also affect the

oncogenic transformation process, via reprogramming and remodelling of carcinogenic gene expression. Common types of cancers caused by EDPs include breast, ovarian and testicular cancer **(Dich *et al.*, 1997; Fleming *et al.*, 1999; Cabello *et al.*, 2003).** In addition to the developmental and reproductive effects, there is also growing evidence that metabolic and cardiovascular disorders may be linked with EDPs. Endocrine disruptors may act directly or indirectly as obesogens, by promoting adipogenesis through control/fostering of lipid accumulation **(Grun and Blumberg, 2007**) or through energy balance through shifting towards calorie storage by modifying basal metabolic rate, altering gut microbiota **(Snedeker and Hay, 2012)**, promoting food storage and altering hormonal control **(Blumberg, 2011)** of appetite and satiety **(Heindel, 2011).** Endocrine disruptors also contribute to the manifestation of metabolic syndrome through inflammatory processes via cytokines/adipokines, producing the effects of metabolic imbalance. ROS and other oxidative stress biomarkers generated by EDPs impair eNOS and other anti-atherosclerotic enzymes triggering pro-atherosclerotic events by afflicting vascular tones **(Giacco and Brownlee, 2010).**

I.3.1 Imidacloprid

Imidacloprid is a colourless crystal or beige powder with a characteristic bad odour **(Tomlin, 2006)**. This systemic insecticide with systematic name (1[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazoli-dinimine) is an active ingredient in several insecticide brand products such as Admire®, Confidor®, Gaucho®, Premise®, Marathon®, Attakan®, Dinacacao®, Imida® and Parastar®. In these products, it can exist as the only active ingredient or mixed with other active ingredients of the same class or different classes. Parastar® is a mixture containing equal amount of imidacloprid and lambda cyhalothrin. Imidacloprid-based formulations are available as dustable powder, granules, seed dressing, soluble concentrate, suspension concentrate, and wet powder. This insecticide is used for controlling sucking insects such as fleas, aphids, whiteflies, termites, soil insects and some beetles **(Tomlin, 2006)**. It is most commonly used on rice, cereal, maize, potatoes, vegetables, sugar beet, fruit, cotton, hops and turf as foliar and seed treatment. It is also used in indoor and outdoor insect control, home gardening and pet products. It was first registered in the United States of America in 1994 as the first chemical in its chemical class by Bayer Crop Science (**Hovda and Hooser, 2002)**. Acute and chronic toxicity studies carried out by the Environmental Protection Agency (EPA) have classified it as both a toxicity class II and class III pesticide (on a scale of I to IV). On this scale I refer to extremely toxic with an oral LD_{50} <5 mg/kg, II being highly toxic with an oral LD_{50} between 5 and 50 mg/kg, III being moderately toxic with an oral LD_{50} between 50 and 500 mg/kg and IV

being slightly toxic with an oral LD_{50} above 500mg/kg. Imidacloprid has been found to be highly toxic to bees **(Dively *et al.*, 2015)** and moderately toxic to rats **(U.S EPA, 2007).** The median lethal dose in rats has been shown to vary across studies. Oral lethal dose values were estimated to be 450 mg/kg for both sexes in one study **(Tomlin, 2006)** and, 500 and 380 mg/kg in males and females respectively in another study **(WHO, 2004).** Symptoms of acute exposure would be similar to nicotinic signs and are expected to be diarrhoea, fatigue, twitching, salivation, convulsions, cramps, and muscle weakness including the muscles necessary for breathing. Symptoms can last for five days following exposure. Chronic or long-term toxicity of imidacloprid is linked to reproductive and mutagenic effects at relatively high doses. Studies with laboratory rats fed with imidacloprid over two years resulted in decreased body weight and increased thyroid lesions. Elevated blood cholesterol levels and stress to the liver were also observed in dogs fed imidacloprid for 1 year. It is categorized as a "Group E" carcinogen by the EPA.

Several studies have reported a dysregulation in oxidative stress, lipid peroxidation biomarkers and liver injury upon exposure to imidacloprid insecticide in rats **(El-Gendy *et al.*, 2010; Kapoor *et al.*, 2010; Balani *et al.*, 2011; Lohiya *et al.*, 2017). Mohany *et al.*, (2011)** observed a marked increase in serum AST, ALT and ALP after the administration of 0.21 mg/kg body weight of imidacloprid to male albino rats**.** This is suggestive that oxidative metabolites and/or free radicals are produced during its metabolism, leading to cellular and tissue damage. Imidacloprid alters lipid profile by significantly increasing total cholesterol, triglycerides and LDL-cholesterol level while lowering HDL-cholesterol in male rats (**Mehmood *et al.*, 2017**). Same as in animal models, imidacloprid was observed to alter serum lipids in an Indian population (**Pothu *et al.*, 2019**). Moreover, imidacloprid has been shown to directly induce insulin resistance in adipocytes, hepatocytes and myotubes (**Kim *et al.*, 2013)**. The authors observed that imidacloprid inhibited insulin-stimulated glucose uptake in these cell lines through the reduction of PKB phosphorylation. Their results suggested that imidacloprid potentially impairs downstream targets of AKT such as GLUT4 translocation in myotubes and adipocytes, as well as glycolysis and glycogen synthesis in hepatocytes (**Kim *et al.*, 2013)**.

I.3.2 Chlorpyrifos

Chlorpyrifos with systematic name O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphoro-thioate is a white crystal with a slightly skunky odour (rotten egg). It is the active ingredient of various

insecticides with names Dursban®, Lorsban®, L-Drint-20® which exist in the solid form or solution. This insecticide is utilized extensively in agriculture and for residential pest control throughout the world **(Saulsbury *et al.*, 2009).** Target pests of chlorpyrifos include mushroom flies, aphids, spider mites, caterpillars and white flies in greenhouse, outdoor fruit and vegetable crops **(Ambali, 2009).** Humans and other organisms are exposed to this substance by dermal, oral and inhalation mode. In rats, chlorpyrifos appears to be much toxic to females than males upon oral exposure with LD_{50} of 135mg/kg and 163mg/kg for female and male Wistar rats respectively **(McCollister *et al.*, 1974)**. Like other organophosphate insecticides, chlorpyrifos acts primarily by an irreversible inhibition of AChE enzyme leading to ACh accumulation and excessive stimulation of cholinergic receptors with consequences, paralysis and death of organism. More so, chlorpyrifos has been shown to affect other biochemical pathways completely different from those involving the AChE enzyme. Some pathways identified include markers of oxidative stress, lipid peroxidation and metabolic targets associated to Type 2 DM. Chlorpyrifos has been shown to cause oxidative stress in brain, red blood cells, liver, kidney and other organs of rats **(Ahmed and Zaki, 2009; Baş and Kalender, 2011).** In a similar manner, exposure to chlorpyrifos increased levels of lipid peroxides in the rat liver, kidney, brain, and erythrocytes (**Bagchi *et al.*, 1995; Gultekin *et al.*, 2001; Verma and Srivastava, 2001; Oncu *et al.*, 2002; Tuzmen *et al.*, 2008**). Notwithstanding, hyperglycemia has been observed to be one of the side effects of exposure to chlorpyrifos (**Ambali, 2009**; **Ambali *et al.*, 2011; Hamza *et al.*, 2014**). **Montgomery *et al.*, (2008)** in an epidemiological study found a direct relationship between consistent exposure to chlorpyrifos and incidence of diabetes among pesticide applicators**.**

I.3.3 Lambda-cyhalothrin

Lambda-cyhalothrin is a colourless to beige solid that has a mild odour, low water solubility and is non-volatile **(WHO, 1990).** It is the active ingredient in several brand name products like: warrior®, scimitar®, karate®, demand®, icon®, proactiva®, pacha®, lambdalm®, lambdacal®, matador® and parastar®. These products come in various forms including powders, pellets, liquids, small capsules, and ear tags containing the chemical. Lambda-cyhalothrin is widely used for pest control in agriculture, public health, homes and garden **(Amweg and Weston, 2005).** It is applied on cotton, cereals, various vegetables and fruits to control aphids and other pests. In the public health domain, it is used to control cockroaches, mosquitoes, ticks and flies, which are disease vectors. Initially, it was believed that pyrethroids including lambda-cyhalothrin had a strong toxic effect on insects in

very small doses that are relatively harmless to vertebrates and mammals **(Rundle and Forsyth, 1984).** However, subsequent studies have shown that this group of insecticides are quite toxic to mammals and humans **(Shakoori *et al.,* 1992; Wolansky *et al.,* 2006; Yousef, 2010)**, with the degree of toxicity depending on the dose, method and duration of exposure. The LD_{50} of lambda-cyhalothrin has been determined as 79 mg/kg for male rats **(Kidd and James, 1991).**

Lambda-cyhalothrin has been reported to affect the balance between the antioxidant system and ROS. Several studies have reported its ability to induce oxidative stress *in vivo* and *in vitro* **(El-Demerdash, 2007; Fetoui *et al.,* 2008; 2009; 2010; Abdallah *et al.,* 2012;)**. **Fetoui *et al.,* (2009; 2010)** showed that it significantly increased the level of MDA in the liver and kidney of male adult Wistar rats, whereas the activity of SOD and catalase decreased. In humans, lambda cyhalothrin induced oxidative stress in erythrocytes characterised by high SOD and catalase levels (**Deeba *et al.,* 2017).** Tukhtaev and collaborators (2012) also reported that long-term exposure to lambda-cyhalothrin led to the development of oxidative stress in pregnant female rats and their offspring with the degree in offspring decreasing at the cessation of ingestion of a pesticide or its toxic metabolites in breast milk **(Tukhtaev *et al.,* 2012). Elhalwagy *et al.,* (2015)** observed an increase in the plasma levels of AST, ALT, blood glucose, MDA, and a decrease in plasma insulin level in male rats chronically exposed to the substance. Moreover, the degree of oxidative stress was further enhanced when it was used in combination with other pesticides **(El-Demerdash, 2011; 2012).** Lambda-cyhalothrin in combination with the organophosphate, fenitrothion, significantly decreased the activity of GST, SOD and catalase in male Sprague–Dawley rat testes (**El-Demerdash *et al.,* 2013)**. In addition, a significant inhibition of AST, ALT and ALP activities and an increase in cellular lipid peroxidation levels were observed upon exposure to lambda-cyhalothrin (**El-Demerdash *et al.,* 2013)**.

I.3.4 Oxamyl

Oxamyl is a crystalline solid that has a slightly sulphurous odour. Its chemical name is N,N-dimethyl-α-methylcarbamoyloxyimino-α-(methylthio) acetamide, and its most common trade naames are Furaplant Super®, Vydate®, Thioxamyl® and DPX 1410®. It is a contact and systemic insecticide, acaricide and nematicide that is used to control chewing and sucking insects (but not wireworms), spider mites, and nematodes in ornamentals, fruit trees, vegetables, cucurbits, beets, bananas, pineapples, peanuts, cotton, soybeans, tobacco, potatoes and other crops. It is absorbed by

the foliage and roots and translocated to other parts of the plant. It is a non-persistent compound with a half-life in soil estimated at a few days to several weeks **(U.S. EPA, 2004).** The primary degradation products are an oximino derivative called methyl-N-hydroxy-N',N'-dimethyl-1-thiooxaminidate and its isomer, and N,N-dimethyloxamic acid. It is highly toxic with an oral lethal dose of oxamyl (90-95% pure) ranging from 2.5 to 5.4 mg/kg in rats depending on the sex and fasting condition **(Kennedy, 1986).** Symptoms of exposure include: soiled fur, lacrimation, salivation, change in pupillary response, slow righting reflex, abnormal gait, impaired locomotion, tremors, no response to tail pinch, splayed limbs, incoordination, laboured breathing, increased urination, decreased forelimb and hind limb grip strength and/or slightly increased hind limb foot splay. Like other carbamate insecticides, oxamyl has been shown to decrease AChE erythrocytes levels in plasma, brain and erythrocytes **(EFSA Scientific Report, 2005).** In addition to inhibiting acetylcholinesterase enzyme level, another effect of oxamyl exposure is modulation of antioxidant levels in honeybee larva **(Prezenska *et al.*, 2019)**

Devastating effects are also recorded in offspring which are exposed to pesticides during pregnancy (*in-utero* exposure) or in breast milk. The most common adverse birth outcome resulting from *in-utero* exposure to pesticide is foetal development characterised by gestational age, birth weight, birth length and head circumference **(Mayhoub *et al.*, 2014)**. Several studies have reported abnormalities in foetal development for both human and animal studies with *in-utero* exposure to pesticides. Chlorpyrifos was associated with decreased birth weight and birth length overall in a mixed population, lower birth weight among African Americans and reduced birth length in Dominicans **(Perera *et al.*, 2003)**. Prenatal exposure to pyrethroids was associated with increased birth weight, birth length, and gestational age, and with preterm birth in a Chinese population **(Xue *et al.*, 2013; Xu *et al.*, 2020).** Adverse foetal development is known to be a good predictor for neonatal morbidity and mortality **(McCormick, 1985; McIntire *et al.*, 1999).** These abnormalities in birth weight (low or high birth weight) are a risk factor for several cardiovascular and metabolic disease including Type 2 DM (**Strauss and Dietz, 1997; Yanney and Marlow, 2004)**.

Despite consistent findings existing on the association of pesticides with oxidative stress and metabolic abnormalities, it is difficult to compare these results due to differences in sample population, route of administration, dose administered and the absence of a positive control group in most studies. Most studies have been carried out *in vivo* and *invitro* in animal models with only few studies in humans. The dose in these chronic toxicity studies range between 1/100 LD-50

(Tukhtaev *et al.*, 2012) and 6/5 LD-50 **(Mehmood *et al.*, 2017).** Experimental studies in most animal models have been carried out with exposure to specific pesticides. This model can only be used to simulate accidental exposure in humans, which only represents a minority of exposure in humans. This is different from chronic exposure in humans which generally occur during pesticide application, food and water sources with inhalation of several pesticides belonging to different classes. These experimental studies rarely quantify the pesticides in biological fluids which is necessary to establish bioaccumulation and biotransformation processes as well as the ability to cross the placenta membrane for developmental studies.

CHAPTER II:

MATERIALS AND METHODS

II.1 MATERIALS

II.1.1 Equipment

Amongst others, the following equipments were used to carry out this study. A spectrophotometer, a centrifuge, a water bath with agitator, a homogeniser, an Enzyme-Linked Immunosorbent Assay (ELISA) plate reader, a micro centrifuge, a heating block, a weighing balance, sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) and western blotting assemblies, glassware, a thermometer, falcon tubes, stirrers and spatula.

II.1.2 Kits and reagents

The pesticides investigated were imidacloprid, chlorpyrifos, oxamyl and a mixture containing lambda cyhalothrin + imidacloprid. These pesticides were purchased from local vendors with common names L-Drint-20® (Chlorpyrifos 20% EC Technical: 21.50%w/w based on 94% w/w a.i), Confidor® (Imidacloprid SL 17.8 % w/w), Parastar® 40 EC (Imidacloprid 20 g/L + Lambda cyhalothrin 20 g/L) and Vydate® 10 G (oxamyl 10%). Parastar® and L-Drint-20® are formulations of pesticides that are commonly used in agriculture in Cameroon while Confidor® is used worldwide **(Pouokam *et al.*, 2017; Matthews *et al.*, 2003).** The reagents used in this study were purchased from local and international vendors and were all molecular grade. Some other reagents were prepared in the laboratory (Appendix 1). Amongst other consumables, kits were used for tittering total cholesterol, triglycerides, HDL-cholesterol, ALT, AST, insulin and antibodies (GLUT4, NFkβ and beta actin). Chemicals also used in the study are D–glucose, Sodium phosphate dibasic anhydrous, Sodium phosphate monobasic dihydrate, Potassium phosphate monobasic anhydrous, Sodium chloride, Sodium carbonate, Potassium sodium tartrate, Copper (II) sulphate pentahydrate, Bovine Serum Albumin (BSA), Fetal Bovine Serum (FBS), Folins' reagent, Tris base, Ethylene Diamine Tetra Acetate (EDTA), Pyrogallol, Hydrogen peroxide, Tri-chloro acetic acid, Thiobarbituric acid, Butylhydroxytoluene, Hydrochloric acid, Sodium pyruvate, 2,4-di nitro phenyl hydrazine (2,4-DNPH), Sodium hydroxide, L-aspartic acid, DL-alanine, 2-oxoglutarate, Sodium azide, reduced glutathione GSH, reduced Nicotinamide adenine dinucleotide phosphate (NADPH), Potassium hydroxide, Ethanol, 1-chloro-2,4-dinitrobenzene.

II.2 METHODS

This was an experimental study which was divided into three main parts, with a preliminary section carried out to determine the lethal dose (LD)-50.

II.2.1: Preliminary section (Determination of lethal dose-50)

The LD_{50} values for imidacloprid and oxamyl were known for female Wistar Rats but observed to vary across studies, while the LD_{50} value of lambda cyhalothrin was only known for male Wistar rats. It was therefore necessary to determine the LD_{50} values for the studied pesticides as no unique value could be attributed as LD_{50} for imidacloprid, chlorpyrifos, imidacloprid + lambda cyhalothrin and oxamyl.

II.2.1.1 Design and site

This was an *in vivo* prospective study with a follow-up period of 14 days. It was carried out at the Biochemistry Research Laboratory of Jamia Hamdard University in New Delhi-India.

II.2.1.2 Study population

The study population consisted of 40 Female nulliparous Wistar rats aged 10 - 12 weeks and weighing between 180 - 200g.

II.2.1.3 Biological materials

No biological material was used for this section

II.2.1.4 Methodology

The study was carried out according to the Organisation for Economic Co-operation and Development (OECD) guideline for chronic studies **(OECD, 2008**). The dose investigated was considered to induce toxicity or death (lethal dose 50). It was determined using the method described by **Chinedu *et al.*, (2013).** This method was chosen because of its accuracy, requiring few animals, simple and less costly when compared with the up-and-down method of the OEDC guideline. The principle of the method is based on the administration of increasing dose of the pesticide by oral gavage with each rat receiving a specific dose and once until mortality is recorded.

Forty-female nulliparous Wistar rats aged 10 - 12 weeks and weighing between 180 - 200 g were provided by the Animal House of Jamia Hamdard University after obtaining ethical clearance and acclimatized for a week. The first dose of each pesticide was chosen based on information from literature suggesting the median lethal dose and the level of toxicity. The animals were housed in a 12-hour light/dark cycle with free access to food and water. After 7 days acclimatization, 16 rats were selected, fasted overnight. From these 16, four (4) rats were administered Imidacloprid with each receiving 390, 400, 410 or 420 mg of imidacloprid /kg rat, 4 others were administered Chlorpyrifos with each receiving 100, 110, 120 or 130 mg chlorpyrifos /kg rat, 4 were given the formulation containing equal amounts of Lambda cyhalothrin + Imidacloprid with each receiving 30, 35, 40 or 45 mg/ kg rat) and a last group of 4 received Oxamyl (2, 2.5, 3.0 and 3.5 mg/kg rat). The rats were observed for one-hour post-administration and then for 10 minutes at 2-hour intervals over a period of 24 hours for behavioural signs of toxicity (changes in skin and fur, eyes and mucous membranes, respiratory and behaviour pattern, with more attention directed towards tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma) and 48 hours for mortality. When none of the signs were recorded after a period of 48 hours, twelve other rats were selected from the remaining 24 and fasted overnight. From these 12, three were administered imidacloprid each receiving either 430, 434 or 438 mg of imidacloprid /kg rat, 3 administered chlorpyrifos each receiving either 132, 134 or 136 mg of chlorpyrifos /kg rat, 3 administered the pesticide mixture with each receiving 50, 55 or 60 mg of lambda cyhalothrin + imidacloprid /kg rat and 3 administered oxamyl with each receiving 3.8, 4.2 or 4.5 mg of oxamyl/ kg rat. Death was recorded in the group exposed to chlorpyrifos, lambda cyhalothrin + imidacloprid and oxamyl. With no death recorded in the imidacloprid-exposed group, a much higher dose was given to three other rats in an increasing manner (442, 444 and 446 mg imidacloprid /kg rat). The minimal dose of each pesticide that resulted in the death of a rat was repeated for confirmation. The LD50 value of each pesticide was calculated using the formula:

$LD_{50} = \frac{MO+M1}{2}$ **Chinedu *et al.*, (2013)** where:

Mo = maximal dose of each pesticide without mortality recorded

M_1 = minimal dose of each pesticide with mortality recorded in one rat.

II.2.2: Part 1 (Female rat study)

II.2.2.1 Study design and site

The study was a prospective study with follow-up covering a period of 2 months (September 2016 to October 2016). Recruitment, follow-up, sample collection, processing and analysis were all performed at the Biochemistry Research Laboratory of Jamia Hamdard University. The analyses performed included total protein, antioxidant enzymes (AST, ALT, SOD, GPx, GR, catalase), reduced glutathione and lipid peroxidation marker (MDA).

II.2.2.2 Study population

The study population included 30-female nulliparous Wistar rats aged 12 - 14 weeks and weighing between 200 - 220 g.

II.2.2.3 Biological material

The biological material used in this part was whole blood collected in dry tubes, the kidney and liver of rats.

II.2.2.4 Methodology

This *in-vivo* experimental study was carried out according to the Organisation for Economic Co-operation and Development (OECD) guideline for chronic studies **(OECD, 2008**). Thirty-female Nulliparous Wistar rats aged 12 - 14 weeks and weighing between 200 - 220 g were provided by the Animal House of Jamia Hamdard University after obtaining ethical clearance. These rats were checked to ensure that they were fertile and having a regular cycle. The rats were acclimatised for 7 days with free access to food (standard normal diet) and water in a 12-hour light/dark cycle. After acclimatisation, the rats were fasted overnight for approximately 12 hours. The next day, the rats were weighed and FBG test was conducted using a glucometer (Accu-Chek active) and glucose strips with blood collected by tail prick. The rats were then divided into five equal groups matched by weight and FBG. Marks were made on the rats using picric acid. This was to identify and distinguish the different groups to which they belonged. They were all mated with male rats in cages with a 2:1 female to male ratio. The mating duration period was continuous and lasted for a maximum of 15 days. To know if an animal was pregnant, the vaginal swap smear and staining with

Methyl Blue were used to examine for the presence of sperms in the smear. A positive result signified gestation and pesticide administration was initiated on the pregnant Wistar rat. Throughout, Group 1 (Control) received distilled water while Groups 2 to 5 received Pesticide 1 (44 mg Imidacloprid per kg body weight/day), Pesticide 2 (13.5 mg Chlorpyrifos per kg body weight/day), Pesticide 3 (a mixture containing 5.6 mg Imidacloprid + 5.6 mg Lambda Cyhalothrin per kg body weight/day) and Pesticide 4 (0.4 mg Oxamyl per kg body weight/day) respectively with gastro-oesophageal probe. These doses, corresponding to 1/10 LD_{50}, were chosen as shown to induce biochemical alterations without causing mortality and considered to be lower than the doses to which humans are exposed during pesticide application and accidental poisoning (through water or food containing pesticides). The freshly prepared solutions were administered at a volume of 10 mL/kg throughout gestation while rats were maintained on normal diet with free access to water. Pesticide administration was discontinued after birth, and animals were kept on normal diet and followed for a month. At the end of follow-up, the rats were fasted overnight and sacrificed by decapitation the next day under anaesthesia. Blood samples were collected into dry tubes and serum prepared and stored at −20°C for alanine transaminase and aspartate transaminase quantification. The rats were dissected, and the liver, kidney, pancreas, brain and heart removed, washed in ice-cold normal saline solution and weighed. A small portion of the liver and one kidney were collected and refrigerated at –80 °C for antioxidant and MDA quantification.

II.2.3: Part 2 (offspring study)

II.2.3.1 Study design and site

The study was a prospective study with follow-up covering a period of 4 months (November 2016 to February 2017). Recruitment, follow-up, sample collection, processing and analysis were all performed at the Biochemistry Research Laboratory of Jamia Hamdard University. The analyses performed included total protein, antioxidant enzymes (AST, ALT, SOD, GPx, GR, catalase), reduced glutathione and lipid peroxidation marker (MDA).

II.2.3.2 Study population

The study population included offspring of pregnant Wistar rats aged 4 weeks and 12 weeks.

II.2.3.3 Biological material

The biological material used in this part was whole blood collected in dry tubes, the kidney and liver of rats.

II.2.3.4 Methodology

At birth, offspring were followed up by weekly recording of body weight and weaned at 4 weeks of age. At this point, eight (8) offspring each (4 males and 4 females) were randomly selected from each group and fasted overnight. The next day, they were decapitated under anaesthesia and blood samples were collected in dry tubes. Serum was prepared and stored at −20°C for alanine transaminase and aspartate transaminase quantification. The rats were dissected; a small portion of the liver and one kidney were removed, washed in ice-cold normal saline solution, and refrigerated at –80 °C for total protein, antioxidant and MDA quantification. The remaining offspring were followed up until 12 weeks and 8 randomly selected and sacrificed (4 males and 4 females per group) after an overnight fast. Similar samples were collected as in the offspring sacrificed at weaning. The study was approved by the Animal Ethics Committee of Jamia Hamdard University and the animals used in this study were treated humanely with regards for the alleviation of suffering.

II.2.4: Part 3 (Female rat and offspring study)

II.2.4.1 Study design and site

The study was a prospective study with follow-up covering a period of 4 months (November 2016 and February 2017). Recruitment, follow-up, sample collection, processing and analysis were all performed at the Biochemistry Research Laboratory of Jamia Hamdard University. The analyses performed included glucose quantification, lipid profile analyses (total cholesterol, HDL-C and triglyceride quantification), insulin ELISA, Western blotting analysis of NFkβ and GLUT4, and histopathological studies on the liver, kidney and pancreas.

II.2.4.2 Study population

The study population included 30 female Wistar rats, their offspring aged 4 weeks and 12 weeks.

II.2.4.3 Biological material

The biological material used in this part was whole blood collected in dry tubes, the kidney, liver, pancreas and muscle of rats.

II.2.4.4 Methodology

Samples were collected from the 30 female adult Wistar rats, 8 randomly selected offspring sacrificed at weaning and 8 randomly selected offspring sacrificed at adult age. Blood samples were collected in dry tubes and serum prepared and stored at -20^{0}C for glucose, lipid profile and insulin quantification. Muscles samples of the bicep femoris in parents and adult offspring were collected and stored at -80^{0}C for GLUT4 and NFkβ protein expression analysis. Section of liver, kidney and pancreas were collected in formalin for histological analysis

II.2.5 Experimental analyses

II.2.5.1 Preparation of tissue homogenates

Homogenates of liver and kidney were prepared as follows. Liver and kidney samples were weighed into Eppendorf tubes. Nine volumes of chilled Phosphate buffer (0.1M, pH 7.4) were added and the contents ground with a tissue homogeniser (Heidolph DIA X 900) on ice. The homogenates were centrifuged at 10,000 rpm for 15 minutes at 4ºC and the supernatants collected for total protein, antioxidant enzyme and MDA measurements.

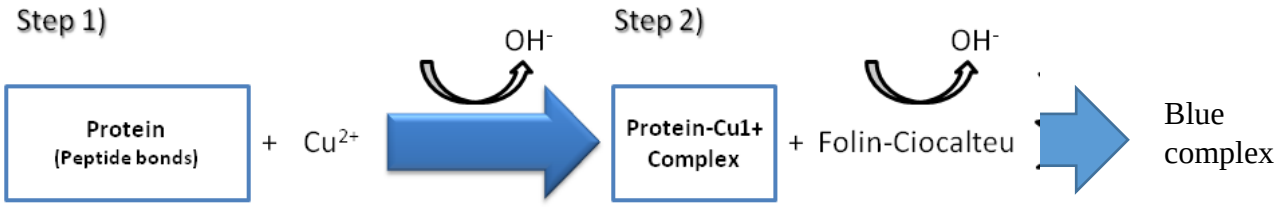
II.2.5.2 Total protein quantification

The quantification was carried out by a modified version of the method described by **Lowry *et al.*, (1951)**.

Principle

Under alkaline conditions, divalent copper ions form a complex with peptide nitrogen bonds of proteins and are reduced to monovalent copper ions. The monovalent copper ions and the radical groups of tyrosine, tryptophan, and cysteine reduce Folin Ciocalteau phosphomolybdic phosphotungstic acid to hetero-polymolybdenum blue. This coloured product absorbs light at a

wavelength of 660 nm with the intensity of the coloration being directly proportional to the amount of protein present.



BSA standard solution (1mg/mL) was serially diluted to give solutions of different concentrations (Figure 12) in labelled standard tubes.

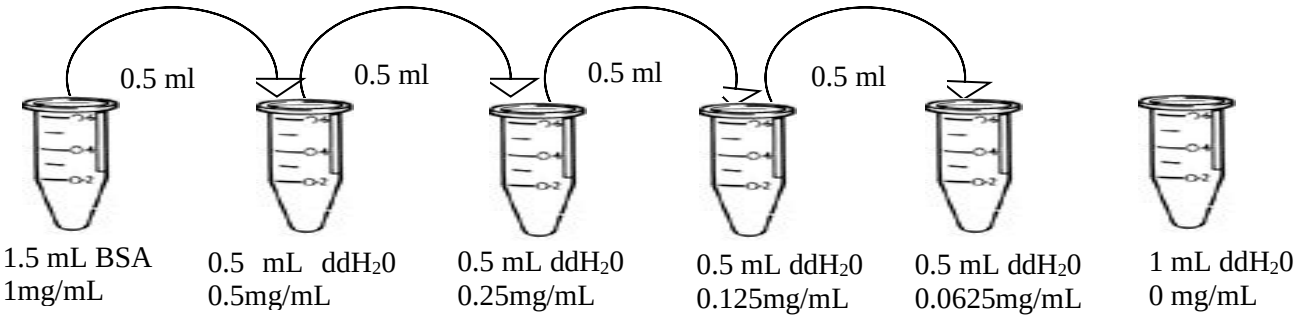


Figure 12: Bovine Serum Albumin dilutions

Sample tubes were labelled and 100 µL of each sample diluted to 1 mL with distilled water. In each of the tubes, starting with blank containing 1mL ddH_2O, standards prepared by dilutions and samples (0.9 mL ddH_2O + 0.1mL Sample), 4.5 mL of Lowry's solution were added and incubated for 10 minutes in the dark at room temperature and pressure (Table II). After the first incubation, 0.5 mL of 1N Folin's Reagent were added and further incubated for 30 minutes in the dark at rtp as described in Table II.

Table II: Total protein quantification procedure

Tubes	Blank	Standards	Sample
ddH_2O (mL)	1	-	0.9
Standard solution (mL)	-	1	-
Sample (mL)	-	-	0.1
Lowry's Solution (mL)	4.5	4.5	4.5
Incubation	10 minutes in the dark		
Folin's Reagent (mL)	0.5	0.5	0.5
Incubation	30 minutes in the dark		

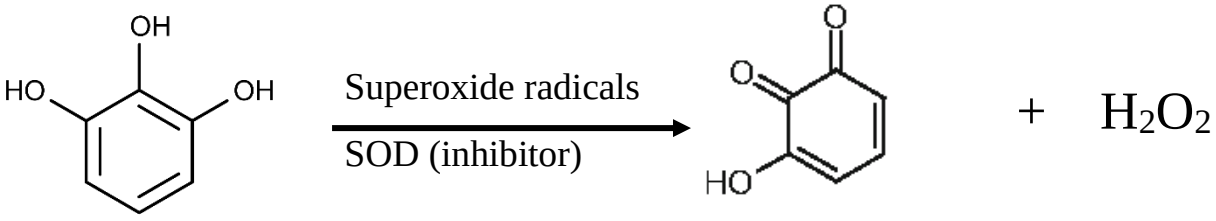
After the second incubation, the optical density (OD) was measured at 660 nm in a spectrophotometer against the blank tube. A standard curve of OD versus concentration of BSA was plotted and used to calculate the concentration of proteins in the samples by extrapolation (Appendix 2).

II.2.5.3 Superoxide Dismutase Quantification

This was carried out using a method modified from that of **Marklund and Marklund (1974).**

Principle

Pyrogallol is auto oxidised in an alkaline medium in the presence of superoxide radicals to produce hydrogen peroxide and pyrogallol-ortho-quinone which absorbs at a wavelength of 420 nm. This auto-oxidation is 50% in the presence of EDTA at pH 8.5. SOD competes with pyrogallol for superoxide radicals thereby inhibiting the auto-oxidation of pyrogallol and reducing the signal. Thus, a low signal at 420 nm correlates with high levels of SOD enzyme activity.



Pyrogallol Pyrogallol-ortho-quinone

Method

The spectrophotometer was set on the kinetic program to record every 30 seconds at a wavelength of 420 nm for 180 seconds. It was adjusted to read zero with the blank reagent containing Tris-EDTA buffer pH 8.5. For each sample, 0.1 mL was mixed with 2.8 mL of the buffer solution. Pyrogallol (0.1 mL) was added, mixed rapidly by inversion and the OD was measured using the above-mentioned conditions. A control tube was also prepared by replacing the sample with 0.1 mL of double distilled water.

Expression of results

A unit of SOD corresponds to the amount of SOD that will result in 50% inhibition of the oxidation of pyrogallol to pyrogallol-ortho-quinone for one minute. SOD activity was calculated as follows

$$\% \text{ Inhibition of pyrogallol autoxidation} = \frac{\Delta OD \text{ control} - \Delta OD \text{ test}}{\Delta OD \text{ control}} \times 100$$

$$\text{SOD Activity (U/mL)} = \frac{\% \text{ inhibition of pyrogallol autoxidation}}{50}$$

$$\text{SOD Activity (U/mg protein)} = \frac{\text{SOD activity } (\frac{U}{mL})}{\text{Protein concentration } (\frac{mg}{mL})}$$

Where ΔOD_{test} is the difference in absorbance of the test sample for 60 seconds

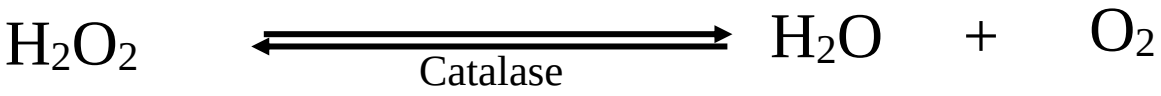
Where $\Delta OD_{control}$ is the difference in absorbance of the control sample for 60 seconds

II.2.5.4 Catalase quantification

This was carried out using a method slightly modified from that of **Aebi (1984).**

Principle

Hydrogen peroxide which absorbs light at a wavelength of 240 nm is a substrate of catalase. In the presence of catalase, hydrogen peroxide is converted into water and oxygen leading to a decrease in the absorbance over time. The decrease in absorbance in the presence of samples measured at 240 nm is proportional to the amount of catalase present in the sample.



Method

The spectrophotometer was set on the kinetic program to record every 30 seconds at a wavelength of 240 nm for 120 seconds. The spectrophotometer was adjusted to read zero with the blank reagent (Phosphate buffer). Each sample was diluted to 1mL with Phosphate buffer (20 µL sample:980 µL buffer) and transferred into the cuvette. One mL of phosphate buffer and 1 mL of hydrogen peroxide solutions were added to the diluted sample and rapidly mixed by inversion. The cuvette was placed inside the spectrophotometer and the absorbance recorded using the kinetic program immediately upon addition of hydrogen peroxide.

Expression of results

The activity of catalase was calculated using the formula:

$$\text{Activity of catalase (U/mg protein)} = \frac{(\Delta OD / \text{min}) \times Vt}{\varepsilon 240 \times d \times Vs \times Ct}$$

Where:

ΔOD/min is the difference between the absorbance at an interval of 1 min

Vt is the total volume of the reaction mixture in mL (3 mL)

ε240 is the milli molar extinction coefficient of hydrogen peroxide (0.0436 $mM^{-1}cm^{-1}$)

d is the path length of the cuvette (1 cm)

Vs is the volume of the sample used in mL (0.02 mL)

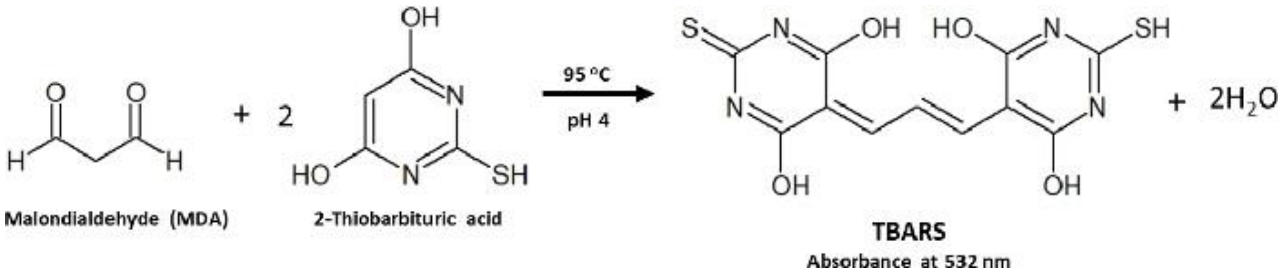
Ct is the total concentration of the protein in mg/mL

II.2.5.5 Malondialdehyde quantification

This was carried out by the measurement of Thiobarbituric Acid Reactive Substrate (TBARS) using a modified method of **Ohkawa *et al.*, (1979)**.

Principle

This assay is based on the reaction of the chromogenic reagent, 2-thiobarbituric acid (TBA) substrate with MDA at 25°C. One molecule of MDA reacts with 2 molecules of TBA to yield a violet chromophore which absorbs at a wavelength of 532 nm. The intensity of the coloration is directly proportional to the amount of MDA present.



Method

One hundred microliters of each sample were added into a Pyrex test tube, followed by 400 µL of TBA substrate reagent mixture, and the tubes were closed tightly. The mixture was heated at 100ºC for 15 minutes. The cork was removed, and the tubes were placed in an ice bath for cooling. The mixture was then centrifuged at 1500 g for 5 minutes. The OD of the supernatant was read at 532 nm. The concentration of MDA was calculated using its molar extinction coefficient ($\varepsilon = 1{,}53 \times 10^5$ M^{-1} cm^{-1}) as follows:

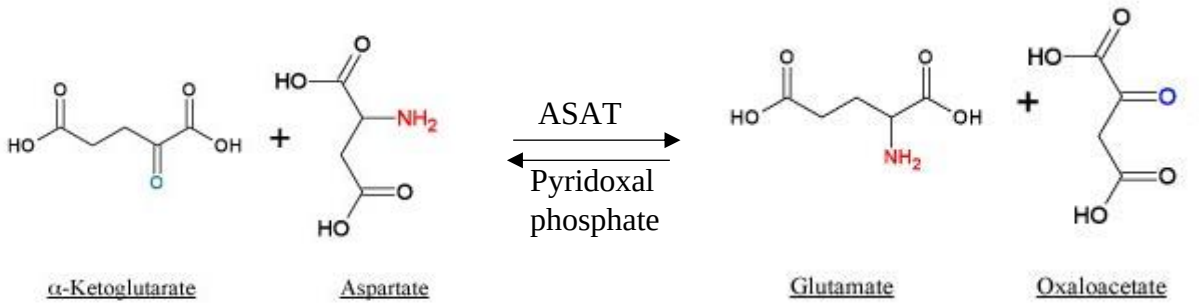
$$\text{Concentration of MDA } (\mu M) = \frac{OD}{\varepsilon} \text{ x } 1{,}000{,}000 \times \text{dilution factor}$$

II.2.5.6 Aspartate transaminase quantification

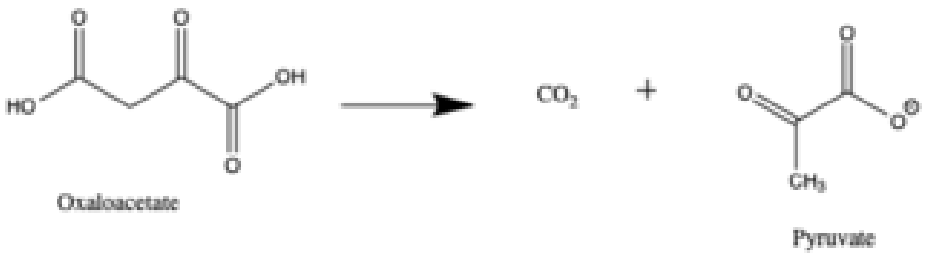
This was carried out using the method described by **Reitman and Frankel (1957)** with slight modifications**.**

Principle

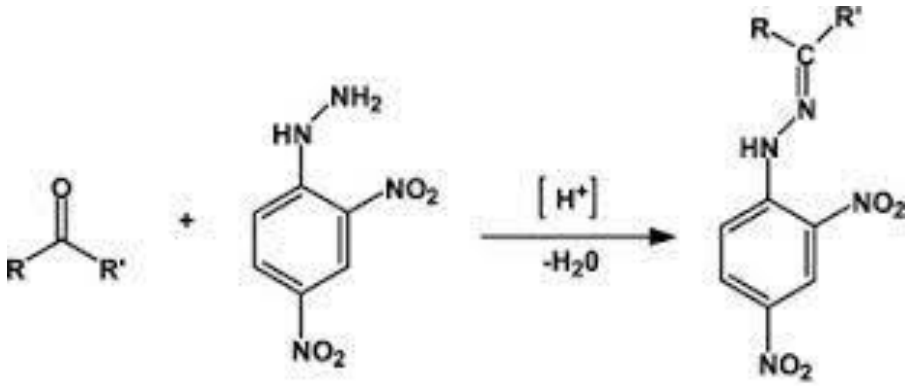
AST catalyses the reversible transamination of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate. Oxaloacetate spontaneously decarboxylates to form pyruvate under strong acidic conditions. Pyruvate reacts with 2,4-dinitrophenylhydrazine in an alkaline medium to form pyruvate hydrazone which absorbs light at a wavelength of 510 nm with the rate of change in absorbance being directly proportional to the AST activity in the sample.



α-Ketoglutarate Aspartate Glutamate Oxaloacetate



Oxaloacetate Pyruvate



Pyruvate 2,4-DNPH Pyruvate hydrazone

Method

One tube was labelled as blank and the others as samples. One hundred µL of the AST substrate solution were introduced into each tube. They were pre-incubated at 40 °C for 10 minutes in a water bath. Twenty microliters of samples were added into each sample tube while 20 µL of physiological saline were added into the blank tube. The tubes were gently mixed using a homogeniser (Vortex) and incubated for 60 minutes at 40°C. When removed from the water bath, 100 µL of the colouring reagent (2,4 DNPH) were added into each tube. The tubes were further incubated at room temperature for 20 minutes, and the reaction stopped by adding 1000 µL of 0.4 N NaOH. Their ODs were read against that of the blank tube in a spectrophotometer at a wavelength of 510 nm.

Calibration curve

The solutions were pipetted into the individual tubes as shown in Table III. The standard solution was prepared at a concentration of 1100mg/dL

Table III: Standard for ALT/AST quantification

Tube number	Blank	Standard 1	Standard 2	Standard 3	Standard 4
Physiological saline (mL)	0.10	0.10	0.10	0.10	0.10
AST or ALT Substrate (mL)	0.50	0.45	0.40	0.35	0.30
Standard solution (mL)	-	0.05	0.10	0.15	0.20
2,4-DNPH (mL)	0.50	0.50	0.50	0.50	0.50

Legend: AST = Aspartate Transaminase, ALT = Alanine Transaminase, DNPH = Di-Nitro Phenyl Hydrazine

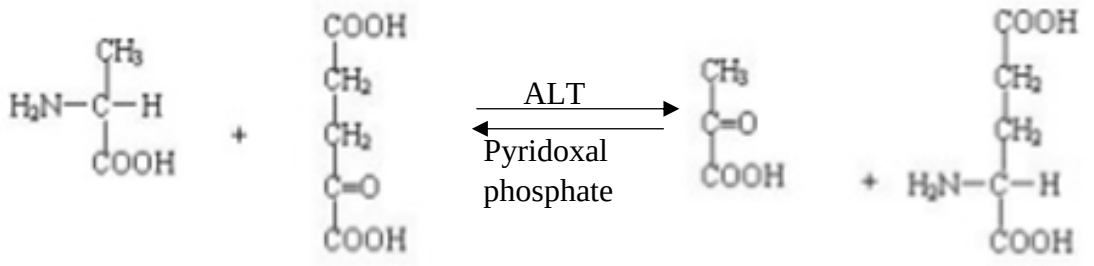
Each tube was mixed and incubated for 20 minutes at rtp. Into each of the tubes, 5 mL of 0.4N NaOH solution were added and incubated for 10 minutes at rtp. The ODs were then read at 510 nm, calibrating with the blank tube. A graph of OD versus the concentration of pyruvate was plotted (Appendix 2) and the concentration of AST in each sample extrapolated.

II.2.5.7 Alanine transaminase quantification

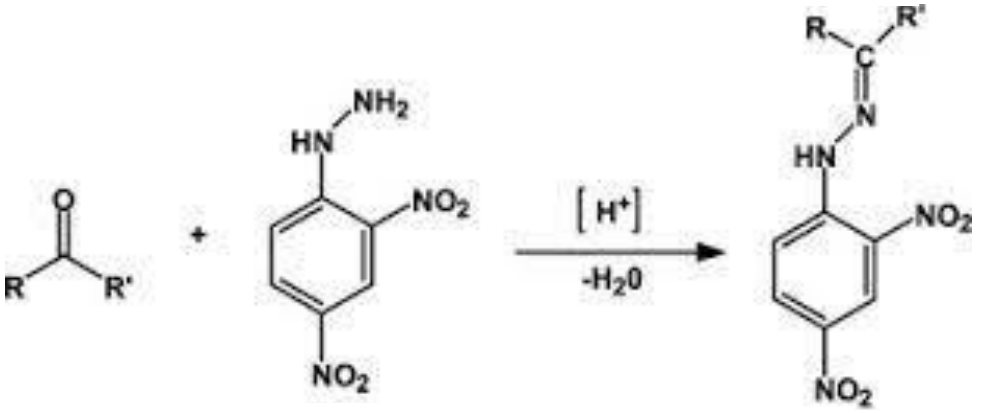
This was carried out using the method described by **Reitman and Frankel (1957**) with slight modifications.

Principle

ALT catalyses the transfer of an amino group from L-alanine to 2-oxoglutarate to form pyruvate and L-glutamate. Pyruvate reacts with 2,4-dinitrophenylhydrazine in an alkaline medium to form pyruvate hydrazone which absorbs light at a wavelength of 510 nm with the rate of change in absorbance being directly proportional to the ALT activity in the sample.



L-alanine 2-oxoglutarate Pyruvate L-glutamate



Pyruvate 2,4-Dinitrophenyl hydrazine Pyruvate hydrazone

Method

One tube was labelled as blank and the others as samples. One hundred µL of the ALT substrate solution were introduced into each tube. The tubes were pre-incubated at 40 °C for 10 minutes in a water bath. Twenty microliters of samples were added into each sample tube while 20 µL of physiological saline were added into the blank tube. Each tube was gently mixed using a homogeniser (Vortex) and incubated for 30 minutes at 40°C. On removal from the water bath, 100 µL of the colouring reagent (2,4 DNPH) were added, incubated at room temperature for 20 minutes

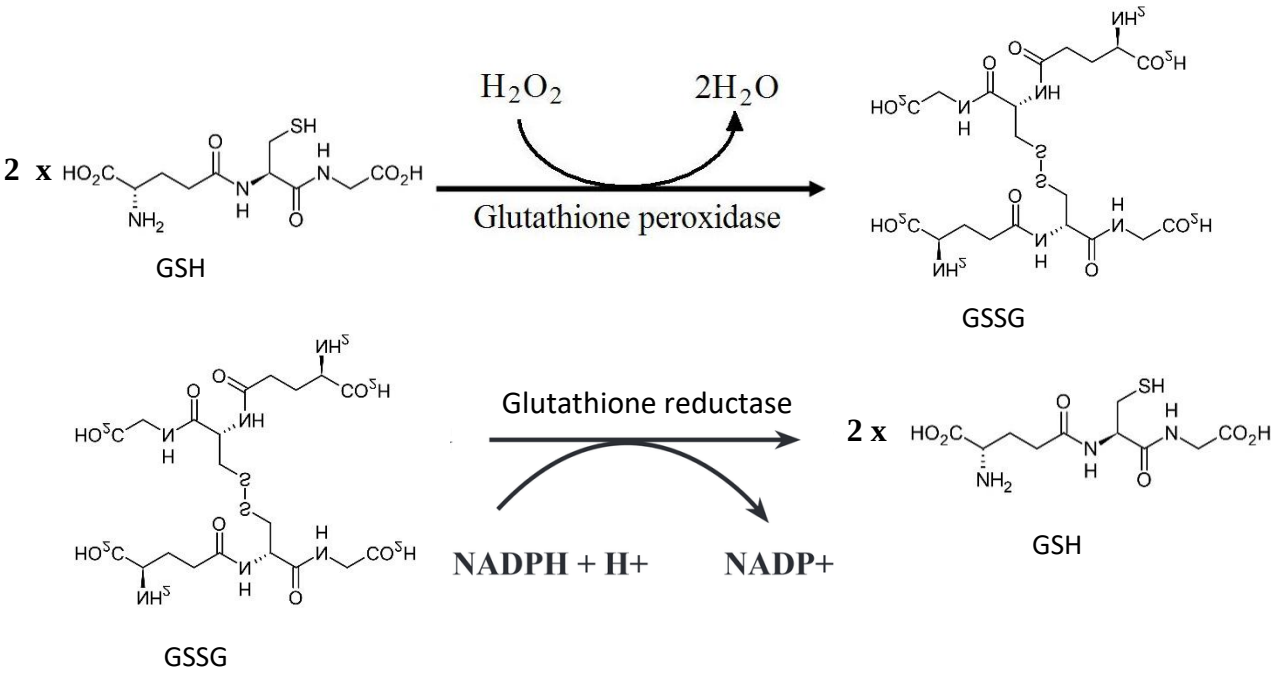
and the reaction stopped by adding 1000 µL of 0.4 N NaOH. Their ODs were read against that of the blank tube in a spectrophotometer at a wavelength of 510 nm. The concentration of ALT in each sample was determined by extrapolation from the standard curve (Appendix 2).

II.2.5.8 Glutathione peroxidase quantification

This was carried out using the method described by **Wendel (1980).**

Principle

Glutathione peroxidase catalyses the oxidation of reduced glutathione (GSH) in the presence of hydrogen peroxide to oxidised glutathione (GSSG) with the concomitant formation of water. GSSG formed oxidises NADPH to NADP and is in turn reduced back to GSH. The decrease in the absorbance at 340 nm characteristic of the disappearance of NADPH is directly proportional to the amount of enzyme present in the sample.



Method

The spectrophotometer was set on the kinetic program to record every 30 seconds at a wavelength of 340 nm for 180 seconds. The blank was read with the buffer solution (50 mM sodium phosphate monobasic anhydrous + 0.4 mM EDTA, pH 7.0). Three mL of the reaction mixture (7.0 mg of sodium azide, 33. 2 mg GSH, and 1.08 mg NADPH Reduced in 100 mL buffer) were placed in the

cuvette and 50 µL of hydrogen peroxide (0.042%) added. Fifty uL of sample were then added, mixed by inversion and the OD read. The activity of the enzyme was calculated as follows:

$$\text{Enzyme activity (units/mL)} = \frac{(\Delta \text{OD}/\text{min}) \times \text{Vt} \times 2}{6.22 \times \text{Vs}}$$

$$\text{Enzyme activity (units/mg protein)} = \frac{\text{Enzyme activity } (\frac{\text{units}}{\text{mL}})}{\text{protein concentration } \left(\frac{\text{mg}}{\text{mL}}\right)}$$

Where

ΔOD/min is the difference between the absorbance within a period of 1 min

2 = 2 µmoles of GSH produced per µmole of ß-NADPH oxidized

Vt = Total volume (in millilitres) of assay

Vs = Volume of sample (in millilitres) used

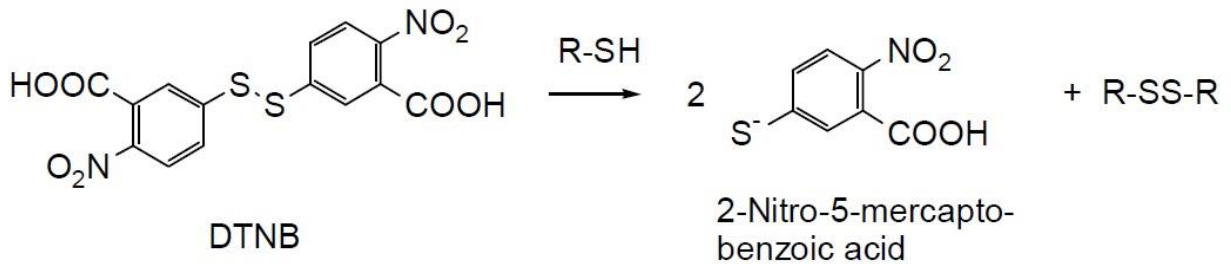
6.22 = Micro molar extinction coefficient of ß-NADPH at 340 nm

II.2.5.9 Reduced glutathione quantification

The quantification of GSH was carried out by the method of **Moron *et al.*, (1979)** with slight modifications.

Principle

This method is based on the reaction between reduced glutathione and 5-5' dithiobis-2- nitro benzoic acid (DTNB) to give a coloured compound that absorbs light at 412 nm with the intensity of the coloration being directly proportional to the amount of GSH present.



DTNB

2-Nitro-5-mercapto-benzoic acid

Method

Dilutions of reduced glutathione (GSH) were prepared from the stock GSH solution (200 µg/mL) to serve as standard (Table IV).

Table IV: Dilutions of stock for GSH quantification

Tube	**Blank**	**STD 1**	**STD 2**	**STD 3**	**STD 4**	**STD 5**
GSH stock (mL)	0.0	0.2	0.4	0.6	0.8	1.0
ddH_20 (mL)	1.0	0.8	0.6	0.4	0.2	0.0

Legend: GSH = Reduced glutathione, ddH_20 = double distilled water, STD = Standard

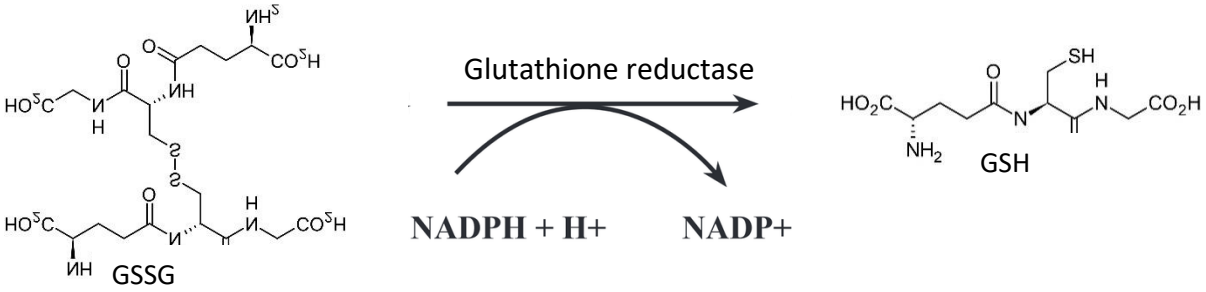
Tubes were labelled with the corresponding sample identity and 200 µL of each sample was diluted to 1000 µL with phosphate buffer. Into each of the tubes (blank, standard and sample), 1000 µL of phosphate buffer was added followed by 1000 µL of DTNB. Each of the tube was incubated and the OD read between 2 and 10 minutes at 412 nm in a spectrophotometer. A graph of OD = f (concentration) of standard was used to determine the concentration of GSH in samples by extrapolation (Appendix 2).

II.2.5.10 Glutathione reductase quantification

This assay was performed with a method modified from that of **Mavis and Stellwagen, (1968).**

Principle

In the presence of glutathione reductase, GSSG and β-NADPH are reduced and oxidized respectively to GSH and β-NADP as shown in the equation below. A decrease in the OD measured at 340 nm, characteristic of the disappearance of β-NADPH, is directly proportional to the amount of glutathione reductase present in the sample.



Method

The spectrophotometer was set on the kinetic program to record every 30 seconds at a wavelength of 340 nm for 180 seconds. The buffer solution (100 mM Potassium dihydrogen phosphate monobasic anhydrous + 3.4 mM EDTA, pH 7.6) was used to auto zero the spectrophotometer. In a cuvette, 2.9 mL of the reaction mixture (0.65 mL distilled water, 1.5mL buffer, 0.1 mL GSSG, 0.35 mL ß-NADPH and 0.3 mL BSA) were added and mixed. Then 0.1 mL of the sample was added, rapidly mixed by inversion and the OD measured. The activity of the enzyme was calculated as follows.

$$\text{Enzyme activity (units/mL)} = \frac{(\Delta \text{OD}/\text{min}) \times \text{Vt} \times \text{df}}{\varepsilon 240 \times \text{Vs}}$$

$$\text{Enzyme activity (units/mg protein)} = \frac{\text{Enzyme activity } (\frac{\text{units}}{\text{mL}})}{\text{protein concentration } \left(\frac{\text{mg}}{\text{mL}}\right)}$$

Where:

ΔOD / min is the difference between the absorbance at an interval of 1 min

Vt = Total volume (in millilitres) of assay

df = Dilution Factor Vs = volume of sample (in millilitres) used.

ε240 = Millimolar extinction coefficient of glutathione -2,4 – dinitrobenzene (6.22) at 340 nm

II.2.5.11 Glucose Quantification

The concentration of glucose was measured in the serum using the glucose oxidase colorimetric method according to the manufacturer's protocol (ab65333 – Glucose Assay kit).

Principle

The enzyme hexokinase catalyses the reaction between glucose and adenosine triphosphate (ATP) to form glucose-6-phosphate (G-6-P) and adenosine diphosphate (ADP). In the presence of nicotinamide adenine dinucleotide (NAD), G-6-P is oxidized by the enzyme glucose-6-phosphate dehydrogenase to 6-phosphogluconate and reduced nicotinamide adenine dinucleotide (NADH) which absorbs light at a wavelength of 510nm. The increase in NADH concentration is directly proportional to the glucose concentration.

Experimental procedure

The wells of the ELISA plate were labelled as sample and standard. Fifty micro litters of the reagent mixture were pipetted into all the wells. 50uL of glucose standard (1nmol/uL) and 50uL of samples were pipetted into their respective wells. The ELISA plate was incubated at rtp for 30 minutes in the dark and the OD read at 570nM.

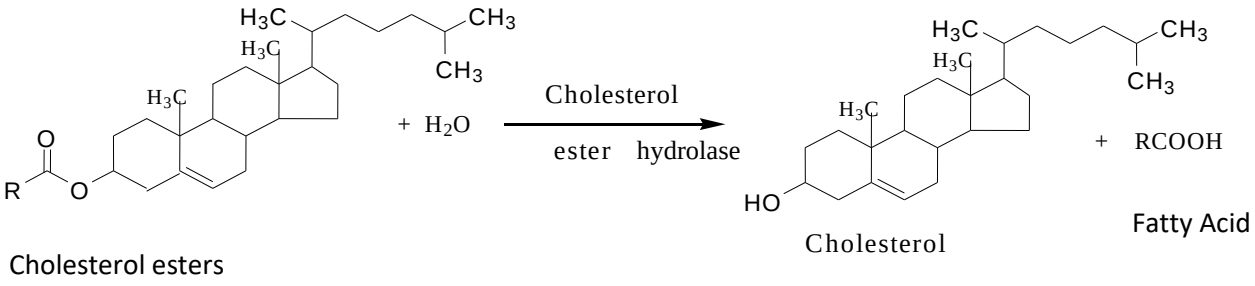
$$\text{Glucose (mg/dL)} = \frac{\text{OD sample}}{\text{OD standard}} \text{ x concentration of standard (mg/dL)}$$

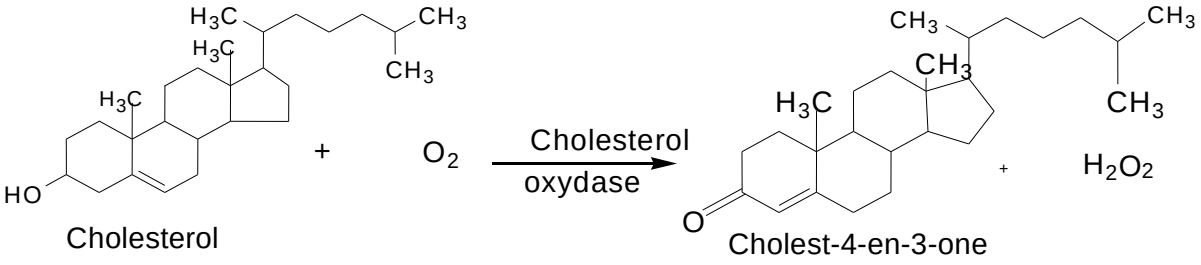
II.2.5.12 Total cholesterol quantification

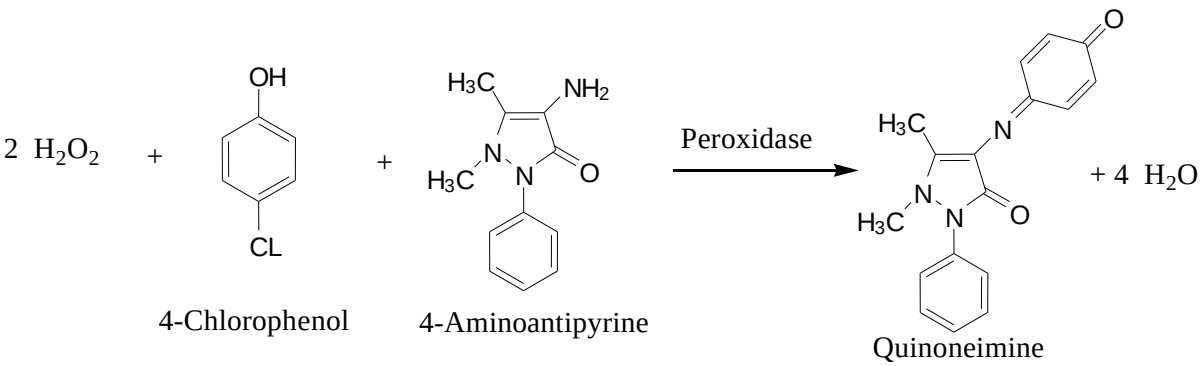
The concentration of total Cholesterol in the serum was measured using the Cholesterol Kit CHRONO LAB, as previously described by **Allain *et al.*, (1974)**.

Principle

Cholesterol esters are hydrolysed to cholesterol by Cholesterol ester hydrolase. The cholesterol produced is then oxidized by Cholesterol oxidase to cholest-4-en-3-one with the production of hydrogen peroxide, which reacts with 4-aminophenazone and a phenol in the presence of peroxidase to yield a quinone-imine dye that absorbs light at a maximum wavelength of 505 nm. The intensity of the coloration of this quinone-imine is directly proportional to the concentration of Cholesterol in the sample.







Experimental procedure

A set of three tubes was labelled as blank, standard and sample. One millilitre of the Cholesterol Kit CHRONOLAB reagent was added into each of the tubes. Ten µL of double- distilled water, 10 µL

of standard Cholesterol solution (200 mg/dL), and 10 µL of sample were added into the blank, standard and sample tubes respectively (Table V).

Table V: Total cholesterol quantification procedure

Tube label	Blank	Standard	Sample
Cholesterol Reagent Kit	1 mL	1 mL	1 mL
Double distilled water (ddH$_2$0)	10 µL	-	-
Standard solution (200 mg/dL)	-	10 µL	-
Sample solution	-	-	10 µL

All the tubes were incubated at room temperature for 30 minutes and their ODs read against that of the blank tube in a spectrophotometer at a wavelength of 505 nm. The concentration of total Cholesterol in each sample was calculated using the following formula.

$$\text{TC (mg/dL)} = \frac{\text{OD sample}}{\text{OD standard}} \text{ x concentration of standard (mg/dL)}$$

II.2.5.13 HDL-Cholesterol quantification

The concentration of HDL-Cholesterol in the serum was measured using an enzymatic colorimetric method modified from that described by **Jabbar *et al.* (2006)**.

Principle

All lipoproteins containing Apolipoprotein B (VLDL, LDL, Chylomicrons) are precipitated in the serum with phosphotungstate in the presence of magnesium ions. After incubation and centrifugation, the concentration of HDL-Cholesterol is then measured in the clear supernatant using the same principle as for total Cholesterol.

Experimental Procedure

One hundred micro-litres of the precipitant solution were added into tubes each containing 100 µL of the sample solutions. After mixing well, the tubes were centrifuged at 4000 rpm for 20 minutes. Ten µL of the resulting supernatant were used as sample for the quantification of HDL-Cholesterol as follows: A set of three tubes were labelled as blank, standard, and sample. One millilitre of the Cholesterol Kit CHRONO LAB reagent was added into each of them. Then 10 µL of double-distilled

water, 10 µL of standard HDL-Cholesterol solution (50 mg/dL), and 10 µL of the sample were added into the blank, standard and sample tubes respectively (Table VI).

Table VI: HDL-Cholesterol quantification procedure

Tube label	Blank	Standard	Sample
Cholesterol Reagent Kit	1 mL	1 mL	1 mL
Doubled distilled water (ddH_20)	10 µL	-	-
Standard solution (50 mg/dL)	-	10 µL	-
Sample solution	-	-	10 µL

All the tubes were incubated at room temperature for 30 minutes and their ODs read against that of the blank tube in the spectrophotometer at a wavelength of 505 nm. The concentration of HDL-Cholesterol in each sample was calculated using the following formula.

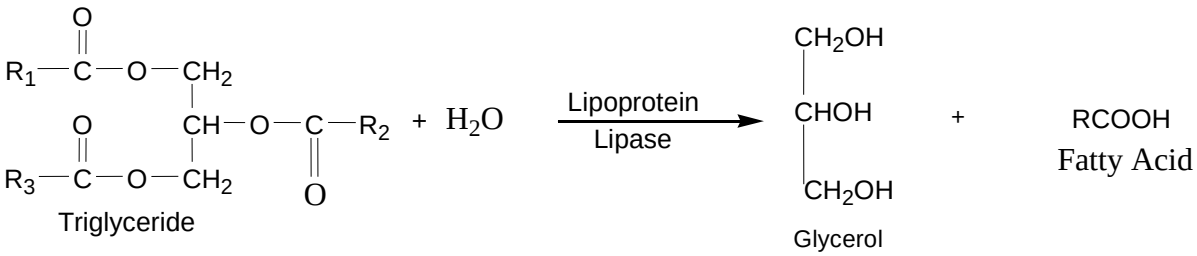
$$\text{HDL-C (mg/dL)} = \frac{\text{OD sample}}{\text{OD standard}} \text{ x concentration of standard (mg/dL)}$$

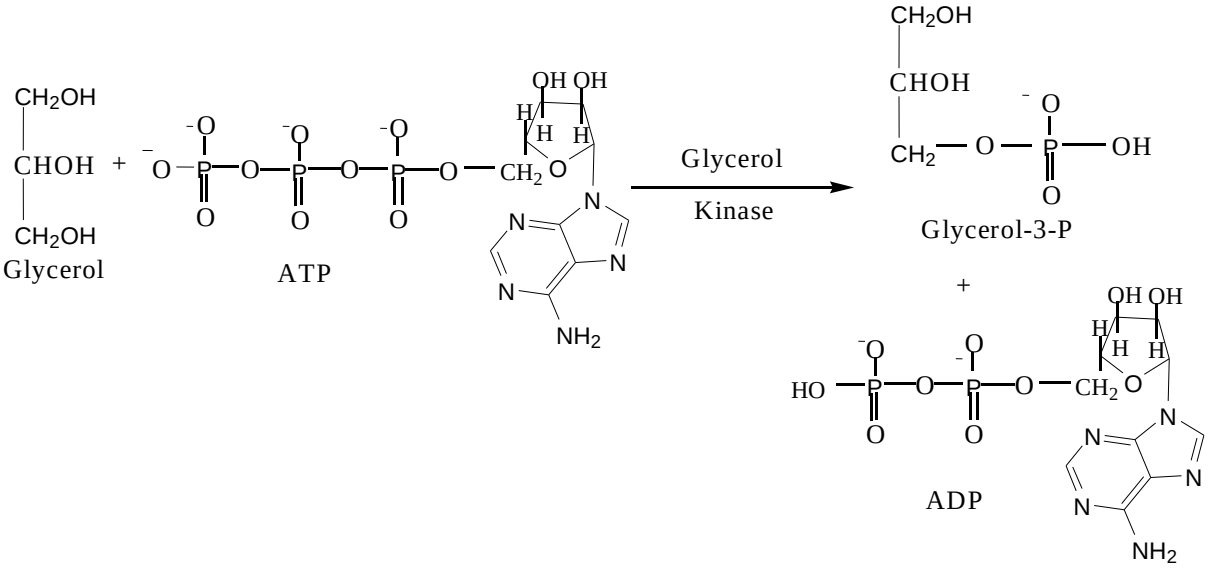
II.2.5.14 Triglyceride quantification

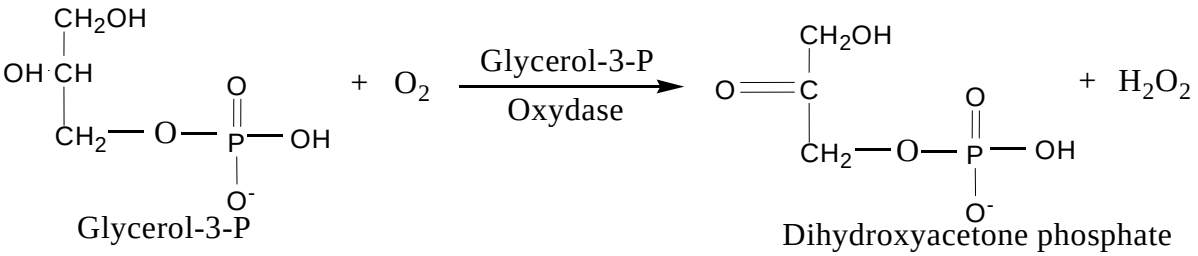
The concentration of TG in the serum was measured using the Triglyceride Kit CHRONO LAB as previously described by **McGowan *et al.*** (**1983)**.

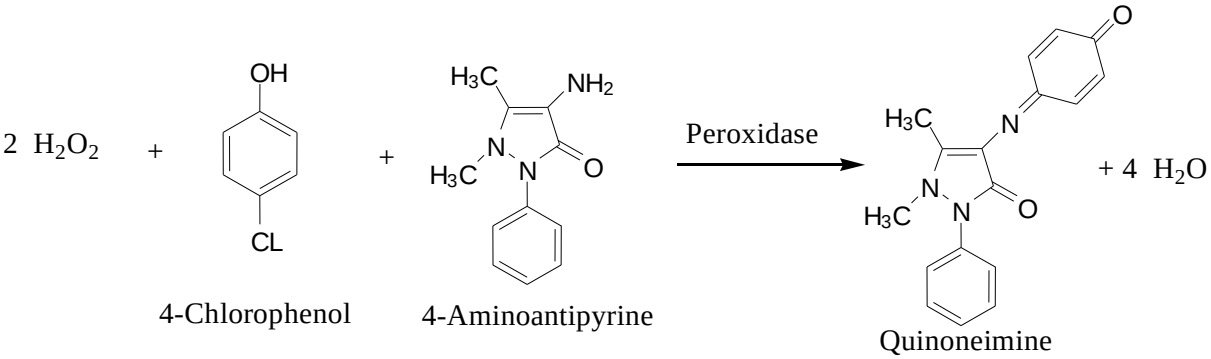
Principle

Triglycerides are hydrolysed by Lipoprotein Lipase into glycerol and FFA. The glycerol is then phosphorylated by Glycerol Kinase in the presence of ATP and Mg^{2+} into Glycerol-3-phosphate which is further oxidised by Glycerol Phosphate oxidase to yield Dihydroxyacetone Phosphate and hydrogen peroxide. A peroxidase finally catalyses the combination of the hydrogen peroxide to 4-aminophenazone in the presence of p-chloro-phenol to yield a Quinone that absorbs light at a maximum wavelength of 505 nm. The intensity of the coloration of this quinone is directly proportional to the concentration of TG in the sample.


Triglyceride
Lipoprotein
Lipase
Glycerol
RCOOH
Fatty Acid


Glycerol
ATP
Glycerol
Kinase
Glycerol-3-P
ADP


Glycerol-3-P
Glycerol-3-P
Oxydase
Dihydroxyacetone phosphate


4-Chlorophenol
4-Aminoantipyrine
Peroxidase
Quinoneimine

Experimental procedure

A set of three tubes was labelled as blank, standard, and sample. One millilitre of the TG Kit CHRONO LAB reagent was added into each of them. Then 10 µL of double-distilled water, 10 µL of standard TG solution (200 mg/dL), and 10 µL of sample were added respectively into the tubes labelled blank, standard and sample (Table VII).

Table VII: Triglyceride quantification procedure.

Tube label	Blank	Standard	Sample
Reagent KIT	1 mL	1 mL	1 mL
Doubled distilled water (ddH$_2$0)	10 µL	-	-
Standard solution (200 mg/dL)	-	10 µL	-
Sample solution	-	-	10 µL

All the tubes were incubated at room temperature for 30 minutes and their ODs read against that of the blank in a spectrophotometer at a wavelength of 505 nm. The concentration of TG in each sample was calculated using the following formula.

$$\text{TG (mg/dL)} = \frac{\text{OD test}}{\text{OD standard}} \text{ x concentration of standard (mg/dL)}$$

Calculations

The concentration of LDL-Cholesterol was calculated by the equation of **Friedewald *et al.*, 1972**. LDL cholesterol cannot be calculated when TG exceeds 400mg/dL.

$$\text{LDL-Cholesterol (mg/dL)} = (\text{TC (mg/dL)} - \text{HDL-Cholesterol (mg/dL)}) - \frac{\text{TG (mg/dL)}}{5}$$

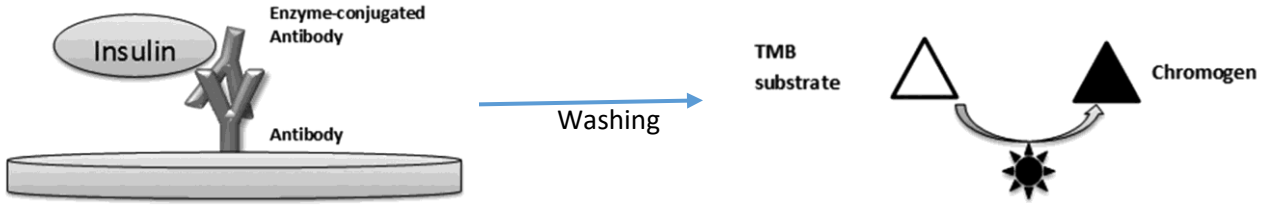
Atherogenicity index (AI) was calculated using the formula $AI = \text{Log}_{10} \frac{\text{Triglycerides } (\frac{mg}{dL})}{\text{high density lioprotein cholesterol } (\frac{mg}{dL})}$ (**Niroumand *et al.*, 2014**).

II.2.5.15 Insulin quantification in plasma

Insulin quantification was performed using a sandwich ELISA method according to the manufacturer's protocol (ALPCO Rat Insulin ELISA kit).

Principle

Insulin binds specifically to a monoclonal antibody coated on an ELISA plate. When added, insulin binds to a conjugate antibody fixed on the ELISA plate. After washing to remove the unbound antibody, a substrate is added which reacts with the enzyme to give a coloured product that absorbs light at 450 nm. The intensity of the coloration is directly proportional to the concentration of insulin present in the sample.



Experimental procedure

All reagents were equilibrated to room temperature and solutions diluted to 1X. The microplate was labelled in triplicates for standard, control and test samples and incubated at room temperature for 15 minutes. Ten µL each of the standard, control and sample in triplicates were pipetted into their respective pre-labelled wells and 75 µL of the working strength conjugate was added. The microplate was then covered with a plate sealer and incubated at room temperature for 2 hours while shaking at 800 rpm on a microplate shaker. The content of each well was then decanted, and the microplate washed 6 times with 350 µL of wash buffer per well using a microplate washer. During washing and after the final wash, the plate was inverted and tapped on an absorbent paper towel to remove residual wash buffer and bubbles. One hundred µL of TMB substrate were then added into each well. The microplate was covered with a plate sealer and incubated for 15 minutes at room temperature while shaking at 800 rpm on a microplate shaker. This was followed by the addition of 10 µL of the stop solution into each well and the plate was shaken gently to mix the contents. The microplate was then placed on a reader and the optical density read at 450 nm. A graph of concentration of standard = f (OD) standard was plotted and used to determine the concentration of insulin in each sample by extrapolation (Appendix 2).

Calculations

Insulin resistance was determined by the homeostasis model assessment (HOMA-IR) using the formula: HOMA-IR = $\frac{\text{Fasting Glucose(mg/dL)}\times\text{Fasting Insulin(mU/L)}}{405}$ (**Matthews *et al.*, 1985**).

Beta cell function was determined by HOMA-β using the formula: HOMA-β = $\frac{360\times\text{Fasting Insulin }(\frac{\text{mU}}{\text{L}})}{\text{Fasting Glucose }\left(\frac{\text{mg}}{\text{dL}}\right)-63}$ (**Matthews *et al.*, 1985**).

II.2.5.16 Glucose Transporter 4 and Nuclear Factor Kappa Beta quantification

The Western Blotting method was employed for the quantification of GLUT4 and NFkβ proteins in nuclear fractions. Protein extracts were prepared from muscle samples, separated by SDS PAGE, transferred onto a nitrocellulose membrane, incubated with primary and secondary antibodies and developed with Luminol. Four samples were randomly selected from the Female Wistar Rats and 6 samples from the adult offspring of group 1 (control), group 2 (Imidacloprid) and group 3 (Chlorpyrifos) for western blotting analysis (**Machado *et al*., 1993**).

Extraction of proteins

Experimental procedure

Protein extraction buffer A (sucrose, HEPES, magnesium chloride, potassium chloride, glycerol, EDTA, DTT and PMSF) and Protein extraction buffer B (HEPES, magnesium chloride, sodium chloride, glycerol, EDTA, DTT and PMSF) were prepared (Appendix 1) and proteinase inhibitor added into each in the ratio 1:99. In 100 mg muscle sample was added 400 uL of buffer A. The muscle was chopped and homogenise on ice. The mixture was allowed to stand for 15 minutes and 2.5 uL of Nonidet P-40 added. The mixture was then centrifuged at 2000 g for 15 minutes at 4 ºC and the supernatant collected as cytosolic fraction. The pellet was re-suspended in 100 uL of cold buffer B and incubated for 30 minutes on ice. The mixture was then centrifuged at 20,000 g for 15 minutes at 4 ºC and the supernatant collected as nuclear protein. Protein concentration of each fraction was determined by Lowry's method as described previously.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Principle

This is an analytical method used to separate components of a protein mixture based on their size and molecular weight. In an electric field, the proteins denatured and rendered negatively charged by SDS will migrate towards the positively charged electrode (anode). They will separate by a molecular sieving effect based on size with their speed of migration being inversely proportional to their molecular weight.

Experimental procedure

This was carried out as described by Machado and collaborators in 1993 with slight modifications **(Machado *et al*., 1993).** All reagents were assembled and the required solutions (polyacrylamide, 10 % Separating Gel, 5 % Stacking Gel, Electrophoresis Buffer, Sample Loading Buffer, Stain and Destain solution) prepared (Appendix 1). The setup was assembled, and the Separating Gel poured into the cassette. The mixture was allowed to solidify for approximately 20 minutes. The combs were fitted, the Stacking Gel added and allowed to set for approximately 1 hour. Alongside this, 10 µL of 5 × Loading Buffer were mixed with 40 µL of each sample (nuclear protein extract). The mixture was heated at 100ºC in a boiling water bath for 7 to 10 minutes and placed on ice to cool down. After solidification of the gel, the Electrophoresis Buffer was added, and the comb was removed. Twenty µL of samples were loaded into each well, along with 4 µL of Molecular Weight Marker (middle lane). The cassette was placed in the gel tank and filled with the Electrophoresis Buffer. The power was turned on and the gel was run first at 95 volts for 30 minutes and then at 120 volts for 2 hours. After the separation, the power was turned off, the gel removed from the cassette and the presence of proteins verified by Coomassie Blue staining.

Coomassie blue staining

The gel was placed in the staining dish containing the Fixing Solution (50 % methanol and 10 % glacial acetic acid) and fixed for 1 hour with gentle agitation. The Fixing Solution was removed and the Staining Solution (0.1 % Coomassie Brilliant Blue R–250, 50 % methanol and 10 % glacial acetic acid) added and incubated for 20 minutes with gentle agitation. The presence of blue lines (bands) represented the presence of protein. When the protein bands were observed, the Staining Solution was replaced by the Destain Solution (20% methanol + 10% acetic acid) and incubation

was left to take place with gentle agitation. The Destain Solution was changed several times until the background of the gel was fully destained.

Western blotting

Principle

The method is based on building an antibody: protein complex through the specific binding of primary and secondary antibodies to proteins immobilized on a membrane and detecting the bound antibodies by one of several detection methods. Proteins separated by SDS-PAGE are transferred onto a nitrocellulose membrane and blocked with blocking reagent during incubation. The membrane is incubated with the primary antibody which binds with the protein of interest and later with a secondary antibody that is specific to the primary antibody and has a covalently attached enzyme. Upon addition of a chromogenic substrate, a coloured reaction will occur, with the intensity of the colouration being directly proportional to the amount of protein present.

Experimental procedure

This experiment was carried out as described by Towbin and collaborators in 1979 with slight modifications (**Towbin *et al.*, 1979).** All reagents were assembled and the required solutions (PBS-T, PBS, Transfer Buffer, Blocking Solution, Ponceau Red) prepared (Appendix 1). The gel from the SDS PAGE was removed and soaked together with the nitro cellulose membrane in the Transfer buffer. Both were assembled in the wet transfer device in a "sandwich" of negative electrode/sponge/3 Whatmann filter paper/gel/membrane/3 Whatmann filter paper/sponge/positive electrode. The "sandwich" was made in the Transfer Buffer, making sure that air bubbles were removed between the gel and membrane by rolling them out with a 15 mL test tube. The assembly was then fitted into the gel tank and filled with the Transfer Buffer. The device was plugged on to electricity and the transfer effected at 95 volts for 2 hours. After unplugging the device, the membrane was removed, incubated with Ponceau Red (for 5 minutes at rtp) with the side containing the proteins facing upward. The presence of bands indicated that the proteins have been transferred from the gel onto the membrane. The membrane was incubated with the Destain Solution on a magnetic shaker to wash off the Ponceau Red Stain. After complete destaining, 5% BSA was added and incubated for 45 minutes at rtp. The membrane was then gently rinsed with PBS-T and incubated with the Primary Antibody (GLUT4, NFkβ or β-actin) for 2 hours at rtp or overnight at 4 ºC with gentle shaking. This was followed by washing with PBS-T upon agitation at 15-, 5-, 5-

and 5-minute intervals. The last washing solution was discarded, and the membrane was incubated with Secondary Antibody for 90 minutes at rtp with constant shaking. The washing step was repeated with PBS-T at similar time intervals. At the end, the membrane was removed and spread on a flat surface. One mL of the Developer Solution was poured on it and the exposure was set at 30, 60 and 90 seconds for NFkβ and β-actin while GLUT4 was exposed for 2, 3 and 5 minutes. The images were analysed with the Image J Software during which the images were converted into quantitative data (relative density).

II.2.5.17Histopathology

Histopathological sections of liver, kidney and pancreas were prepared. After fixation of the tissues in formalin, each was removed and cut into transverse sections using a scalped blade. The sections were then washed with running tap water for approximately 45 minutes to remove formalin and transferred for processing. The steps included dehydration, clearing, infiltration, embedding, sectioning, mounting and staining.

Dehydration

This is the first step of tissue processing which enables the removal of water from the samples. This process was carried out by passing the tissue through a series of increasing alcohol concentrations (70, 80, 85 and 90% for 1 hour each and 3 × 100% for 2 hours).

Clearing

This serves as a transition stage between dehydration and infiltration. The tissues were cleared by rendering them transparent so that they could easily pass into the infiltration medium. This was done by passing them into a bath of xylene (2 × 2 hours).

Infiltration

Infiltration enables the saturation of tissue cavities and cells with a supporting substance. The tissues were transferred and kept in a hot bath (60 - 63°C) containing paraffin for 2 hours for complete infiltration.

Embedding

This enables the formation of a solid support around the tissue for easy sectioning. Each tissue was removed with the help of a pair of forceps and placed carefully in the right orientation at the centre of a mould. Hot paraffin was then added gently, and the solution allowed to solidify at rtp and later transferred onto a cooling block for further solidification. Each block was kept at least overnight before the next step.

Sectioning

The mould was removed, and excess paraffin taken off using a blade. The thickness of the section was set at 10 microns and the block was clamped in the microtome carrier so that the upper and lower surfaces were parallel to the knife edge. The blade was moved towards the block and locked to prevent movement. It was then unlocked, and the wheel rotated to enable the cutting of the sections into the desired sizes.

Mounting

The identity of the tissue was engraved on one end of a clean and dry slide. The sections produced were transferred into 50% ethanol solution for 45 seconds and later into water at 40°C for 1 – 2 minutes to open them and exposure the tissues. The section was then arranged at the centre of the slide in a line perpendicular to its long axis, transferred onto a heating block for 15 minutes for evaporation of the paraffin. The slides were then kept for at least 24 hours before staining

Staining

Staining was carried out to enable the visualisation of the different cell organelles under a light microscope. The Haematoxylin and Eosin (H and E) staining method was employed. The first step was deparaffinisation, where the slides were fitted into a staining box and dipped in xylene solution (3 × 3 minutes). The box was then transferred into xylene/absolute alcohol 1:1 mixture (3 minutes), absolute alcohol (2 × 3 minutes) and 95% alcohol (2 × 3 minutes) for hydration. The slides were then rinsed with distilled water 2 – 3 times and stained with Haematoxylin (6 – 8 minutes) by immersing them in the solutions. The sections were then washed under running tap water until the water was no longer coloured (approximately 5 minutes), and then dunked 2 – 3 times in an acid alcohol mixture (1% HCl in 70% ethanol) until they turned pink. They were again washed under running tap water (3 – 5 minutes), dunked slowly 5 – 6 times in ammonia water (1 mL NH_4OH in 1L of water) and again rinsed 3 – 5 times with tap water. The slides were there transferred into the

Eosin Y stain for 2 minutes and blotted rapidly. The next step was dehydration where they were passed through increasing concentrations of alcohol (70% for 2 minutes, 85% for 2 minutes and 100% for 2 minutes × 2). The tissues were then cleared in xylene/ethanol (1:1) mixture for 2 minutes and finally in pure xylene (2 minutes × 3). When removed from the last solution, the excess xylene was drained off. One drop of permount was added at the centre of the slide and the cover slip lowered at an angle over the section. The slides were kept between 24 and 48 hours for drying. Excess permount was cleaned from the slide and cover slip using a razor blade and the slides visualised with a microscope.

II.2.6 Statistical analyses

The optical densities (ODs) obtained were entered into an Excel spread sheet (Microsoft Office Excel 2013). The concentrations and ODs of the standards were used to plot a standard curve from which the concentration of each sample was then calculated. The activity of each sample was further calculated using its concentration, molar extinction coefficient, sample volume and total volume of reaction mixture where necessary.

Western blot images were opened in Image J in 8-bit mode. A rectangle was drawn across the three images corresponding to the three samples and the lanes plotted. After plotting the lanes of the three samples, their peaks were labelled, expressed as a percentage of the total size of all highlighted peaks. This was carried out for the proteins of interest (GLUT4 and NFkβ) and the control protein (β-actin). For each gel the first lane was chosen as the standard and the relative density for each peak calculated as percentage value of peak divided by percentage value of standard. The relative density for gene of interest (GLUT4 or NFkβ) was divided by the relative density of β-actin to obtain the level of expression compared to the normalisation gene. These values were recorded for the control, chlorpyrifos and imidacloprid groups.

The results were imported from an Excel sheet into GraphPad prism version 5 for analysis. Categorical variables were expressed as numbers and percentages while continuous variables were expressed as mean ± SEM. Paired t-test was used to compare fasting blood glucose collected before and after exposure in female Wistar rats. ANOVA test was used to compare continuous variables between the 5 groups, with Bonferroni post-test used to compare the data between the control and the different exposed groups. All analyses were carried out at 95 % Confidence Intervals (CI) and $p < 0.05$ was statistically significant.

CHAPTER III:

RESULTS AND DISCUSSION

III.1. RESULTS

III.1.1 Determination of Median Lethal Dose.

The maximal dose of each pesticide without mortality recorded (Mo) and the minimal dose that resulted in mortality (M_1) were recorded (Table VIII). The median lethal dose (LD_{50}) was calculated from these two doses using the formula $LD_{50} = \frac{MO+M1}{2}$. The dose to be administered was chosen as 1/10 of the LD_{50} of each pesticide.

Table VIII: Median lethal doses of studied pesticides

Pesticide	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
M_0 (mg/ kg)	438	134	55	3.8
M_1 (mg/ kg)	442	136	57	4.2
LD_{50} (mg/ kg)	440	135	56	4.0

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, Mo = Highest dose without mortality, M_1 = Minimum dose with associated mortality, LD = Lethal Dose.

III.1.2 Effect of *in-vivo* oral exposure of pregnant rats to pesticides on biochemical markers of liver function and oxidative stress

III.1.2.1 Reproductive outcome, weight variation and organ weight in parent rats

The average gestation length of the rats per group varied between 23 - 27 days (Table IX). In all the different groups (exposed and control) some of the rats did not get pregnant even after a prolong period of mating (15 days). In each group, the average number of pups ranged between 6 - 8 pups/dam and the average birth weight ranged between 5.95 – 7.09 grams (Table IX).

Table IX: Effect of pesticides on reproductive outcome of female Wistar rats.

Group	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
Average Gestational length (days)	22±1	23±2	22±1	22±2	24±3
Number of pregnancies recorded	4	5	2	3	3
Average number of pups/ dam	7.75±0.48	6.4±0.43	8.5±1.5	6.67±0.67	8.00±0.58
Number of male pups	15	17	11	12	14
Average birth weight of pups (grams)	6.2 ± 0.2	5.9 ± 0.4	6.7 ± 0.4	5.8 ± 0.4	6.4 ± 0.3

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl. Data are reported as mean ± SEM and number for 6 animals.

The weights of the dams were observed to increase throughout the study (Figure 13). However, no significant difference was observed between the Pesticide-exposed groups and the control group.

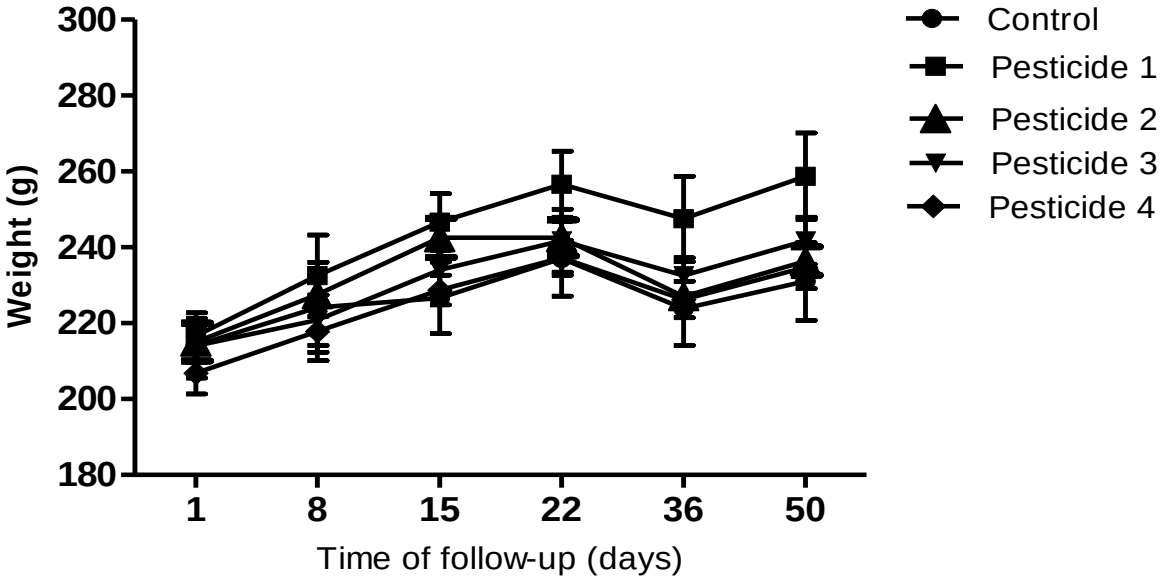


Figure 13: Effect of exposure to pesticides on body weight of female Wistar rats.
Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl. Data are reported as mean ± Standard Error of Mean (SEM) for 6 animals.

After sacrifice and dissection of the animals, the liver, kidneys, pancreas, brain and heart were removed, washed, dried on tissue paper and weighed. The weights of these organs in the exposed groups were not significantly different from those of the control group (Table X).

Table X: Effect of pesticides on organ weight of female Wistar rats

Parameter	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
Liver (g)	7.76 ± 0.37	8.36 ± 0.45	7.35 ± 0.86	7.80 ± 0.44	7.21 ± 0.28
Kidney (g)	1.62 ± 0.06	1.62 ± 0.06	1.53 ± 0.12	1.43 ± 0.13	1.47 ± 0.12
Pancreas (g)	0.68 ± 0.12	0.64 ± 0.08	0.54 ± 0.12	0.63 ± 0.09	0.61 ± 0.08
Brain (g)	1.63 ± 0.11	1.81 ± 0.12	1.64 ± 0.09	1.61 ± 0.09	1.71 ± 1.02
Heart (g)	0.90 ± 0.02	0.99 ± 0.08	0.92 ± 0.10	0.96 ± 0.08	0.92 ± 0.06

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl. Data are reported as mean ± SEM for 6 samples compared using ANOVA and Bonferroni post-test.

III.1.2.2 Kidney and liver antioxidant enzyme, and malondialdehyde levels in control and exposed groups

Alterations in the level of antioxidant enzyme activities were recorded in the liver (Table XI) and kidney (Table XII) of parent rats exposed to pesticides when compared with the control group. Liver SOD was significantly lower in rats exposed to Pesticides 1 (1.2U/mg protein lower), 2 (1.5U/mg protein lower) and 3 (2U/mg protein lower), while a significantly higher value was recorded in rats exposed to Pesticide 4 (2.5U/mg protein higher) when compared to the control group having an enzymatic activity of 3.9U/mg protein. Exposure to all four pesticides led to a significant decrease in liver catalase and liver GSH of parent rats. Pesticides 1 and 2 significantly increased liver GPx levels (7.4U/mg protein and 7.6U/mg protein higher respectively) while Pesticides 3 and 4 significantly lowered its levels (12U/mg protein and 12.4U/mg protein lower respectively). Liver GR was significantly reduced in rats exposed to Pesticide 4 (2U/mg protein lower) while liver MDA was significantly increased in rats exposed to Pesticide 2 (5.2µM).

Table XI: Effect of pesticides on antioxidant enzymes and malondialdehyde levels in liver of female Wistar rats

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
SOD (U/mg protein)	3.9 ± 0.4	2.7 ± 0.3 *	2.4 ± 0.2 *	1.9 ± 0.4 **	6.4 ± 0.4 **
CAT (U/mg protein)	24.5 ± 1.9	14.7 ± 1.4 **	15.0 ± 1.7 **	3.5 ± 0.8 ***	12.7 ± 1.9 **
GPx (U/mg protein)	14.8 ± 1.2	22.2 ± 1.9 *	24.4 ± 2.8 *	2.8 ± 0.5***	2.4 ± 0.1 ***
GSH (µg/mL)	26.7 ± 2.4	18.9 ± 1.9 *	17.3 ± 2.4 *	3.8 ± 0.2 ***	3.6 ± 0.3 ***
GR (U/mg protein)	3.1 ± 0.5	1.7 ± 0.6	1.9 ± 0.5	2.2 ± 0.4	1.1 ± 0.3 *
MDA (µM)	0.7 ± 0.06	5.3 ± 0.3	5.9 ± 0.6 ***	0.6 ± 0.1	0.6 ± 0.1

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, SOD = Superoxide Dismutase, CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GR = Glutathione Reductase, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM for 6 samples compared using ANOVA and Bonferroni post-test.

In the kidney, SOD was significantly higher in Pesticide 1 (1.4U/mg protein higher) rats while GPx was significantly lower in those exposed to Pesticide 4 (6.6U/mg protein higher). Similarly, GSH was significantly higher rats exposed to Pesticide 1 (10.2U/mg protein higher) and Pesticide 2 (8.8U/mg protein higher). Moreover, rats exposed to Pesticides 1, 3 and 4 had significantly higher MDA levels (1.4μM, 2.5μM and 4μM higher respectively) when compared to the control group. Kidney catalase levels were significantly higher in Pesticides 3 and 4 rats (21U/mg protein and 18U/mg protein higher respectively) while the GR levels were significantly lower in rats exposed to Pesticides 3 (18.6U/mg protein lower) and 4 (21.7U/mg protein lower) compared to the control group (Table XII).

Table XII: Effect of pesticides on antioxidant enzymes and malondialdehyde levels in kidney of female Wistar rats

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
SOD (U/mg protein)	1.4 ± 0.3	2.8 ± 0.4 *	2.0 ± 0.3	1.3 ± 0.2	1.2 ± 0.2
CAT (U/mg protein)	14.9 ± 6.4	22.4 ± 0.9	31.7 ± 4.6	35.9 ± 1.9 *	32.9 ± 2.5 *
GPx (U/mg protein)	20.8 ± 2.2	20.6 ± 1.4	21.1 ± 1.1	15.54 ± 2.6	14.2 ± 1.4 *
GSH (μg/mL)	19.3 ± 1.4	29.5 ± 1.7 **	28.1 ± 2.9 *	15.0 ± 1.8	16.8 ± 1.6
GR (U/mg protein)	48.4 ± 3.4	50.1 ± 2.7	48.5 ± 3.0	29.8 ± 4.9 *	26.7 ± 3.1 **
MDA (μM)	1.0 ± 0.1	2.4 ± 0.3*	1.6 ± 0.3	3.5 ± 0.6 ***	5.0 ± 0.3 ***

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, SOD = Superoxide Dismutase, CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GR = Glutathione Reductase, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM for 6 samples compared using ANOVA and Bonferroni post-test.

III.1.2.3: Serum alanine transaminase and aspartate transaminase in control and exposed groups

Aspartate transaminase (AST) and alanine transaminase (ALT) levels were significantly higher in female Wistar rats exposed to pesticide 1 ($p < 0.001$ for AST and ALT) and pesticide 2 ($p < 0.001$ for AST and ALT) when compared to the control group (Figure 14).

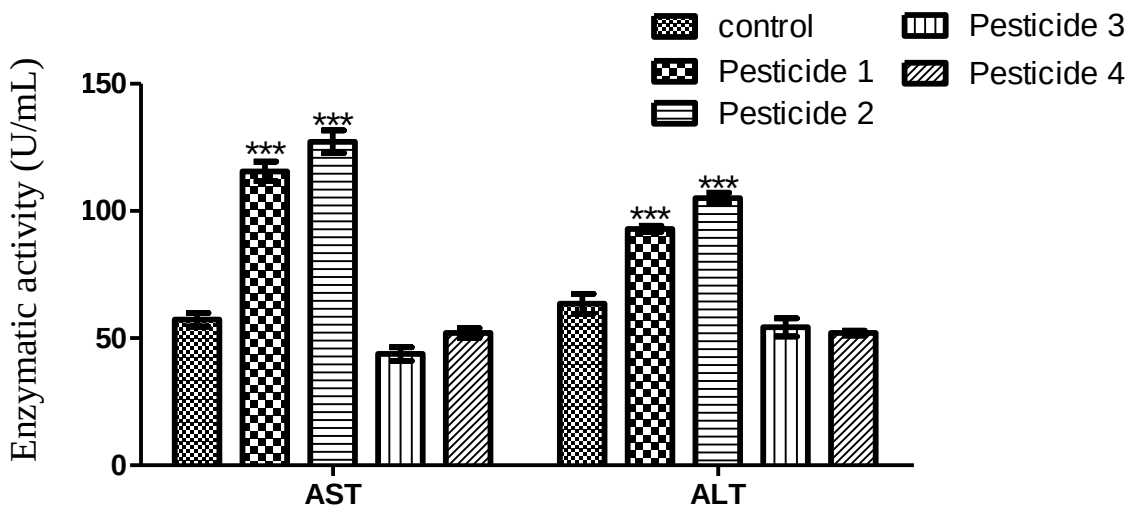


Figure 14: Effect of pesticides on aspartate transaminase and alanine transaminase levels in female Wistar rats.
Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, *** = $p < 0.001$ compared to the control group. Data are reported as mean ± SEM for 6 samples compared using ANOVA and Bonferroni post-test.

III.1.3: Effect of *in-utero* exposure of offspring to pesticides on biochemical markers of liver function and oxidative stress

III.1.3.1 Weight of rats and organs of offspring sacrificed at weaning and adulthood.

At birth, offspring were followed up and their weight recorded on weekly bases. At weaning 8 offspring were randomly selected and sacrifice, while another 8 were sacrificed at adult age (12 weeks). The average weights of the rats sacrificed at weaning was observed to increase throughout the follow-up period in all the groups with no significant difference between the exposed groups and control group (Figure 15).

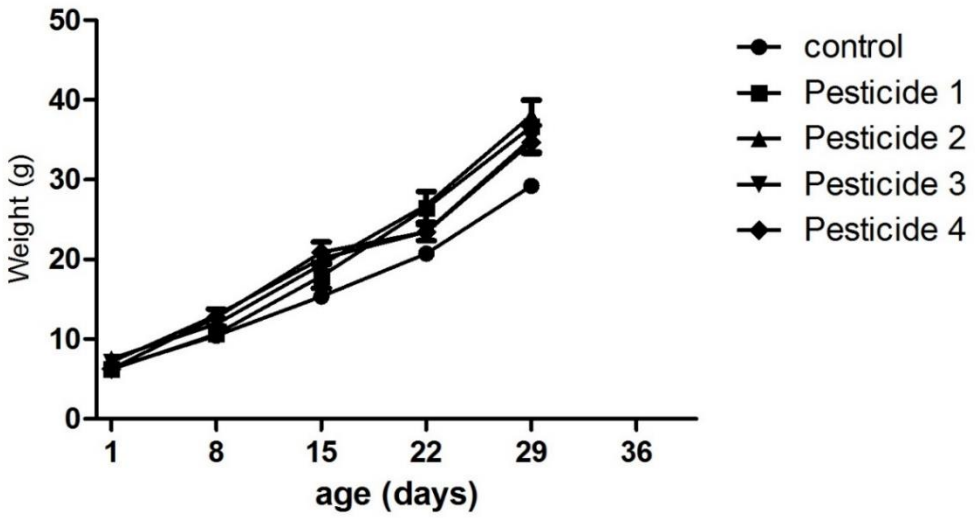


Figure 15: Effect of exposure to pesticides on body weight of offspring sacrificed at weaning.
Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, compared to the control group. Data are reported as mean ± SEM for 8 animals compared using ANOVA and Bonferroni post-test.

In a similar manner, offspring sacrificed at adult age recorded an increase in body weight in all the groups with no significant difference between the offspring of exposed groups when compared to the control group (Figure 16).

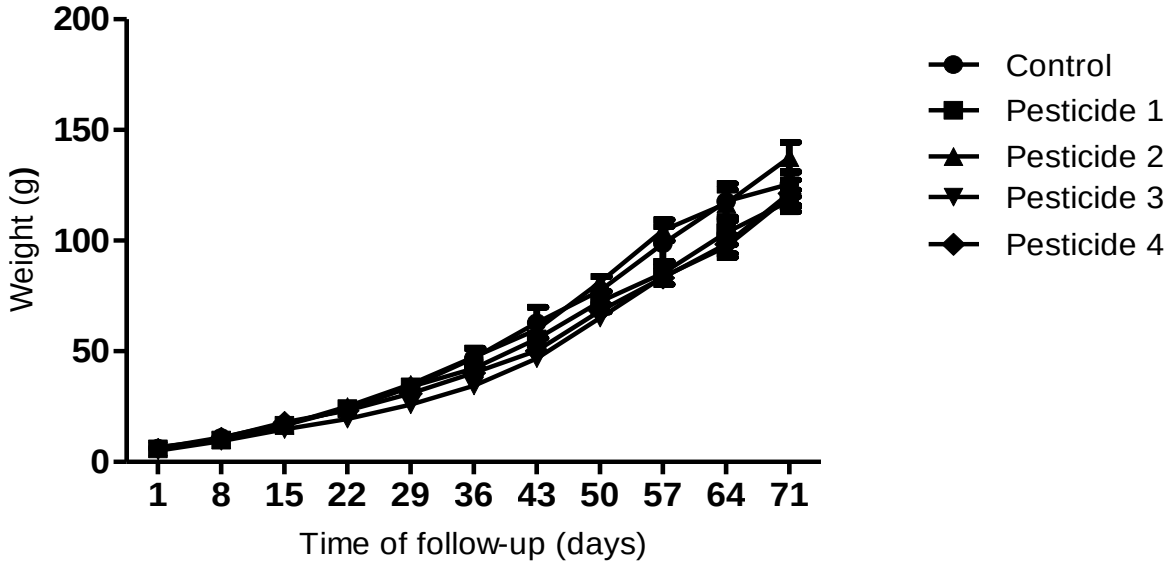


Figure 16: Effect of exposure to pesticides on body weight of offspring sacrificed at adult age.

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl. Data are reported as mean ± SEM for 8 animals compared using ANOVA and Bonferroni post-test.

After sacrifice and dissection of the animals, the liver, kidney, pancreas, brain and heart were removed, washed, dried on a tissue paper and weighed. In offspring sacrificed at weaning, the weights of the liver, kidney and brain were significantly higher in offspring of rats exposed to pesticide 2 when compared to the control group. No significant difference was observed in the weights of the other organs in exposed groups compared to the control group (Table XIII).

Table XIII: Effect of pesticides on organs weight in offspring of rats sacrificed at weaning

Organ	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
Liver (mg)	1.96 ± 0.10	2.41 ± 0.24	2.55.0 ± 0.13 **	2.31 ± 0.11	2.10 ± 0.15
Kidney (mg)	0.56 ± 0.03	0.62 ± 0.05	0.69 ± 0.04 *	0.58 ± 0.03	0.61 ± 0.04
Pancreas (mg)	0.21 ± 0.04	0.29 ± 0.06	0.22 ± 0.02	0.22 ± 0.02	0.21 ± 0.03
Brain (mg)	1.36 ± 0.05	1.45 ± 0.02	1.58 ± 0.05 *	1.41 ± 0.06	1.50 ± 0.04
Heart (mg)	0.26 ± 0.02	0.29 ± 0.02	0.32 ± 0.02	0.27 ± 0.02	0.31± 0.03

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM for 8 samples compared using ANOVA and Bonferroni post-test.

There was no significant difference in the weights of these organs between the exposed and control groups in offspring sacrificed at adult age (Table XIV).

Table XIV: Organ weight in control and exposed offspring of rats sacrificed at adult age

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
Liver (mg)	4.97 ± 0.24	4.86 ± 0.33	5.65 ± 0.38	4.85 ± 0.25	5.02 ± 0.30
Kidney (mg)	1.21 ± 0.06	1.04 ± 0.06	1.29 ± 0.09	1.08 ± 0.08	1.26 ± 0.07
Pancreas (mg)	0.45 ± 0.02	0.42 ± 0.06	0.49 ± 0.06	0.48 ± 0.03	0.44 ± 0.04
Brain (mg)	1.57 ± 0.06	1.61 ± 0.07	1.68 ± 0.10	1.59 ± 0.06	1.62 ± 0.08
Heart (mg)	0.58 ± 0.03	0.58 ± 0.03	0.67 ± 0.04	0.59 ± 0.028	0.61 ± 0.03

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl. Data are reported as mean ± SEM for 8 samples ANOVA and Bonferroni post-test.

III.1.3.2 Liver antioxidant enzymes, and malondialdehyde levels in offspring

In offspring sacrificed at weaning, liver antioxidant enzyme activities and MDA levels were significantly altered in pesticides exposed rats compared to the control group (Table XV). Liver SOD dropped significantly in rats exposed to Pesticides 3 and 4 (0.6U/mg protein each) when compared to the control group. Liver catalase and MDA levels were significantly higher in rats exposed to Pesticide 1 (4.9U/mg protein) and Pesticide 2 (1.5µM) respectively when compared with the control group.

Table XV: Effect of pesticides on antioxidant enzymes and malondialdehyde levels in liver of offspring sacrificed at weaning

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
SOD (U/mg protein)	1.9 ± 0.2	1.7 ± 0.2	1.9 ± 0.2	1.3 ± 0.2 *	1.3 ± 0.2 *
CAT (U/mg protein)	9.2 ± 0.9	14.1 ± 1.8 *	8.6 ± 0.7	11.9 ± 2.5	13.4 ± 5.8
GPx (U/mg protein)	28.2 ± 2.3	29.8 ± 4.9	26.7 ± 3.1	27.2 ± 2.3	33.5 ± 1.4
GSH (µg/mL)	21.4 ± 3.3	15.0 ± 1.8	16.8 ± 1.6	15.5 ± 2.6	14.2 ± 1.4
GR (U/mg protein)	7.1 ± 3.7	1.8 ± 0.3	1.4 ± 0.4	1.3 ± 0.2	1.2 ± 0.2
MDA (µM)	3.5 ± 0.4	3.5 ± 0.6	5.0 ± 0.3 *	4.4 ± 1.1	3.3 ± 0.4

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, SOD = Superoxide Dismutase, CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GR = Glutathione Reductase, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM for 8 samples compared using ANOVA and Bonferroni post-test.

Alterations in liver (Table XVI) antioxidant enzyme activities and MDA levels were recorded in adult offspring of pesticide-exposed rats when compared with those of the control group. Liver SOD was significantly lower in rats exposed to Pesticide 1 (20.4U/mg protein) and Pesticide 2 (19.8U/mg protein). Liver catalase was significantly higher in rats exposed to Pesticide 1 (5U/mg protein) and Pesticide 2 (6.1U/mg protein) while a significantly lower catalase level (6.5U/mg protein) was recorded in the group exposed to Pesticide 4. GPx levels significantly dropped in Pesticide 1 (55.2U/mg protein), Pesticide 2 (55.4U/mg protein) and Pesticide 4 (21.5U/mg protein) groups. Similarly, liver GSH dropped significantly in Pesticide 1 (9.4µg/mL), 3 (28.2µg/mL) and 4 (28.1µg/mL) groups. Likewise, significantly lower liver GR levels were recorded in offspring of parent rats exposed to Pesticides 3 (1.2U/mg protein) and 4 (1U/mg protein). A one-fold increase in liver MDA was recorded in offspring of rats exposed to Pesticides 1 (4.2µM) and 2 (4.5µM).

Table XVI: Effect of pesticides on antioxidant enzymes and malondialdehyde levels in liver of offspring sacrificed at adult age.

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
SOD (U/mg protein)	23.3 ± 3.8	2.9 ± 0.3 **	3.5 ± 0.4 **	19.3 ± 1.4	16.6 ± 1.0
CAT (U/mg protein)	16.5 ± 1.0	21.5 ± 1.2 **	22.6 ± 0.7 ***	17.9 ± 2.5	10.0 ± 2.2 *
GPx (U/mg protein)	70.9 ± 4.4	15.7 ± 1.9 ***	15.5 ± 1.3 ***	55.7 ± 8.7	49.4 ± 4.5 **
GSH (µg/mL)	30.9 ± 3.3	21.5 ± 1.9 *	24.3 ± 1.7	2.7 ± 0.2 ***	2.8 ± 0.2 ***
GR (U/mg protein)	2.9 ± 0.4	2.5 ± 0.6	1.9 ± 0.3	1.7 ± 0.3 *	1.9 ± 0.2 *
MDA (µM)	5.8 ± 1.4	10.0 ± 1.2*	10.3 ± 0.9*	5.6 ± 0.1	6.1 ± 0.2

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, SOD = Superoxide Dismutase, GPx = Glutathione Peroxidase, GST = Glutathione S Transferase, GSH = Reduced Glutathione, GR = Glutathione Reductase, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM for 8 samples compared using ANOVA and Bonferroni post-test.

III.1.3.3 Kidney antioxidant enzymes, and malondialdehyde levels in offspring

Alterations in kidney antioxidant enzyme activities and MDA levels were recorded in offspring sacrificed at weaning and adult offspring of pesticide-exposed rats when compared with those of the control group. In the kidney of offspring sacrificed at weaning (Table XVII), SOD was significantly higher in rats exposed to Pesticides 1 (2.2U/mg protein) and 2 (2.9U/mg protein). Similarly, GPx was significantly higher in rats exposed to Pesticides 2 (7.7U/mg protein) and 4 (4.1U/mg protein) compared to the control group. GSH and MDA levels were significantly higher in the kidney of all pesticide-exposed groups compared to the control group. GR was significantly higher in the kidney of rats exposed to Pesticides 1 (7.8U/mg protein) and 4 (3.5U/mg protein) compared to the control group.

Table XVII: Effect of pesticides on antioxidant enzymes and malondialdehyde levels in kidney of offspring sacrificed at weaning.

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
SOD (U/mg protein)	2.8 ± 0.6	5.0 ± 0.4 *	5.7 ± 0.6 **	4.6 ± 1.1	2.9 ± 0.5
CAT (U/mg protein)	10.7 ± 5.6	24.6 ± 13.7	48.9 ± 29.4	8.0 ± 1.0	8.3 ± 0.8
GPx (U/mg protein)	4.2 ± 0.5	13.4 ± 5.8	11.9 ± 2.5 *	7.0 ± 2.2	8.3 ± 0.9 **
GSH (µg/mL)	33.5 ± 1.4	69.1 ± 6.5 **	75.7 ± 4.9 ***	52.0 ± 5.9 *	53.2 ± 7.0 *
GR (U/mg protein)	3.3 ± 0.4	11.1 ± 2.4 *	6.9 ± 1.4	6.1 ± 1.2	6.8 ± 1.2 *
MDA (µM)	0.3 ± 0.02	0.9 ± 0.2 *	1.6 ± 0.1 ***	0.9 ± 0.1 *	0.8 ± 0.1 **

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, SOD = Superoxide Dismutase, CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GR = Glutathione Reductase, *** = $p < 0.001$ **, = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM for 8 samples compared using ANOVA and Bonferroni post-test.

In the kidney of rats sacrificed at adult age, SOD was significantly higher in offspring of Pesticide 3 (1.2U/mg protein) and Pesticide 4 (1.9U/mg protein) exposed rats (Table XVIII). Kidney catalase was significantly higher (9.5U/mg protein) while GSH was significantly lower (11.8µg/mL) in offspring of parent rats exposed to Pesticide 2. Moreover, kidney GPx and MDA levels were significantly higher in offspring of parent rats exposed to Pesticide 1 (2.9U/mg protein and 1µM respectively) and Pesticide 2 (5U/mg protein and 2.6µM respectively) than the control group. GR was significantly higher in offspring of rats exposed to Pesticide 2 (5.3U/mg protein) and significantly lower in offspring of Pesticide 3 (18.6U/mg protein) and Pesticide 4 (19.2U/mg protein) exposed rats (Table XVIII).

Table XVIII: Effect of pesticides on antioxidant enzymes and malondialdehyde levels in kidney of offspring sacrificed at adult age.

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
SOD (U/mg protein)	3.8 ± 0.3	3.1 ± 0.2	3.4 ± 0.3	5.0 ± 0.4 *	5.7 ± 0.6 *
CAT (U/mg protein)	17.4 ± 2.5	21.2 ± 1.5	26.9 ± 2.9 *	24.6 ± 13.7	48.9 ± 29.4
GPx (U/mg protein)	7.5 ± 0.6	10.4 ± 0.5 **	12.5 ± 0.8 ***	13.4 ± 5.8	11.9 ± 2.5
GSH (µg/mL)	26.4 ± 3.3	21.5 ± 1.6	14.6 ± 3.3 *	17.1 ± 4.7	20.7 ± 4.1
GR (U/mg protein)	31.8 ± 1.2	34.3 ± 1.9	37.1 ± 1.4 *	13.2 ± 1.6 ***	12.6 ± 1.4 ***
MDA (µM)	2.2 ± 0.2	3.2 ± 0.3*	4.8 ± 0.8*	2.4 ± 0.5	2.6 ± 0.5

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, SOD = Superoxide Dismutase, GPx = Glutathione Peroxidase, GST = Glutathione S Transferase, GSH = Reduced Glutathione, GR = Glutathione Reductase, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM for 8 samples compared using ANOVA and Bonferroni post-test.

III.1.3.4: Serum alanine transaminase and aspartate transaminase in offspring

Alanine transaminase was significantly higher in weaned offspring of rats exposed to Pesticide 1 ($p<0.05$), Pesticide 2 ($p<0.01$), Pesticide 3 ($p<0.05$) and Pesticide 4 ($p<0.01$) compared to the control group (Figure 17), while the difference in AST level was not statistically significant.

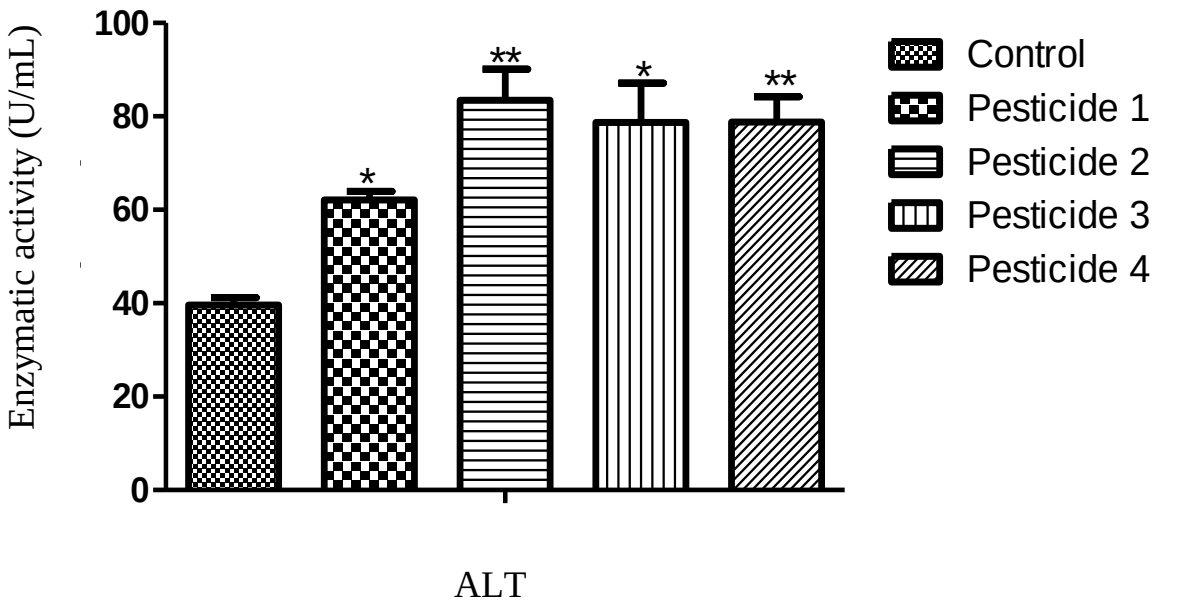


Figure 17: Effect of pesticides on alanine transaminase activity in offspring sacrificed at weaning
Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, ALT = Alanine transaminase, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM for 8 samples compared using ANOVA and Bonferroni post-test.

In offspring sacrificed at adult age, plasma ALT and AST were significantly higher in all exposed groups compared to the control group (Figure 18).

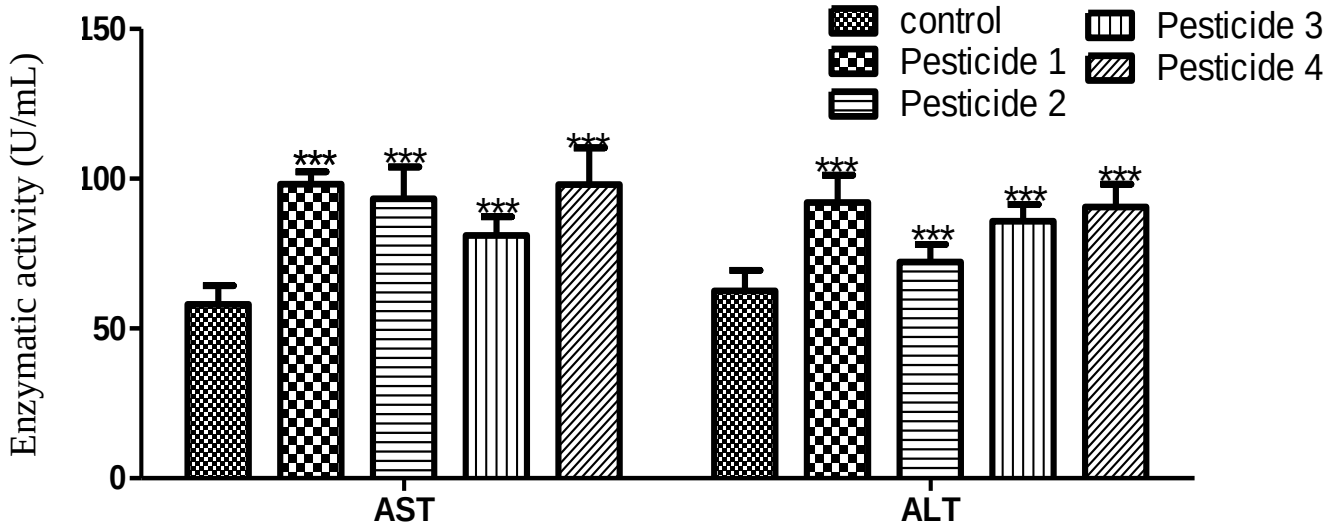


Figure 18: Effect of pesticides on aspartate transaminase and alanine transaminase levels in offspring sacrificed at adult age

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, *** = $p < 0.001$ compared to the control group. Data are reported as mean ± SEM for 8 samples compared using ANOVA and Bonferroni post-test.

III.1.4. Metabolic pathways affected by oral and in-utero exposure of pregnant rats and their offspring to pesticides.

III.1.4.1 Metabolic parameters in female Wistar rats and their offspring

The metabolic parameters investigated were blood glucose, insulin, and lipid profile. Insulin sensitivity and secretion were assessed as HOMA-IR and HOMA-β calculated from fasting glucose and fasting insulin values. In parent rats, the fasting blood glucose (FBG) before exposure to the various pesticides was similar in all groups (79.6-82.0 mg/dL). At the end of the follow-up, FBG and insulin were significantly higher in rats exposed to pesticide 1 (19.4 mg/dL and 5.7mU/mL for FBG and insulin respectively), pesticide 2 (23.6mg/dL and 5.3mU/mL for FBG and insulin respectively) and pesticide 4 (46.8mg/dL and 7.5mU/mL for FBG and insulin respectively) compared to the control group (Table XIX). Comparing the FBG before and after exposure, a minor reduction was recorded in the control group (4mg/dL) and rats exposed to pesticide 3 (1.4mg/dL). However, in the other pesticides exposed groups, there was a significant increase in FBG levels (16.2mg/dL, 18mg/dL and 42.6mg/dL for pesticides 1, 2 and 4 respectively) when compared to the control group. HOMA-IR values were significantly higher in the above-mentioned groups (1.6, 1.5 and 2.8 respectively) while no difference in HOMA-β values was recorded. Equally, an alteration in lipid profile parameters was recorded in pesticide-exposed rats (Table XIX). The animals exposed to pesticide 1 had a significant increase in total cholesterol (43.2mg/dL) and LDL-cholesterol (50.4mg/dL) levels while those exposed to pesticide 2 had a significant increase in LDL-cholesterol (29.9mg/dL) levels. HDL-cholesterol was significantly lower in rats exposed to pesticides 1 (13.2mg/dL), 2 (3.7mg/dL) and 4 (5mg/dL) when compared to the control group.

Table XIX: Effect of pesticides on FBG, insulin, HOMA-IR, HOMA-β and lipid profile in female Wistar rats

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
FBG 1 (mg/dL)	80.4 ± 5.4	79.6 ± 6.6	82.0 ± 4.2	80 ± 2.4	80.6 ± 2.8
FBG 2 (mg/dL)	76.4 ± 4.7	95.8 ± 4.6 *,a	100.0 ± 5.6 *,a	78.6 ± 4.6	123.2 ± 7.5 **,a
Insulin (mU/mL)	4.6 ± 0.6	10.3 ± 1.4 *	9.9 ± 1.8 *	6.8 ± 1.4	12.1 ± 4.1 **
HOMA-IR	0.8 ± 0.2	2.4 ± 0.4**	2.3 ± 0.4*	1.3 ± 0.3	3.6 ± 0.5 **
HOMA-β	141.8 ± 63.1	133.4 ± 32.5	103.9 ± 23.9	156.9 ± 40.5	72.4 ± 45.3
TC (mg/dL)	83.3 ± 7.4	126.5 ± 5.9 **	103.5 ± 8.1	81.5 ± 7.7	107.6 ± 5.6
TG (mg/dL)	69.5 ± 5.0	86.8 ± 7.2	77.8 ± 4.7	47.3 ± 4.5	64.5 ± 6.5
HDL – C (mg/dL)	17.5 ± 1.0	14.3 ± 0.5 *	13.8 ± 0.9 *	18 ± 0.7	12.5 ± 0.6 *
LDL – C (mg/dL)	49.1 ± 7.9	99.5 ± 6.6 **	79.0 ± 8.5 *	55.4 ± 7.2	70.5 ± 6.8
AI	0.60 ± 0.07	0.78 ± 0.12	0.75 ± 0.07	0.42 ± 0.08	0.71 ± 0.10

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, FBG 1 = Fasting Blood Glucose before exposure, FBG 2 = Fasting Blood Glucose after exposure, HOMA-IR = Homeostasis Model Assessment of Insulin Resistance, HOMA-β = Homeostasis Model Assessment of Beta Cell Function, TC = Total Cholesterol, TG = Triglycerides, HDL–C = High Density Lipoprotein Cholesterol, LDL–C = Low Density Lipoprotein Cholesterol, AI = Atherogenicity Index, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM with n = 6 compared using ANOVA and Bonferroni post-test. For FBG before and after treatment, a paired t-test was used, a = $p < 0.05$ comparing before exposure and after exposure.

In offspring sacrificed at weaning, a significant alteration was recorded in FBG, insulin and HOMA-IR in at least one of the pesticide exposed groups compared to the control group, while HOMA-β was not significantly different (Table XX). FBG was significantly higher in offspring of rats exposed to Pesticide 1 (44.4mg/dL), Pesticide 2 (15.1mg/dL), Pesticide 3 (19.2mg/dL) and Pesticide 4 (22.4mg/dL). Fasting insulin was significantly higher in offspring of rats exposed to Pesticide 1 (7.9mU/mL), Pesticide 2 (7.3mU/mL) and Pesticide 4(7.7mU/mL). HOMA-IR was significantly higher in offspring of all exposed groups compared to the control group. In a similar manner, a significant increase in lipid profile parameters (total cholesterol and LDL cholesterol) was observed in offspring of the exposed groups compared to the control group (Table XX). Total cholesterol and LDL cholesterol increased in offspring of rats exposed to Pesticide 1 (25.7mg/dL for TC and 24.9mg/dL for LDL-C), Pesticide 2 (20mg/dL TC and 19.7mg/dL for LDL-C), Pesticide 3 (15.5mg/dL for TC and 18mg/dL for LDL-C) and Pesticide 4 (16.6mg/dL for TC and 19.9mg/dL for LDL-C) when compared to the control group.

Table XX: Effect of pesticides on FBG, insulin, HOMA-IR, HOMA-β and lipid profile in offspring sacrificed at weaning.

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
FBG (mg/dL)	65.9 ± 3.6	110.3 ± 4.6 ***	81.0 ± 4.9 *	85.0 ± 4.6 **	88.3 ± 4.6 **
Insulin (mU/mL)	10.1 ± 1.1	18.0 ± 1.1 ***	17.4 ± 1.4 **	12.4 ± 1.2	17.8 ± 1.3 ***
HOMA-IR	1.7 ± 0.2	4.9 ± 0.4 ***	3.5 ± 0.4 **	2.6 ± 0.2 *	3.9 ± 0.4 *
HOMA-β	241.6 ± 30.9	152.3 ± 23.4	315.5 ± 65.4	348.0 ± 163.4	433.1 ± 192.0
TC (mg/dL)	106.8 ± 2.6	132.5 ± 5.5 **	126.8 ± 4.1 **	122.3 ± 4.4 *	123.4 ± 5.2 *
TG (mg/dL)	75.3 ± 4.2	86.0 ± 3.8	82.8 ± 4.2	77.0 ± 3.7	77.4 ± 3.3
HDL – C (mg/dL)	57.3 ± 5.2	55.8 ± 4.4	56.0 ± 4.1	53.9 ± 4.5	55.3 ± 4.0
LDL – C (mg/dL)	34.5 ± 3.8	59.4 ± 7.9 *	54.2 ± 5.3 *	52.5 ± 5.6 *	54.4 ± 4.6 *
AI	0.13 ± 0.05	0.19 ± 0.05	0.17 ± 0.05	0.15 ±0.05	0.15 ± 0.05

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, FBG = Fasting blood glucose, HOMA-IR = Homeostasis Model Assessment of Insulin Resistance, HOMA-β = Homeostasis Model Assessment of Beta Cell Function, TC = Total Cholesterol, TG = Triglycerides, HDL–C = High Density Lipoprotein Cholesterol, LDL–C = Low Density Lipoprotein Cholesterol, AI = Atherogenicity Index, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM with n = 8 compared using ANOVA and Bonferroni post-test.

In offspring sacrificed at adult age, FBG, insulin and HOMA-IR values were significantly higher in rats exposed to Pesticide 1 (22mg/dL, 11.8mU/mL and 1.5 respectively) and Pesticide 2 (18.3mg/dL, 13.4mU/mL and 2 respectively) when compared with the control group. Equally, alterations in lipid profile were recorded in adult offspring of pesticide-exposed groups compared with the control group. A significant increase in TC level was recorded in offspring of rats exposed to Pesticides 1 (71.5mg/dL) and 2 (32.6mg/dL). TG was significantly higher in offspring of rats exposed to Pesticides 1 (24.3mg/dL), 2 (31.2mg/dL) and 3 (24.5mg/dL). LDL-Cholesterol was significantly higher in the Pesticide 1 (61.1mg/dL) group while no difference was noted in HDL-Cholesterol in all exposed groups when compared with the control group (Table XXI).

Table XXI: Effect of pesticides on FBG, insulin, HOMA-IR, HOMA-β and lipid profile in offspring sacrificed at adult age.

Sample	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
FBG (mg/dL)	83.7 ± 6.2	105.7 ± 3.5 *	102.0 ± 4.8 *	93.0 ± 2.9	83.8 ± 4.2
Insulin (mU/mL)	7.6 ± 1.5	19.4 ± 1.3 ***	21.0 ± 1.1 ***	9.5 ± 1.4	10.5 ±1.7
HOMA-IR	1.7 ± 0.3	3.2 ± 0.3 **	3.7 ± 0.7 *	2.2 ± 0.5	2.2 ± 0.2
HOMA-β	132.2 ± 4.5	163.6 ± 5.4	193.0 ± 3.8	114.4 ± 3.4	181.8 ± 4.1
TC (mg/dL)	106.7 ± 7.7	178.2 ± 7.5 ***	139.3 ± 8.8 *	120.9 ± 6.8	114.7 ± 5.9
TG (mg/dL)	74.6 ± 2.8	98.9 ± 8.1 *	105.8 ± 6.2**	99.1 ±3.4 *	83.8 ± 7.5
HDL – C (mg/dL)	48.9 ± 2.2	52.8 ± 3.8	45.8 ± 3.8	56.9 ± 2.7	54.9 ± 3.2
LDL – C (mg/dL)	42.8 ± 6.3	106.9 ± 7.4 ***	70.9 ± 11.1	44.15 ± 5.4	42.4 ± 6.3
AI	0.18 ± 0.01	0.27 ± 0.03*	0.33 ± 0.03**	0.24 ± 0.01**	0.18 ± 0.03

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, Pesticide 3 = Imidacloprid + Lambda Cyhalothrin, Pesticide 4 = Oxamyl, FBG = Fasting Blood Glucose, HOMA-IR = Homeostasis Model Assessment of Insulin Resistance, HOMA-β = Homeostasis Model Assessment of Beta Cell Function, TC = Total Cholesterol, TG = Triglycerides, HDL–C = High Density Lipoprotein Cholesterol, LDL–C = Low Density Lipoprotein Cholesterol, AI = Atherogenicity Index, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ compared to the control group. Data are reported as mean ± SEM with n = 8 compared using ANOVA and Bonferroni post-test.

III.1.4.2 Glucose Transporter 4 and Nuclear Factor Kappa Beta in female Wistar rats and their offspring

In dams, the expression of GLUT4 (Figure 19) and NFkβ (Figure 20) was evaluated in the control, imidacloprid and chlorpyrifos-exposed groups. These two exposed groups were chosen as they were observed to produce more biochemical alterations compared to the rats exposed to Oxamyl and the formulation containing Imidacloprid + Lambda cyhalothrin.

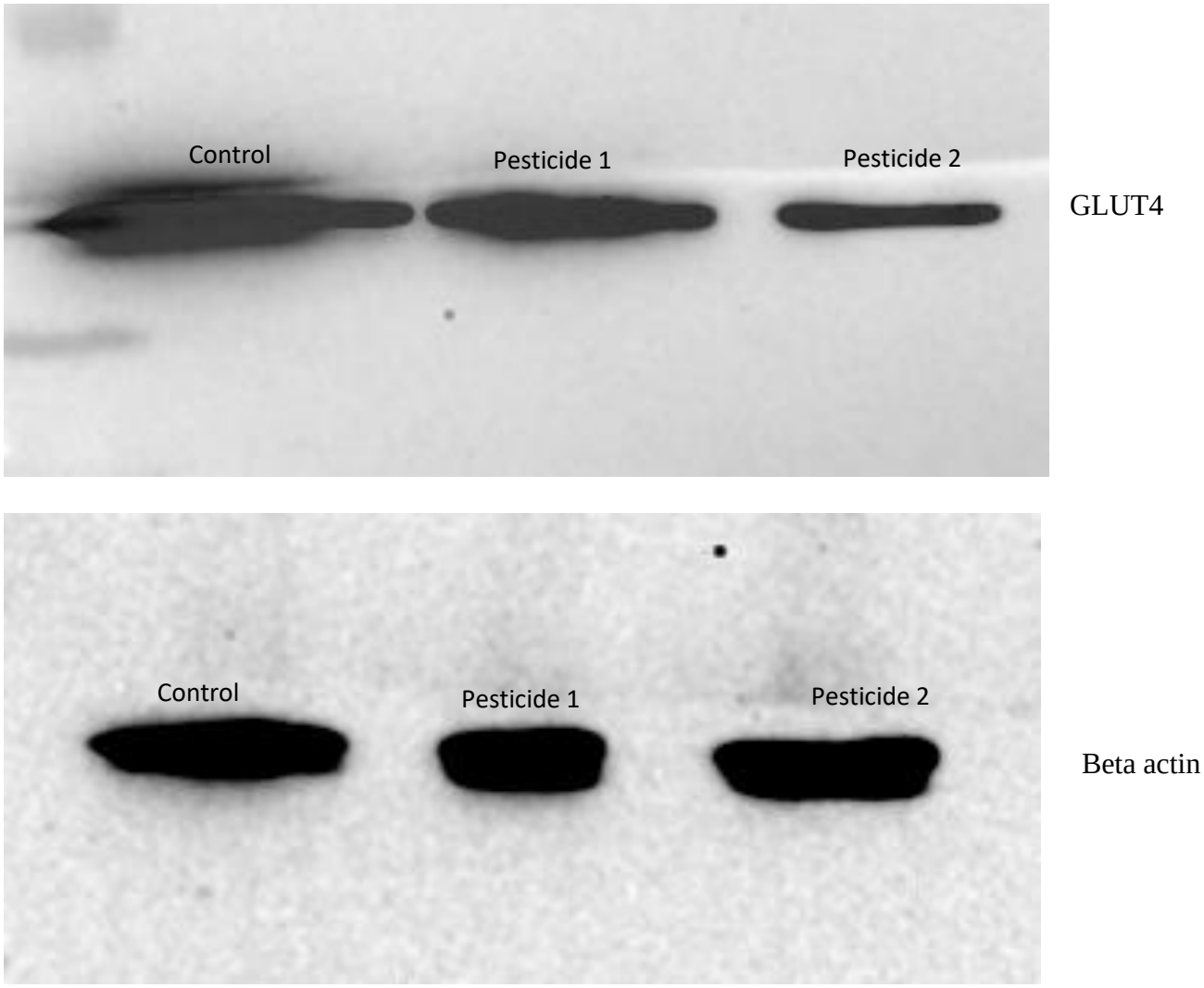
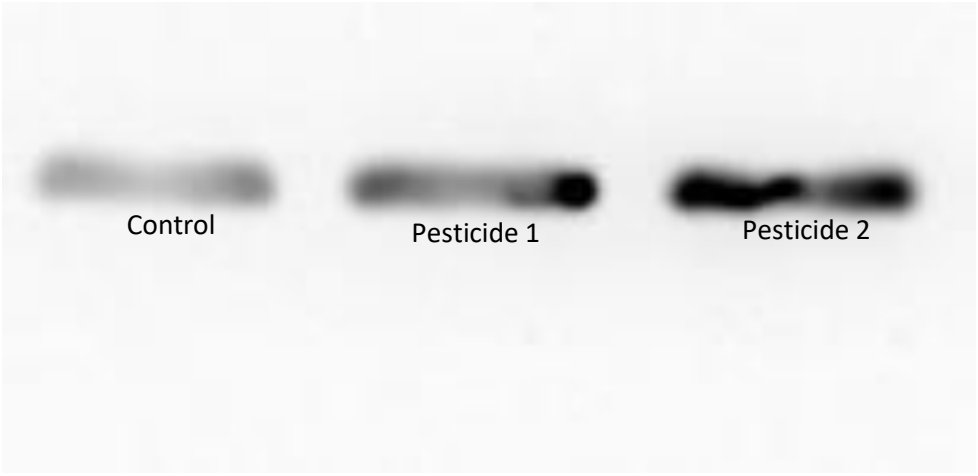
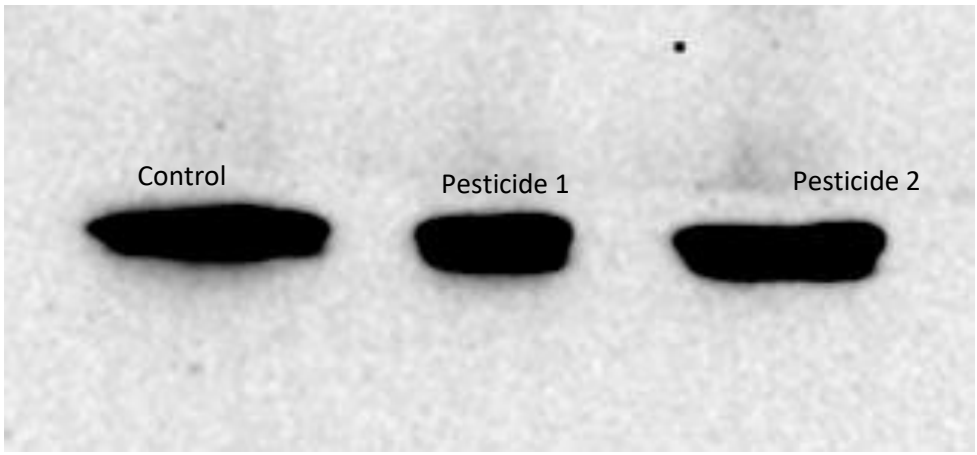


Figure 19: GLUT 4 and β-actin expression in female Wistar rats
Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos



Nuclear factor kappa β



Beta actin

Figure 20: NFKβ and β-actin expression in female Wistar rats
Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos

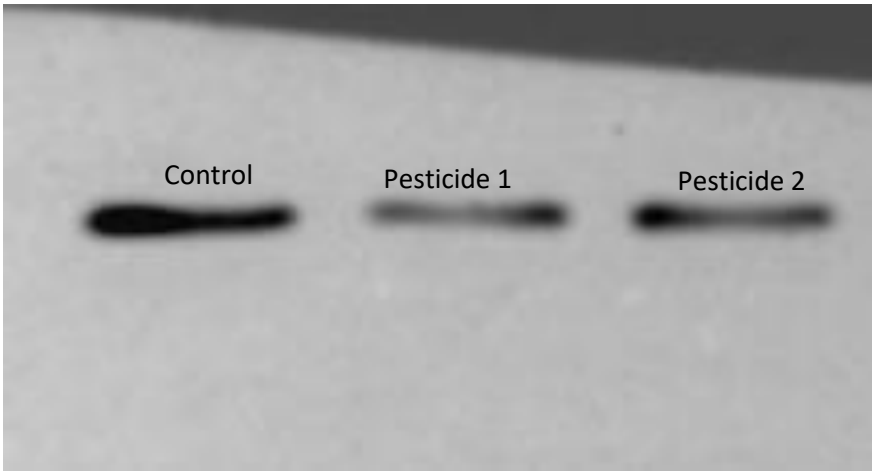
The expression of GLUT4 was significantly lower in Imidacloprid and Chlorpyrifos exposed groups compared to the control group, while NFkβ expression was significantly higher in Imidacloprid and the Chlorpyrifos exposed groups compared to the control group (Table XXII).

Table XXII: Expression level of glucose transporter 4 and nuclear factor kappa beta in female Wistar rats.

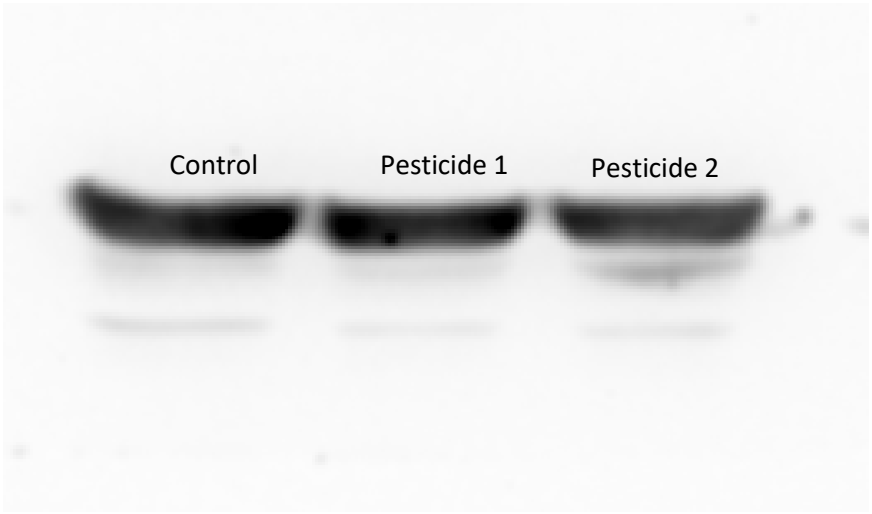
Sample	Control	Pesticide 1	Pesticide 2
GLUT4 expression	1.10 ± 0.15	0.59 ± 0.07**	0.31 ± 0.13***
NFkβ expression	1.51 ± 0.45	3.35 ± 0.66**	2.90 ± 0.38*

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, GLUT4 = Glucose transporter 4, NFkβ = Nuclear Factor Kappa Beta, *** = $p<0.001$, ** = $p< 0.01$, * = $p<0.05$ compared to the control group. Data are reported as mean ± SEM for 4 animals compared using ANOVA and Bonferroni post-test.

GLUT4 (Figure 21) and NFkβ (Figure 22) proteins were also expressed in adult offspring of control and exposed rats.



Glucose transporter 4



Beta actin

Figure 21: GLUT4 and β-actin expression in adult offspring of control and exposed rats.
Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos.

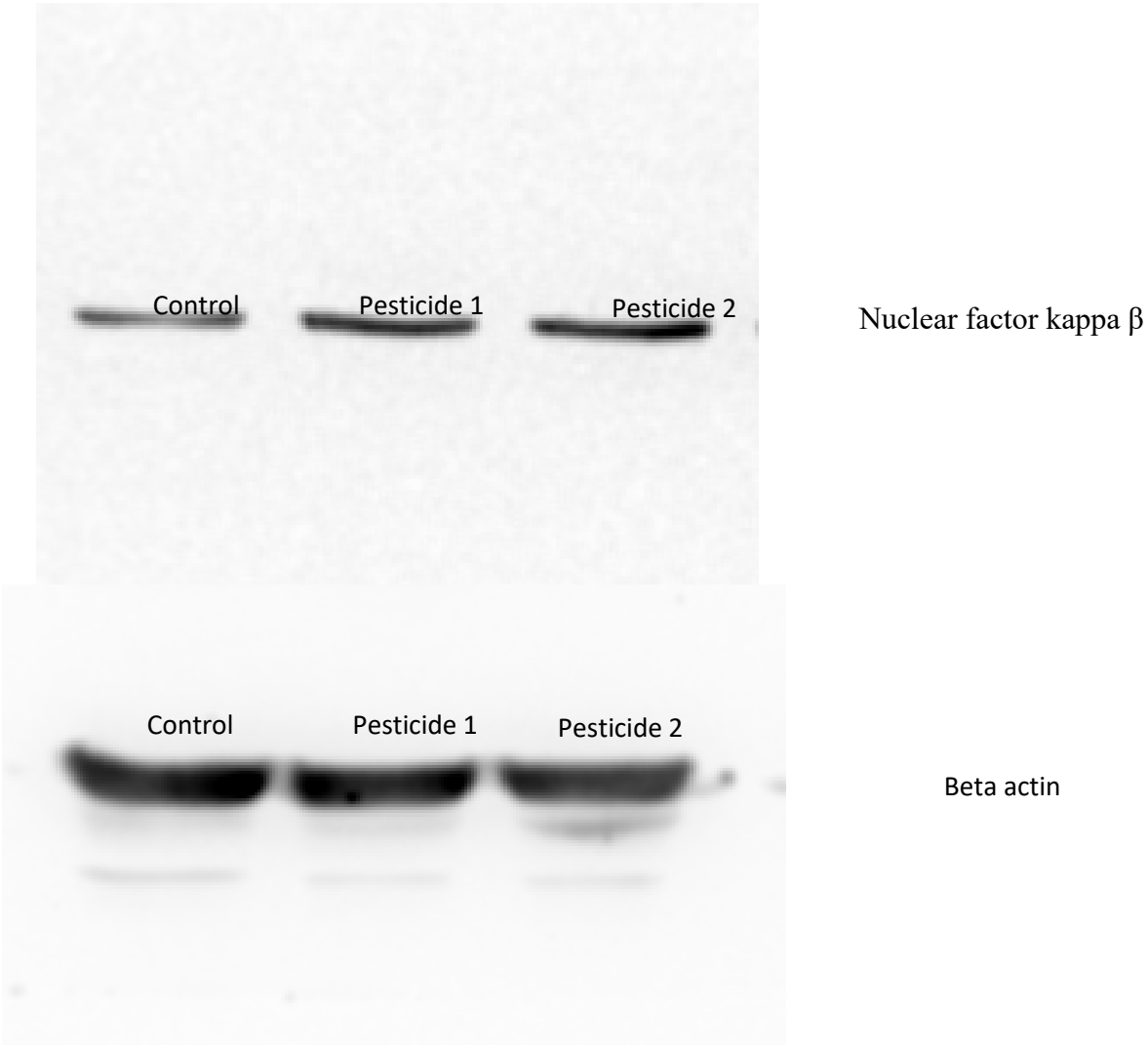


Figure 22: NFkβ and β-actin expression in adult offspring of control and exposed rats.
Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos.

The expression of GLUT4 was significantly lower in Imidacloprid and Chlorpyrifos exposed groups compared to the control group while the expression of NFkβ was significantly higher in Imidacloprid and Chlorpyrifos exposed rats compared to the control group (Table XXIII).

Table XXIII: Expression level of glucose transporter 4 and nuclear factor kappa beta in rats.

Sample	Control	Pesticide 1	Pesticide 2
GLUT4 expression	1.20 ± 0.21	0.71 ± 0.18**	0.79 ± 0.14*
NFkβ expression	0.85 ± 0.17	1.45 ± 0.30*	2.05 ± 0.25***

Legend: Pesticide 1 = Imidacloprid, Pesticide 2 = Chlorpyrifos, GLUT4 = Glucose transporter 4, NFkβ = Nuclear Factor Kappa Beta, *** = p<0.001, ** = p< 0.01, * = p< 0.05 compared to the control group. Data are reported as mean ± SEM for 6 rats compared using ANOVA and Bonferroni post-test.

III.1.4.3 Histology results

III.1.4.3.1 Effect of pesticides on liver histology

After staining with Haematoxylin and Eosin (H&E), the functional units observed on a light microscope with a magnification of 400 x were: hepatocytes, hepatic artery, portal vein and branches of the bile duct. In the control group of parents and offspring, all these features were observed to be normal. The hepatocytes of the liver were observed to be normal, each with a well-defined eosinophilic cytoplasm. The hepatic cells were large and polyhedral in shape with mono and binucleated nucleus. They contained plates, separated by hepatic sinusoids which are lined by endothelial cells and Kupffer cells and drain into the central vein. However, in the liver of the treated rats, there were histological damages observed. The liver showed mono-nuclear inflammatory cell infiltration and hyperplasia. Moreover, proliferation in Kupffer cells, dilated blood sinusoids and cytoplasmic vacuolization were also observed in the liver of the treated groups (Figure 23).

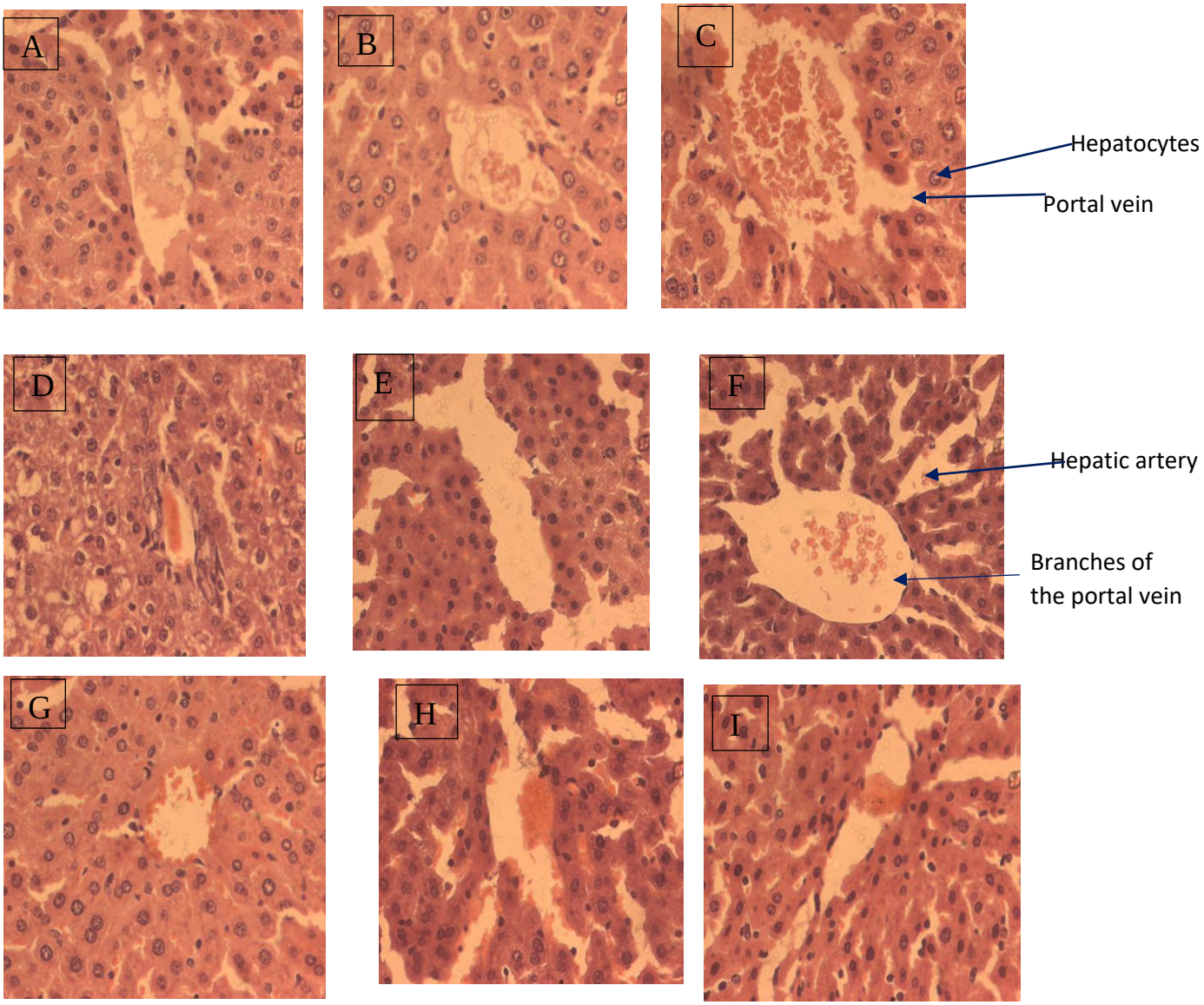


Figure 23: Representative histological images of rat liver examined under light microscope.
Legend: A = Control dams, B = Imidacloprid-treated dams, C = Chlorpyrifos-treated dams, D = Control 6-week offspring, E = 6-week offspring of dams treated with Imidacloprid, F = 6-week offspring treated with Chlorpyrifos, G = Control 12-week offspring, H = 12-week offspring of dams treated with Imidacloprid, I = 12-week offspring treated with Chlorpyrifos,

III.1.4.3.2 Effect of pesticides on kidney histology

The kidney was captured using a microscope with a magnification of 400 x. In the control rats of parents and offspring, the histoarchitecture of the glomeruli and renal tubules was observed to be normal. The podocytes were also observed to be normal in both the control and exposed rats and offspring. However, necrosis was observed in 4-week offspring exposed to Pesticide 1 and Pesticide 2. The kidney sections of rats exposed to Pesticide 1, Pesticide 2, and offspring of rats exposed to pesticide 1 were characterised by degeneration of glomerulus and renal tubules, vacuolization of renal

tubules, deposition of eosin positive substances in the glomeruli and renal tubules, as well as infiltration of the leucocytes (Figure 24).

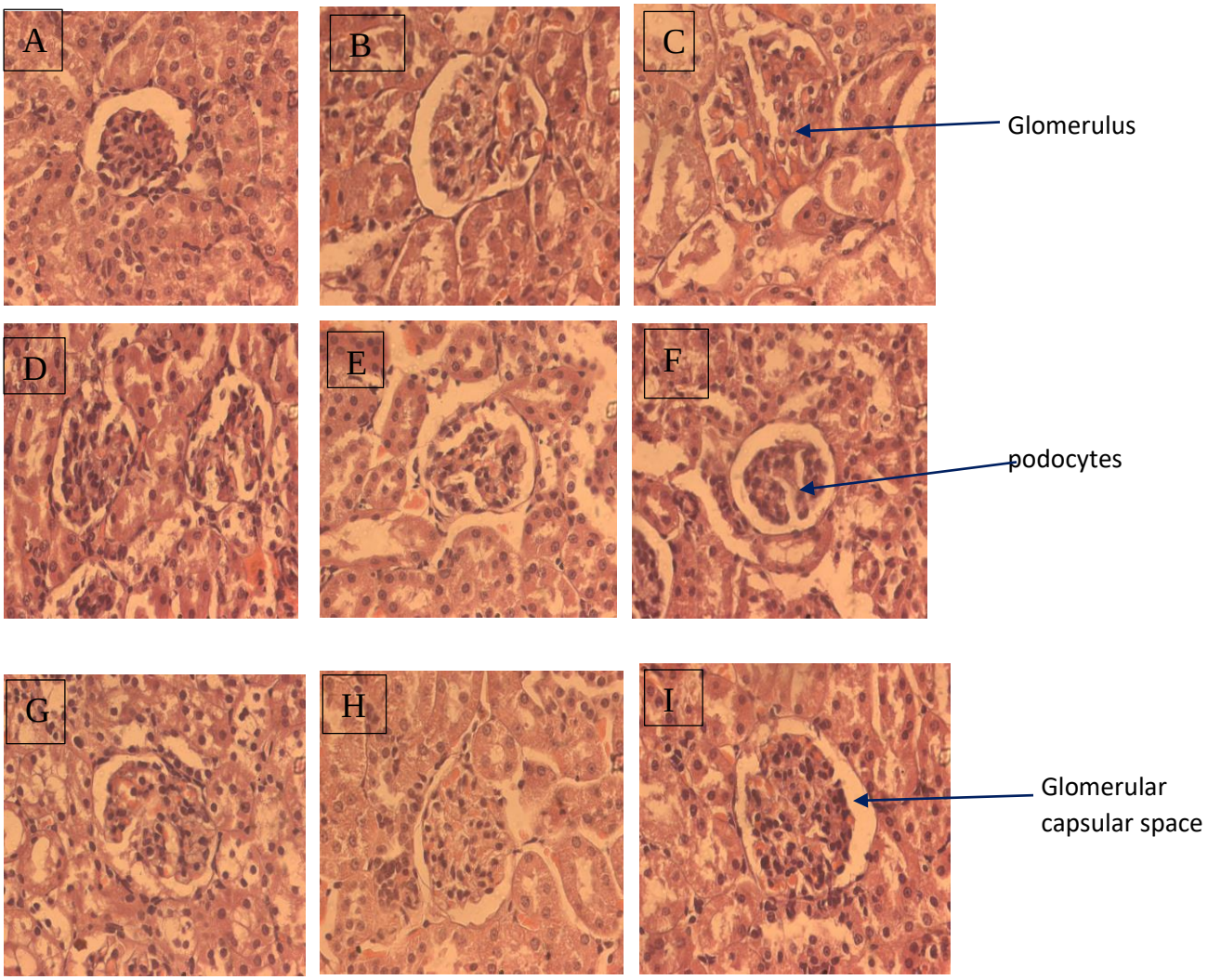


Figure 24: Representative histological images of rat kidney examined under a light microscope
Legend: A = Control dams, B = Imidacloprid-treated dams, C = Chlorpyrifos-treated dams, D = Control 6-week offspring, E = 6-week offspring of dams treated with Imidacloprid, F = 6-week offspring treated with Chlorpyrifos, G = Control 12-week offspring, H = 12-week offspring of dams treated with Imidacloprid, I = 12-week offspring treated with Chlorpyrifos.

III.1.4.3.3 Effect of pesticides on pancreas histology

Light microscopical examination of pancreas with a magnification of 400 x in control groups showed normal architecture of the pancreas in the form of normal islets of Langerhans surrounded by the pancreatic acini with normal interlobular connective tissues. The islets cells were interspersed between the acinar cells and were seen to be lightly stained compared to the acinar cells. The pancreatic lobules were separated by intact intra and inter-lobular connective tissues. However, in rats exposed to Pesticide 2 as well as their offspring, there was degeneration of cells of acini with widening of the

interlobular spaces. Rats exposed to Pesticide 1 and their offspring displayed similar features as the control group (Figure 25).

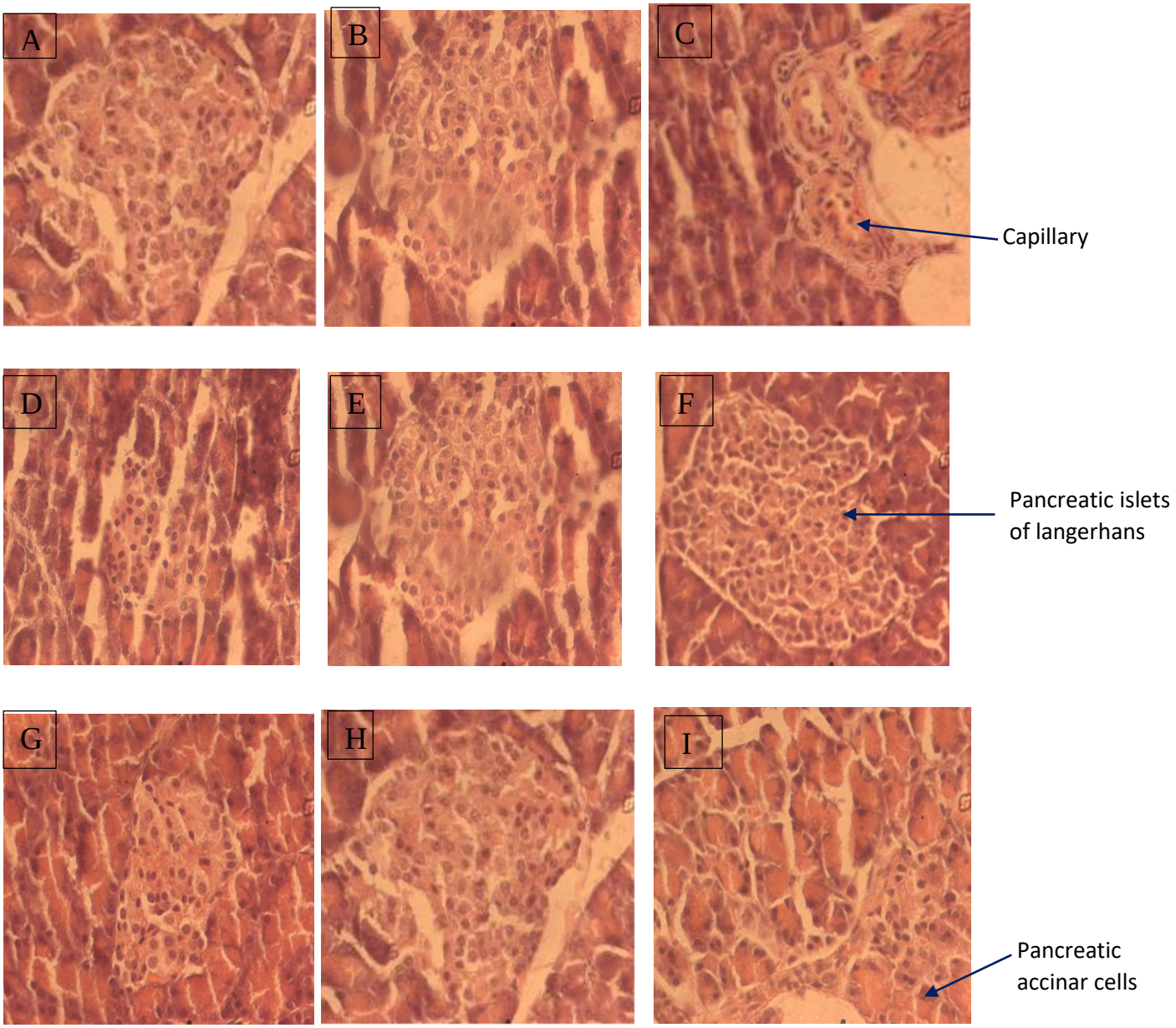


Figure 25: Representative histological images of rat pancreas examined under a light microscope.

Legend: A = Control dams, B = Imidacloprid-treated dams, C = Chlorpyrifos-treated dams, D = Control 6-week offspring, E = 6-week offspring of dams treated with Imidacloprid, F = 6-week offspring treated with Chlorpyrifos, G = Control 12-week offspring, H = 12-week offspring of dams treated with Imidacloprid, I = 12-week offspring treated with Chlorpyrifos.

III.2 DISCUSSION

The first part of the study was to determine the LD_{50} of Imidacloprid, Chlorpyrifos, Imidacloprid + Lambda cyhalothrin and Oxamyl. The results showed that the LD_{50} values of Imidacloprid (440mg/kg), Chlorpyrifos (135mg/kg) and Oxamyl (4mg/kg) were relatively similar to those of other published data (450mg/kg for Imidacloprid **(Tomlin, 2006),** 135 mg/kg for Chlorpyrifos **(McCollister *et al.*, 1974)** and 2.5 – 5.4mg/kg for Oxamyl **(Kennedy, 1986)).** The LD_{50} of the combination containing equal amount of Imidacloprid + Lambda cyhalothrin (56mg/kg) was much lower than that of Lambda cyhalothrin alone observed in male rats (79mg/kg) in the literature **(Kidd and James 1991**). This confirms the fact that the lethal dose of each pesticide for the same route of exposure depends on the sex, the environmental conditions of exposure and the biotransformation process. The second part of the study was to investigate the effects of exposure to studied pesticides on markers of oxidative stress and lipid peroxidation, metabolic markers and the risk of Type 2 DM. The present study shows that all the pesticides investigated induced oxidative stress in female Wistar rats characterised by alterations in circulating levels of at least one antioxidant enzyme in liver and/or kidney tissues. The observed increase in lipid peroxidation as well as high plasma ALT and AST levels in female Wistar rats exposed to Imidacloprid and Chlorpyrifos were confirmatory of tissue damage. Moreover, metabolic markers including plasma glucose, insulin, lipid profile, HOMA-IR and protein expression markers (GLUT4 and NFkβ) were altered in the pesticide exposed groups compared to the control group.

The significantly higher FBG in female Wistar rats exposed to Imidacloprid, Chlorpyrifos and Oxamyl compared to the control group was an indication of the hyperglycaemic effect of these pesticides. This was not surprising in rats exposed to Chlorpyrifos since this pesticide has been shown to increase blood glucose in male rats **(Rahimi and Abdollahi, 2007, Acker and Nogueira, 2012; Hamza *et al.*, 2014**). The possible mechanism of organophosphate induced hyperglycemia has been investigated even though few studies explored Chlorpyrifos on glucose and lipid homeostasis (**Acker and Nogueira, 2012)**. The observed pathways include: stimulation of hepatic gluconeogenesis and glycogenolysis by malathion **(Abdollahi *et al.*, 2004**), activation of hypothalamus–pituitary–adrenal (HPA) axis by Chlorpyrifos (**Rahimi and Abdollahi, 2007**) and increase activities of hepatic gluconeogenesis enzymes, such as tyrosine aminotransferase (TAT) and glucose-6-phosphatase (G6Pase) by acephate **(Joshi and Rajini, 2009**) and monocrotrophos **(Joshi and Rajini, 2012**). The activation of the HPA axis by organophosphate pesticides causes secretion of glucocorticoids from adrenal cortex that in turn increases blood glucose by induction of the gluconeogenesis pathway. Moreover, organophosphate pesticides including chlorpyrifos have been observed to increase the risk of metabolic syndrome by

inducing hypertension, hyperglycemia, hyper insulinemia and dyslipidemia in Cameroonians and Pakistanis (**Leonel Javeres *et al.*, 2021).**

In this study, insulin resistance was recorded in rats exposed to the Imidacloprid, Chlorpyrifos and Oxamyl, characterised by a significant higher HOMA-IR values in exposed rats compared to the control group. This phenomenon (insulin resistance) often precedes hyperglycemia when not properly controlled. In the non-diabetic state, hyperglycemia stimulates insulin secretion to enable glucose uptake into the cells. The state of hyperglycemia is very common immediately after a meal and later returns to normal (normal glucose and insulin levels). However, with hyperglycemia during fasting, there is a possibility of insulin resistance characterized by high blood glucose and high fasting insulin levels as indicated by our results. However, beta-cell function was not altered in pesticides exposed group as the difference in HOMA-β values was not significant when compared to the control group. With hyperglycemia generally associated with gluco-toxicity and lipo-toxicity, dyslipidemia was investigated by measuring the different lipid parameters. Dyslipidemia was recorded in rats exposed to Imidacloprid, Chlorpyrifos and Oxamyl as observed by an alteration in at least one of the lipid profile parameters in these groups. Several studies have observed alterations in lipid profile parameters in rats exposed to Chlorpyrifos and other organophosphate pesticides **(Ibrahim and El-Gamal, 2003; Lasram *et al.*, 2009; Acker and Nogueira, 2012).** Moreover, Lambda cyhalothrin and Imidacloprid have been reported to possess insulin resistant and dyslipidemic effects both *in-vivo* and *in-vitro* (**Kim *et al.*, 2013; Ghosh *et al.*, 2016**).

To investigate the possible mechanisms of insulin resistance, the expression of two proteins was analysed. They included: nuclear factor kappa beta (NFkβ) which regulates the expression of proteins involved in the inflammatory pathways and glucose transporter 4 (GLUT4) which is an important protein playing a primordial role in glucose uptake into cells. NFkβ has been shown to be responsible for the development of insulin resistance through the inflammatory pathway. In the muscle samples of Imidacloprid, Chlorpyrifos and control rats, GLUT4 and NFkβ proteins were expressed. However, the expression of NFkβ protein was significantly higher in rats exposed to Imidacloprid and Chlorpyrifos when compared to the control group. Hyperglycaemia could be responsible for the activation and increase in the expression of NFkβ. This study shows that hyperglycemia resulting from Imidacloprid and Chlorpyrifos exposure could activate the expression of NFkβ protein which in turns affects the inflammatory pathway leading to the development of insulin resistance. On the other hand, GLUT4 protein was highly expressed in the control group when compared to the Imidacloprid and Chlorpyrifos exposed groups. Several studies have reported a decrease in the expression of GLUT4 protein in

diabetic individuals **(Camps *et al.*, 1992).** Generally, a decrease in the expression of GLUT4 protein in insulin sensitive tissues results from low circulating insulin levels in individuals with T2D **(Camps *et al.*, 1992)**. Contrarily, insulin levels were observed to be higher in the exposed groups compared to the control group in our study. This difference could be explained by the phenomenon of insulin resistance in the exposed groups which is the major contributing factor for hyper-insulinemia. In this condition, uptake of glucose into cells is reduced which in turn leads to higher blood sugar levels.

In normal cells, there is a balance between the reactive oxygen species and the antioxidant system. These reactive oxygen species can be produced naturally to control physiological processes including apoptosis, necrosis and phagocytosis or when the cell is exposed to external factors such as environmental contaminants. In the latter case, there often occur a tremendous increase in the levels of ROS and consequently a disturbance of the system resulting to oxidative stress. Initially, there is an increase in the level of antioxidants to counteract the effect of these ROS. However, prolonged exposure leads to depletion of the antioxidant system resulting to cellular damage **(Trevisan *et al.*, 2001).** The two most important antioxidant defense mechanism against oxidative stress are SOD and catalase **(Nasr *et al.*, 2016).** SOD whose catalytic function was discovered by McCord and Fridovich (1969) plays a primordial role in the conversion of potentially dangerous superoxide radical to hydrogen peroxide and oxygen **(McCord and Fridovich, 1969**). The hydrogen peroxide released is further broken down by catalase to water which is a non-toxic molecule. Other scavengers of ROS are glutathione peroxidase (GPx), Glutathione Reductase (GR), glutathione S-Transferase (GST) and reduced Glutathione (GSH). GPx, GR and GST are enzymatic antioxidant while GSH is non-enzymatic antioxidant. GPx and GST are two important enzymes in the phase II reactions of detoxification. They catalyse the conjugation of several xenobiotics with GSH thereby eliminating these toxic substances. Glutathione reductase on the other hand serves in the regeneration of GSH which is an antioxidant reacting with free radicals and organic peroxides. The concentration of these enzymes will either increase or decrease when the cell is undergoing oxidative stress depending on the stage of the condition.

The present study revealed that all the pesticides tested induced oxidative stress in female Wistar rats, characterised by alterations in circulating levels of at least one antioxidant enzyme in liver and/or kidney tissues. The observed increase MDA and high plasma ALT and AST levels in rats exposed to Pesticide 1 and Pesticide 2 were confirmatory of tissue damage. Similar alterations observed in parents were recorded in offspring at weaning and at adult age. Oxidative stress in liver and kidney were observed in offspring of rats exposed to pesticides when compared to the control group characterized

by alterations in antioxidant levels. Moreover, Pesticide 1 and Pesticide 2 were shown to induce lipid peroxidation in offspring of rats exposed to these two chemicals. In a similar manner, *in utero* exposure to all 4 pesticides induced liver injury as observed by a 3-fold increase in plasma ALT and AST when compared to the control group. Our findings in directly exposed animals are in accordance with most previous studies**,** with Lambda-cyhalothrin observed to induce oxidative stress both *in-vivo* and *in-vitro* **(El-Demerdash, (2007); Fetoui *et al.* (2008, 2009) Abdallah *et al.* (2012))**. It significantly increased liver and kidney MDA levels of male adult Wistar rats (**Fetoui *et al.*, 2009, 2010).** Similarly**, Balani *et al.* (2011); El-Gendy *et al*. (2010); Kapoor *et al*. (2010) and Mohany *et al.* (2011)** observed dysregulation in oxidative stress and lipid peroxidation biomarkers in rats exposed to Imidacloprid. Furthermore, Chlorpyrifos was observed to induce oxidative stress as well as increase levels of lipid peroxides in the kidney, liver and red blood cells of rats (**Gultekin *et al.*, 2001; Oncu *et al.*, 2002; Verma *et al.*, 2007; Tuzmen *et al.*, 2008; Ahmed and Zaki, 2009; Baş and Kalender, 2011**). Oxidative stress has also been reported in Cameroonians and Pakistanis exposed to chlorpyrifos amongst other organophosphate pesticides (**Leonel Javeres *et al.*, 2020)**. Notwithstanding, ALT and/or AST levels were significantly increased in plasma of exposed rats and their offspring (**Soujanya *et al.*, 2013; Gopinath *et al.*, 2014**). However, the pesticide mixture (5.6g /kg imidacloprid + 5.6g /kg lambda cyhalothrin) did not affect the liver function enzymes which could be due to the low dose or an antagonistic effect of the combination.

The results on oxidative stress, lipid peroxidation and dysregulation in liver function enzyme levels are in conformity with the histological slide of the liver and kidney. Mononuclear cell infiltration and inflammation observed in the liver and the degeneration of glomerulus and renal tubules could probably result from oxidative stress induced by the pesticides investigated. Moreover, the degeneration of cells of acini could be an early stage of beta cell dysfunction since HOMA-β values were not significantly different between the control and exposed groups.

The mechanism of generation of oxidative stress is related to pesticide biotransformation as in the case of Chlorpyrifos. Biotransformation of Chlorpyrifos by cytochrome P450 generates its metabolites Chlorpyrifos oxon and 3,5,6-trichloro-2-pyridinol. These metabolites being more toxic than the parent compound directly or indirectly generate reactive oxygen species such as highly reactive superoxide radical and hydrogen peroxide (**Verma *et al.*, 2007**). At the onset, there is an increase in the level of antioxidants to counteract the effect of these ROS. However, prolonged exposure leads to depletion of the antioxidant system resulting to cellular damage **(Trevisan *et al.*, 2001**) and oxidative stress. The end stage of oxidative stress is impairment of cellular functions and potential tissue damage which is

characterized by lipid peroxidation (increased MDA levels) and leakage of cytosolic enzymes from hepatocytes with increase in ALT and AST enzyme levels.

What is new in our findings is the oxidative stress, lipid peroxidation and metabolic effects of the carbamate insecticide oxamyl in female Wistar rats as well as their offspring. Several carbamate insecticides such as carbofuran, carbosulfan and cartap have been shown to induce oxidative stress, lipid peroxidation and dyslipidemia in Wistar rats **(Rai *et al.*, 2009; Jaiswal *et al.*, 2013; El-Bini Dhouib *et al.*, 2015).** However, to the best of our knowledge, studies on oxamyl are lacking despite being one of the carbamates widely applied in intensive agriculture and incorporated into the surface soil (10 to 15 cm deep) for the control of insects and nematodes (**Tomlin, 2002).** Our study is therefore one of the 1st to report oxidative stress, lipid peroxidation and metabolic effects of oxamyl exposure in Wistar rats. Another important aspect is the deleterious effects of studied pesticides observed in offspring with *in-utero* exposure that was evidenced at weaning and persisted with similar magnitude at adult age without additional exposure. **Tukhtaev *et al.* (2012**) observed similar alterations in oxidative stress enzymes in offspring of rats with *in-utero* exposure to pesticides. However, they reported that the alterations decreased both in exposed rats and their offspring after cessation of breast milk. The difference could be because of the dose used given that the dose in our study was 10 times higher. Therefore, pesticide exposure at certain doses could induce permanent alteration in exposed organisms. Even though pesticides were not quantified in body fluids of offspring, it is possible that alterations in offspring resulted from foetal exposure since these chemicals have been shown to cross the placenta (**Abdel-Rahman *et al.*, 2002**).

With the scope of the study, it was not feasible to include a positive control group containing rats with Type 2 DM. Given that the pesticides were neither quantified in the biological fluids of parents nor offspring, we cannot affirm that the adverse effects observed in offspring were mainly due to foetal exposure, as alterations in the parents could be transmitted to offspring through foetal programming. Another limitation is the fact that we did not study the second generation necessary to establish putative trans-generational effect or conduct methylation analysis to confirm the effect on the epigenome as well as the expression of other proteins involved in the insulin signalling pathway such as pPKB, p-mTOR, and p-IRS-1. While these remain pertinent and would have complemented the findings, such aspiration would require more financial means beyond those available for the research grant. Therefore, further studies looking at the epigenome to investigate the molecular mechanism as well as the second and third generation to characterize the trans-generational effect are warranted.

CONCLUSION

This study was aimed at investigating the effect of exposure to commonly used pesticides on oxidative stress and metabolic parameters in female Wistar rats and their offspring. Metabolic parameters investigated were oxidative stress, lipid peroxidation, lipid profile, glucose tolerance and the expression of GLUT4 and NFkβ proteins. From the results obtained, the following conclusions could be drawn.

1/10 LD_{50} of Chlorpyrifos, Imidacloprid, Oxamyl and a mixture of Lambda cyhalothrin + Imidacloprid induced oxidative stress by modulating antioxidant enzyme concentration and lipid peroxidation in female Wistar rats.

In-utero exposure to Chlorpyrifos, Imidacloprid, Oxamyl and a mixture of Lambda cyhalothrin + Imidacloprid induced oxidative stress by modulating antioxidant enzyme concentration and lipid peroxidation in offspring of Wistar rats at weaning. These deleterious effects are observed to persist until adulthood after cessation of exposure.

Imidacloprid, Chlorpyrifos and Oxamyl altered the metabolic pathways of glucose and lipid metabolism in female Wistar rats and their offspring at weaning and the deleterious effects were observed to persist in offspring until adulthood after cessation of exposure in offspring exposed to Imidacloprid and Chlorpyrifos.

These findings suggest that exposure to pesticides could be a contributing factor to the worldwide increase in the incidence and prevalence of diabetes. Therefore, future studies are needed to elucidate the current findings in humans. Such studies might inform public health and agricultural policy makers on the need to regulate the use of pesticides especially in a country like Cameroon where the drive to a second-generation agriculture has paved the way for an increase in the use of pesticides sometimes by farmers not wearing any form of protection nor respecting prescribed doses.

PERSPECTIVES

- Quantify pesticides in body fluids of exposed rats and their offspring to determine their bioaccumulation and persistence, and investigate the possibility of exposure through lactation
- Evaluate the effect of tested chemicals on gene expression and epigenetics to establish molecular targets. This is especially taking into consideration the fact that these chemicals have been shown to induce deleterious effects in offspring with *in-utero* exposure therefore suggesting foetal programming of disease.
- Evaluate the expression of other proteins involved in the insulin sensitivity and insulin signalling pathways including pPKB, p-mTOR, and p-IRS-1.
- Carry out similar studies in humans to see if the findings can be translated to the human population. This will provide information on the diseases associated with chronic low dose exposure to pesticides and enable policy makers to regulate on the use of these chemicals.

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Appendix 1: Preparation of reagents in the Laboratory

Normal saline (0.9 % NaCl)

A 100 mL normal saline solution was prepared by weighing 0.9 g of sodium chloride into a clean 100 mL beaker. About 50 mL distilled water is added and stirred until the salt dissolved completely. The solution was then poured into a 100 mL volumetric flask with the help of a funnel and completed to the 100 mL mark with distilled water and the solution stored at rtp.

Phosphate buffer (0.1 M pH 7.4)

Prepare 0.1 M anhydrous sodium phosphate dibasic by dissolving 1469.6 mg of salt in 100 mL solution with distilled water. Prepare 0.1 M sodium phosphate monobasic dehydrate by dissolving 1560.1 mg of the salt in 100 mL with distilled water. Titrate the monobasic salt against the dibasic salt while recording the pH and stop at exactly 7.4. Store the solution at 4°C.

D – Glucose (50 %)

A 100 mL solution was prepared by dissolving 50 g in distilled water and the volume completed to 100 mL with distilled water.

Lowry's solution

A 250 mL Lowry's solution was prepared as follows. About 150 mL distilled water was poured into a 250 mL beaker. Hydrated copper (II) sulphate (650 mg) and sodium potassium tartrate (98.3 mg) were weighed and dissolved in the solution in the beaker by stirring. Sodium hydroxide (500 mg) and sodium carbonate (2.5 g) were added into the reaction mixture. The solution was poured into a 250 cm^3 volumetric flask and the volume completed to the mark with distilled water and stored at rtp.

Tris – EDTA buffer (50 mM Tris + 1 mL EDTA pH 8.5)

A 100 mL Tris – EDTA buffer was prepared as follows. About 75 mL of distilled water was placed in a clean beaker. Tris (607 mg) and EDTA (37.23 mg) were weighed and dissolved in the solvent. The pH was adjusted to 8.5 with concentrated HCl. The solution was then poured into a 100 mL volumetric flask and the volume completed to the mark with distilled water and stored at rtp for immediate use or at 4 °C.

Pyrogallol (20 mM)

A 25 mL pyrogallol solution was prepared by weighing 63 mg of pyrogallol and dissolving in about 10 mL of distilled water. The solution was poured into a 25 mL volumetric flask and the volume completed with distilled water to the mark and the solution stored at rtp.

NB: the solution was used only for a single day

Phosphate buffer (50 mM pH 7.0)

Prepare 0.05 M anhydrous sodium phosphate dibasic by dissolving 734.8 mg of salt in 100 mL solution with distilled water. Prepare 0.05 M sodium phosphate monobasic dehydrate by dissolving 780.05 mg of the salt in 100 mL with distilled water. Titrate the monobasic salt against the dibasic salt while recording the pH and stop at exactly 7.0. Store the solution at 4°C.

Hydrogen peroxide 60 mM

A 50 mL solution was prepared by pipetting 340.1 uL hydrogen peroxide into a 50 mL volumetric flask. Distilled water was added to the mark and the solution stored.

TBA Reagent mixture

A 50 mL solution of the reagent mixture was prepared by dissolving 187 mg of TBA, 10 g of TCA and 5 mg of BHT into about 30 mL distilled water. 12.5 mL of HCl 1 N was added and heated at 40 °C until complete dissolution. The mixture was transferred into a 50 mL volumetric flask and the volume completed to the mark and stored.

GPx reaction mixture

A 100 mL solution was prepared by dissolving 7.027 mg sodium azide, 1.081 mg NADPH reduced, 33.24 mg GSH and 14.88 mg EDTA with 0.05 M phosphate buffer, pH 7.0 to 100mL.

Hydrogen peroxide (0.042 %)

A 25 mL solution was prepared by pipetting 35 uL hydrogen peroxide into a 25 mL volumetric flask. Distilled water was added to the mark and the solution stored.

Potassium dihydrogen phosphate monobasic anhydrous buffer (100 mM pH 6.5)

Dissolve 680.45 mg of potassium dihydrogen phosphate anhydrous and 18.61 mg EDTA in about 40 mL distilled water. Adjust the pH to 6.5 with 1 M KOH and complete the volume to 50 mL with distilled water.

CDNB (30mM)

60.765 mg of CDNB was weighed and added to 9.5 mL distilled water. The volume was completed to 10 mL with absolute alcohol.

Phosphate buffer (0.2 M pH 8.0)

Prepare 2 M anhydrous sodium phosphate dibasic by dissolving 2.9392 g of salt to 100 mL solution with distilled water. Prepare 2 M sodium phosphate monobasic dehydrate by dissolving 3.1202 g of the salt to 100 mL with distilled water. Titrate the monobasic salt against the dibasic salt while recording the pH and stop at exactly 8.0. Store the solution at 4°C.

GSH standard (200 ug/mL)

To prepare a 25 mL solution, 5 mg of GSH was weighed and dissolved with distilled water to obtain a 25 mL solution.

Protein extraction buffer A

A 50 mL solution was prepared as follows. In a beaker containing about 30 mL distilled water, 8.57 mg sucrose, 119.15 mg HEPES pH 7.9, 15 mg anhydrous magnesium chloride, 30 mg potassium chloride, 5 ml glycerol, 18 mg EDTA, 7.7 mg DTT and 8 mg PMSF were added and stirred to dissolve completely. The solution was poured into a 50 mL volumetric flask and the volume completed to the mark with distilled water.

Protein extraction buffer B

A 50 mL solution was prepared as follows. In a beaker containing about 30 mL distilled water, 238.3 mg HEPES pH 7.9, 15 mg anhydrous magnesium chloride, 0.8 mg sodium chloride, 10 ml glycerol, 3.7 mg EDTA, 7.7 mg DTT and 8 mg PMSF were added and stirred to dissolve completely. The

solution was poured into a 50 mL volumetric flask and the volume completed to the mark with distilled water.

Tris HCl (1.5 M, pH 8.8)

A 50 mL solution was prepared by dissolving 9.75 g of Tris in 20 mL of distilled water. The pH was adjusted to 8.8 with 1 N HCl and the volume completed to 50 mL.

Tris HCl (0.5 M, pH 6.8)

A 50 mL solution was prepared by dissolving 3.025 g of Tris in 20 mL of distilled water. The pH was adjusted to 6.8 with 1 N HCl and the volume completed to 50 mL.

SDS (10 %)

Dissolve 0.5 g SDS in 4.5 ml deionized water with gentle stirring and bring to 5 ml. Store at room temperature.

Bromophenol blue (1 %)

A 10 mL solution of bromophenol blue was prepared by mixing 100 mg with water and the volume completed to 10 mL. The solution was then stored at rtp.

30 % Acrylamide solution

Dissolve 30 g acrylamide and 0.8 g N'N'-bis-methylene-acrylamide to 100 mL solution with deionized water. Store in the dark at 4° C.

Ammonium persulphate (10 %)

Dissolve 1 g APS (electrophoresis grade) in 10 mL deionized water

Electrophoresis buffer

A 10 X solution was prepared by dissolving 30.3 g Tris base, 144 g glycine and 10 g SDS in about 800 mL of distilled water. The volume was completed to 1 L with water.

Sample loading buffer

Mix 3.9 mL deionized water, 1.0 mL of 0.5 M Tris pH 6.8, 0.8 mL Glycerol, 1.6 mL of 10 % SDS, 0.4 mL of 2-mercaptoethanol and 0.4 mL of 1% bromophenol blue and store in the freezer. `

Loading gels

32.5 mL Tris buffer pH 8.8 was mixed with 2 mL SDS 10 % and the volume completed to 100 mL with distilled water. The solution was labelled as solution A. Another solution labelled solution B was prepared by mixing 5 mL Tris buffer pH 6.8 with 0.5 mL SDS 10 % and 4.5 mL distilled water. Solution A was to prepare the separating gel while solution B was used to prepare the stackling gel.

Separating gel (10 %)

For 10 % separating gel (10 mL solution), 3.8mL ddH2O, 3.4mL of acrylamide solution was mixed with 2.7 mL of solution A and 3.8 mL of distilled water. 100 µL of 10% APS and 10 µL of TEMED were rapidly added, mixed by pipetting and the gel loaded.

Stacking gel (5 %)

The stacking gel was prepared at a concentration of 5 %. For 5 mL solution, 838 uL of acrylamide solution was mixed with 1.25 mL solution B and 2.88 mL distilled water. 38 uL APS 10 % and 3.8 uL TEMED were rapidly added, mixed by pipetting and the gel loaded

Transfer buffer

A 1 L solution was prepared by dissolving 3.02 g Tris and 14.4 g glycine in about 500 mL of distilled water. Three hundred mL of methanol was added, and the volume completed to 1 L with distilled water.

PBS (10 X)

In a 1 L beaker, 80 g of NaCl, 2 g of KCl, 2 g of KH_2PO_4 and 9.2 g of Na_2HPO_4 were dissolved in about 800 mL distilled water. The solution was poured into a 1 L volumetric flask and the volume completed with distilled water to the mark.

PBST (1 X PBS and 0.1 % tween – 20)

In a 1 L beaker, 100 mL of 10 X PBS was diluted to 1 L with distilled water. One mL of tween – 20 was added and the solution stored at 4 °C.

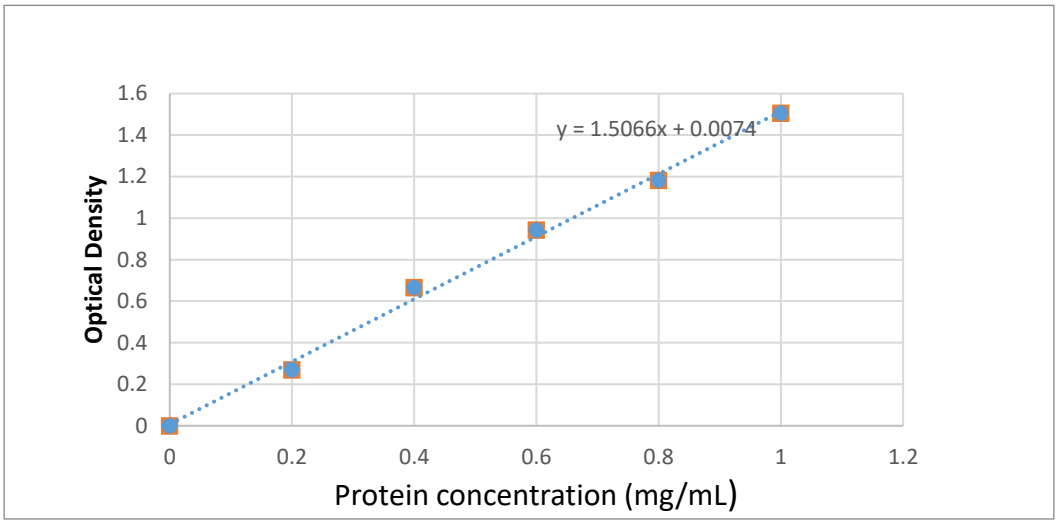
Blocking solution

The blocking solution (5 % BSA) was prepared as follows. In about 30 mL of PBS 1 X, 2.5 g of BSA was added and the mixture swirled gently. At complete dissolution, the volume was completed to 50 mL with PBS 1 X

Appendix 2: Calibration curves

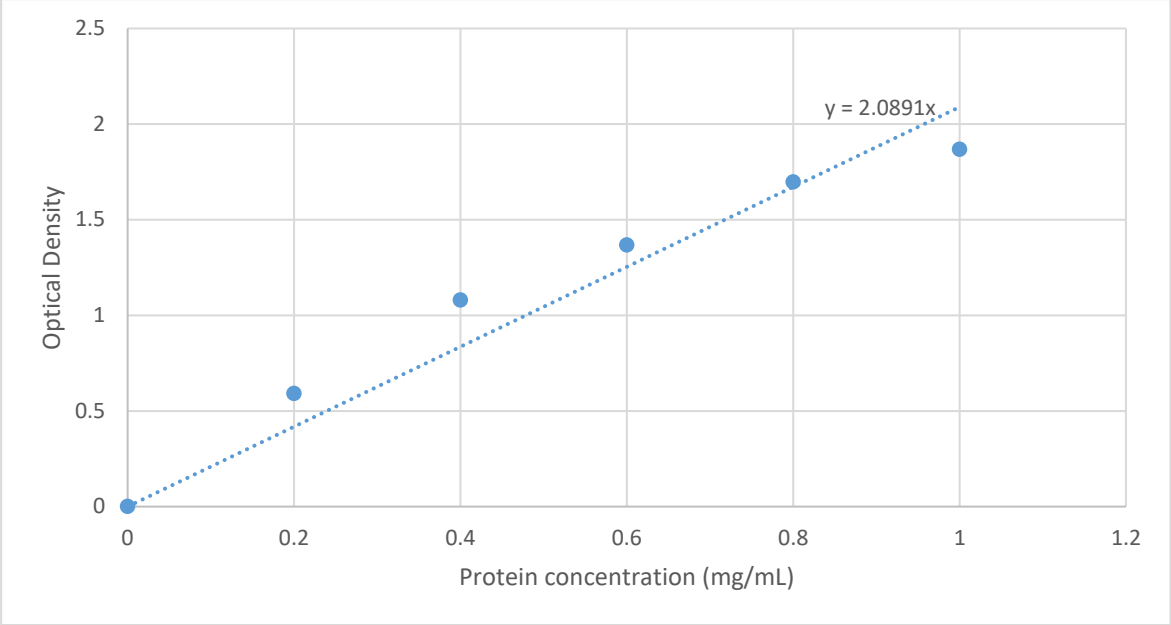
Calibration curve of liver protein: OD = f (BSA concentration in mg/mL)

Tube	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Protein concentration (mg/mL)	0	0.2	0.4	0.6	0.8	1
OD	0	0.269	0.665	0.942	1.182	1.506



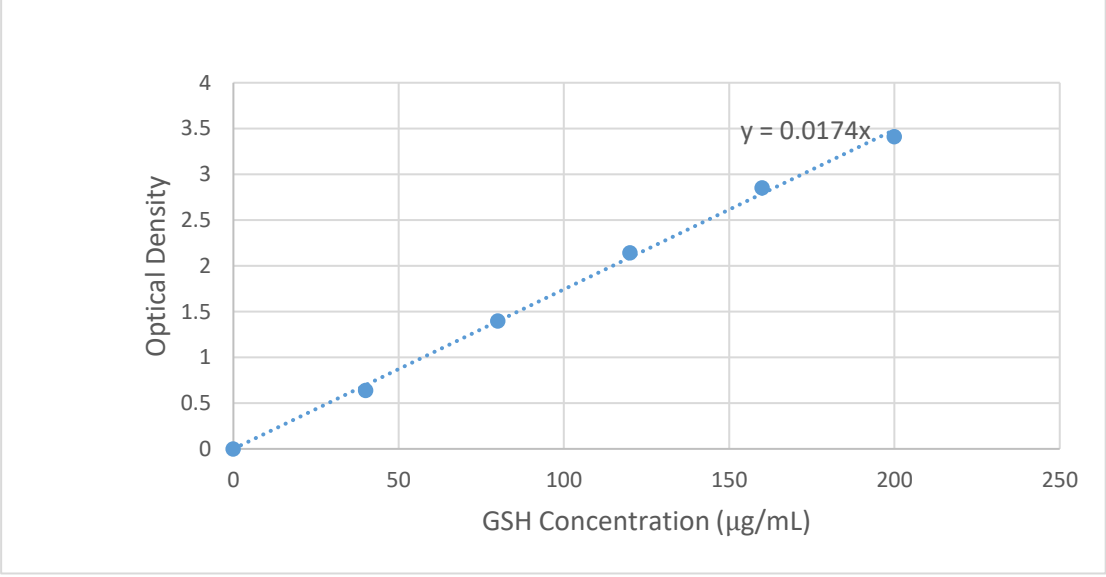
Calibration curve of kidney protein: OD = f (BSA concentration in mg/mL)

Tube	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Protein concentration (mg/mL)	0	0.2	0.4	0.6	0.8	1
OD	0	0.591	1.08	1.367	1.697	1.868



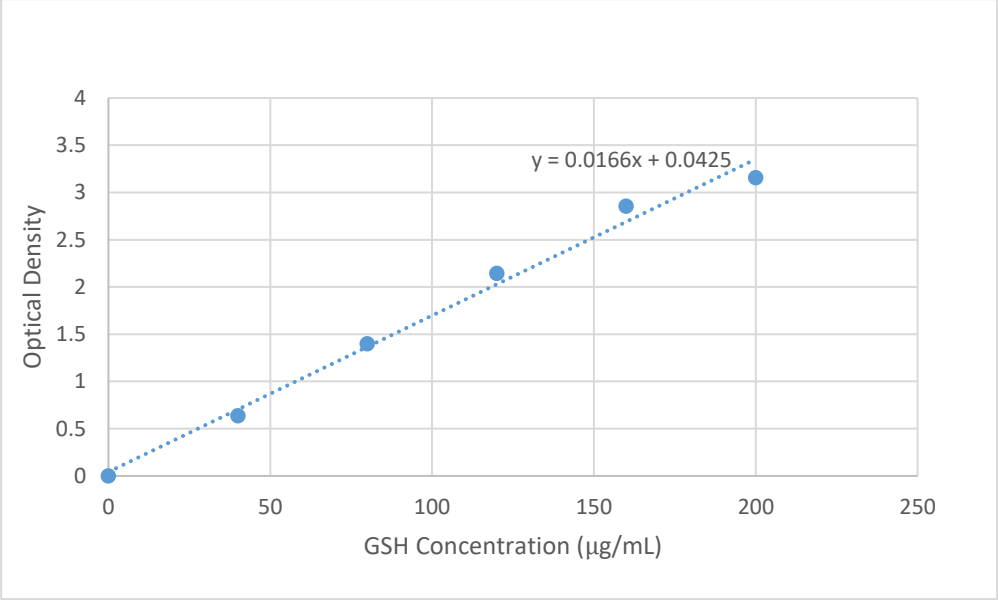
Calibration curve of liver GSH: OD = f (GSH concentration in µg/mL)

Tube	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
GSH Conc. (µg/mL)	0	0.04	0.08	0.12	0.16	0.2
OD	0	0.639	1.397	2.143	2.854	3.100



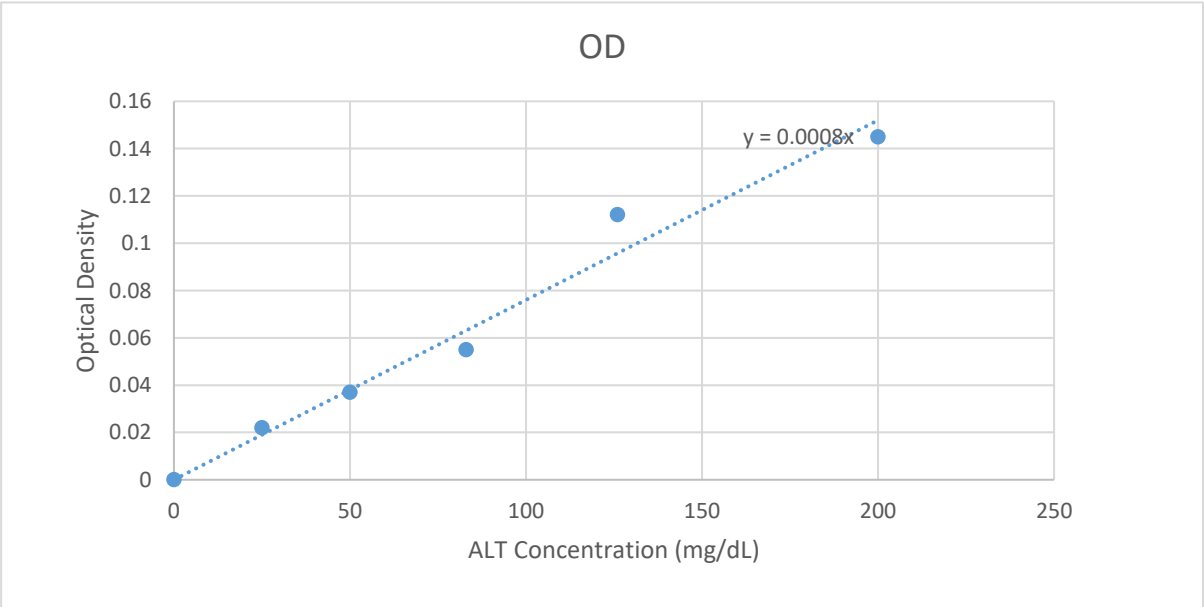
Calibration curve of kidney GSH: OD = f (GSH concentration in µg/mL)

Tube	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
GSH Conc. (µg/mL)	0	40	80	120	160	200
OD	0	0.637	1.397	2.143	2.854	3.155



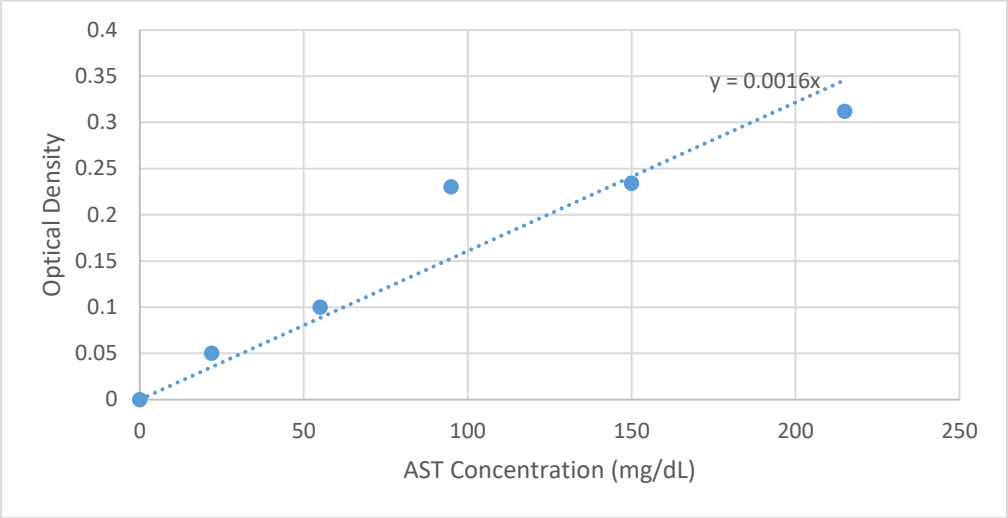
Calibration curve of plasma Alanine transaminase: OD = f (ALT concentration in mg/mL)

Tube	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
ALT standard (mL)	0	0.025	0.05	0.1	0.15	0.2
Conc (mg/dL)	0	25	50	83	126	200
OD	0	0.022	0.037	0.055	0.112	0.145



Calibration curve of plasma Aspartate transaminase: OD = f (AST concentration in mg/dL)

Tube	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
AST standard (mL)	0	0.025	0.05	0.1	0.15	0.2
CONC (mg/dL)	0	22	55	95	150	215
OD	0	0.05	0.1	0.23	0.234	0.312



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DIABETOLOGY & METABOLIC SYNDROME

REVIEW Open Access

Persistent organic pollutants as risk factors for type 2 diabetes

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Abstract

Type 2 diabetes mellitus (T2DM) is a major and fast growing public health problem. Although obesity is considered to be the main driver of the pandemic of T2DM, a possible contribution of some environmental contaminants, of which persistent organic pollutants (POPs) form a particular class, has been suggested. POPs are organic compounds that are resistant to environmental degradation through chemical, biological, and photolytic processes which enable them to persist in the environment, to be capable of long-range transport, bio accumulate in human and animal tissue, bio accumulate in food chains, and to have potential significant impacts on human health and the environment. Several epidemiological studies have reported an association between persistent organic pollutants and diabetes risk. These findings have been replicated in experimental studies both in human (in-vitro) and animals (in-vivo and in-vitro), and patho-physiological derangements through which these pollutants exercise their harmful effect on diabetes risk postulated. This review summarizes available studies, emphasises on limitations so as to enable subsequent studies to be centralized on possible pathways and bring out clearly the role of POPs on diabetes risk.

Keywords: Type 2 diabetes, Persistent organic pollutants, In utero exposure, Insulin secretion, Insulin resistance

Introduction

Type 2 diabetes (T2DM), the most common form of diabetes mellitus, is a complex condition resulting from the interaction between environmental and genetic factors. The global population of adults with diabetes was estimated to be 382 million (8·3%) in 2013 of which 175 million of cases are currently undiagnosed and this figure is predicted to rise to around 592 million (10.1%) by 2035 (International Diabetes Federation [1]).The majority of people with diabetes are aged between 40 and 59 years, and 80% of them live in low- and middle-income countries (IDF, International Diabetes Federation [1]). Higher-than-optimal weight resulting from the combined effects of unhealthy diets and decreased physical activity is a major driver of the ongoing pandemic of T2DM. Mounting evidences have also suggested the contribution of some environmental contaminants to the observed growing T2DM.

Persistent organic pollutants (POPs), one of the classes of environmental contaminants are a variety of chemicals which are either man-made or accidentally produced in industrial processes. The groups of compounds that make up POPs are also classed as PBTs (Persistent, Bio-accumulative and Toxic) or TOMPs (Toxic Organic Micro Pollutants). POPs are organic compounds that are resistant to environmental degradation through chemical, biological, and photolytic processes which enable them to persist in the environment, to be capable of long-range transport, bio accumulate in human and animal tissue, bio accumulate in food chains, and to have potential significant impacts on human health and the environment. POPs are generally classified into five main types which include polychloro-dibenzopara dioxins (PCDD) and polychloro-dibenzo furans (PCBF), polychlorinated biphenyls (PCBs), organo-chlorine (OC) pesticides, and poly-brominated flame retardants. The first three are also known as organo-chlorine compounds. Organo-chlorine compounds were banned in the late 1970s because of their toxicity. Lipid solubility, persistence in the environment and bioaccumulation potential in tissues through the food chain made these compounds to be

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very toxic. The bioaccumulation potential is due to the halogenated structure of the POPs. Human exposure to POPs occurs primarily through the consumption of animal fats like fatty fish, meat and dairy products [2]. There are geographic and socio-economic variations in type and route of exposure. Low and middle income countries population are at higher risk of exposure to POPs, either acute to high concentrations or chronic as long-term exposure to lower concentrations through diet, agriculture, occupation, and accidents especially for substances restricted or banned in developed countries. One of the POPs that is highly used in low and medium countries is dichloro-diphenyltrichloro-ethane (DDT)

DDT is a colourless, crystalline, tasteless and almost odourless organo-chloride which was initially produced and used to control malaria and typhus. It was used during the Second World War to control malaria and typhus among civilians and troops. After the war, DDT was made available for use as an agricultural insecticide. With the discovery of toxicological activity of DDT, its agricultural use was banned in the United States in 1972. By 1991, total ban on the use of DDT, including in disease control was implemented in about 26 countries. Due to the effectiveness of DDT against malaria and the burden of malaria especially in low and medium income countries, DDT even though banned is still used till date. Despite the possible role of DDT in the control of pest, its use should be avoided due to its negative effect on human health. It is classified as moderately toxic by the United States National Toxicology Program. Moreover, a number of studies from the US, Canada and Sweden have found that the prevalence of diabetes increases with serum DDT level. Several recent studies demonstrate a link between in-utero exposure to DDT and its metabolites and developmental neurotoxicity in humans. DDT has also been shown to induce neurological problems such as Parkinson's and Alzheimer's disease.

A review of the evidence on the association between organo-chlorine compounds and diabetes was published in 2005 [3]. However since then, new evidence have emerged to further substantiate such an association and putative mechanisms are increasingly tested, justifying an updated appraisal of existing evidences. Novel reviews have been published on the association between POPs and diabetes but limited to epidemiological studies [4,5]. Accordingly we reviewed the evidence from epidemiological and experimental studies on the association between exposures to persistent organic pollutants and type 2 diabetes risks. Additionally, we discussed the possible metabolic derangements and molecular mechanisms underlying such an association. We also explored evidence that would support the potential for exposure to POPs during foetal life to contribute to increased risk at adult age.

Methods

The main databases used for the search of articles included in this study were PUBMED and HINARI. PUBMED search used the following key words: Type 2 diabetes + POPs, POPs + insulin resistance, POPs + insulin secretion, review + POPs + Diabetes, POPs + in-utero + exposure + diabetes. References from articles relating POPs to type 2 diabetes were used to search for the original articles through HINARI. Our inclusion criteria for epidemiological analyses comprised any cross sectional and prospective study with type 2 diabetes as the outcome published after 2005. For experimental studies, all available data on POPs and diabetes related complications were included. Studies on in-utero exposure to POPs and diabetes were obtained through the Google search engine to complement those from PUBMED.

Evidence from epidemiological studies

Evidence prior to 2005

Evidence from epidemiological studies published before 2005 has been summarised previously by Arisawa et al. in 2005 who included 6 studies confirming an association between exposure to persistent organic pollutants and the risk of type 2 diabetes. These studies of which one was longitudinal and 5 cross sectional were published between 1997 and 2001. The main POPs included were PCDD, polychlorinated dibenzofuranes (PCDF) and PCBs, and exposure was mostly accidental in which individuals used substances containing POPs at work. They confirmed the association between POPs and type 2 diabetes but could not determine a causal relationship in the absence of experimental evidence [3].

Post 2005 epidemiological studies

Since the review by Arisawa et al. several other epidemiological studies have focused on the association between exposure to persistent organic pollutants and the risk of developing type 2 diabetes, using different designs. The characteristic of these studies are summarised in Tables 1 and 2.

Evidence from cross-sectional and case–control studies

Since 2005, at least 20 cross-sectional studies conducted in about 12 countries have been published on the association of POPs with diabetes risk. The number of participants across studies ranged from 65 to 2047, the proportion of men from 30.0% to 64.3% and the proportion with diabetes from 5.8% to 27.4%. Even though with a diabetes prevalence of 11%, the number of diabetes cases was the least (2) in the study of [6]. The association between POPs and diabetes was based on comparing the mean value of POPs in diabetic and non diabetic individuals. Diagnosis of diabetes varied across studies and was based on FPG ≥126 mg/dl, non-fasting glucose

Table 1 Cross sectional studies of the association of POP with diabetes risk

Reference	Location	Population/ setting	Age (years)	Diagnostic criteria of diabetes	Confounders	Total population	Diabetic population	Proportion of men %	Compounds measured	Compounds with statistical significant difference			
										PCBs	Dioxins	OC pesticides	others
[20]	Sweden	Fishermen and their wives from the Swedish east coast	66.5	Self-reported physician-diagnosed diabetes	Age, sex, BMI	380	22	51.6	PCB-153,p,p ′ -DDE	PCB-153 OR = 1.16, 95% CI (1.03-1.32)		p,p'-DDE OR = 1.05, 95% CI 1.01- 1.09).	
[8]	USA	NHANES (1999 – 2002)	≥20	FPG ≥ 126 mg/dl, Non-fasting glucose ≥200 mg/dl, Self-reported physician-diagnosed diabetes	Age, sex, BMI, race/ethnicity, poverty income ratio, WC	2,016	217	NA	PCB-153, HpCDD, OCDD, oxychlordane, DDE, trans-nonachlor	PCB-153 OR = 6.8, 95% CI (3.0–15.5)	HpCDD OR = 2.7, 95% CI (1.3–5.5)	Oxychlordane OR = 6.5 (2.0–21.4) DDE OR = 4.3, 95% CI (1.8–10.2);trans-nonachlor OR = 11.8, 95%CI (4.4–31.3)	
[9]	USA	Mohawk adults (one adult/ household)	57.5	FPG >125 mg/dl, Taking prescribed glucose-lowering medication	Age, sex, BMI, serum lipid levels, smoking history	352	71	38.1	Total PCB, PCB-153, **PCB-74**, DDE, HCB, mirex ,	Total PCB, OR = 3.9,95% CI (1.5–10.6). PCB-153 OR = 3.2, 95% CI (1.3–8.2) PCB-74 OR = 4.9, 95% CI (1.7–13.7)		DDE, OR = 6.4, 95%CI (2.2–18.4) HCB OR = 6.2, 95%CI (2.3–16.9)	
[21]	USA	NHANES (1982– 1984), Hispanic Americans	47	Self-reported physician diagnosed diabetes	Age, sex, BMI, place of birth, education, poverty index	1,303	89	40.5	**p,p ′ -DDT**, p,p ′ -DDE, dieldrin, oxychlordane, β -HCH, HCB, trans-nonachlor			p,p ′ -DDT (OR = 2.3,95% CI(1.1–5.0) , p,p ′ -DDE, OR = 2.63, 95% CI (1.2–5.8) β -HCH, OR = 2.7, 95% CI (0.9–8.2), oxy chlordane OR = 3.3, 95% CI (1.2–8.7) trans nonachlor OR = 3.4, 95% CI (1.4–7.9)	
Lee et al. [61]	USA	NHANES (1999 – 2002)	≥20	FPG ≥ 126 mg/dl, non-fasting glucose ≥ 200 mg/dl, Self-reported physician-diagnosed diabetes	Age, sex, BMI, race/ethnicity, poverty income ratio, WC	1,721	179	NA	PCDDs (3), PCDFs (3), dioxin-like PCBs (4), nondioxin-like PCBs (5),OC pesticides (4)	Dioxin-like PCBs OR = 15.7, 95% CI (3.4–71.2)		Oxychlordane, OR = 4.6, 95% CI (2.0–10.4),trans nonachlor, β –HCH Or = 7.0, 95% CI (2.7–18.1); DDT OR = 2.9, 95% CI (1.5–5.6),	
[23]	Sweden	Swedish fishermen's wives	44	Self-reported diabetes	Age and BMI	544 women	16	0.0	CB-153 and p,p-DDE			CB-153; OR = 1.6, 95% CI (1. 0–2. 7) and p,p-DDE OR = 1.3, 95% CI (1. 1–1.6)	

Table 1 Cross sectional studies of the association of POP with diabetes risk *(Continued)*

[7]	USA	National Health and Nutrition Examination Survey 2003–2004	69	Fasting plasma glucose ≥126 mg/dl non-fasting plasma glucose ≥200 mg/dl , taking insulin or an oral agent.	Age, sex, race, poverty income ratio, and BMI	1,367	156	47.3	PBB-153 PBDE-28 PBDE-47 PBDE-99 PBDE-100 PBDE-153				PBB-153 OR = 1.9, 95% CI (0.9–4.0)
[26]	Japan	Survey (2002 – 2006)	44	Self-reported physician diagnosed diabetes, non-diagnosed participants with plasma HbA 1c >6.1%	age, gender, log (BMI), smoking habit, regional block, residential area, and survey year	1374	65	45.6	7 PCDDs, 10 PCDFs, 12 dioxin-like PCBs	12 dioxin-like PCBs OR = 6.82, 95% CI (2.59–20.1)	7 PCDDs,10 PCDFs OR = 2.21, 95% CI (1.02–5.04),		
[30]	Taiwan	Yu Cheng cohort (1993 – 2003)	55.5	Self-reported physician-diagnosed diabetes	age, BMI, cigarette smoking, and alcohol intake	748	144	41.0	Total PCB	Total PCB in women [OR 2.1,95% CI (1.1– 4.5)			
[62]	South Korea	Community-based health survey	55.6	FPG ≥ 126 mg/dL, taking antidiabetic medication	age, sex, BMI, alcohol consumption, and cigarette smoking.	80	40	52.5	β-hexachlorocyclohexane, HCB, heptachlor epoxide, p,p′-DDE, p,p′-DDD, p,p′-DDT, o,p′-DDT, oxychlordane, trans-nonachlor, and mirex			p,p′-DDE OR = 12.7, 95% CI (1.9–83.7),**p,p′-**DDT OR = 10.6, (1.3–84.9), o,p′-DDT OR = 12.3, 95% CI (1.3–113.2);oxychlordane, OR = 26.0, 95% CI (1.3–517.4); trans-nonachlor OR = 8.1, 95% CI (1.2–53.5) and heptachlor epoxide OR = 3.1, 95% CI (0.8–12.1)	
[19]	Slovakia	heavily polluted Slovakian district of Michalovce and two reference districts (Svidnik and Stropkov)	48	FPG >7.0 mmol/l (all participants) and 2 h glucose >11.1 mmol/l (OGTT, 60% of participants)	Age, sex, BMI	2,047	296	40.8	PCBs (15), p,p′-DDE, p,p′-DDT, HCB, β -HCH	Total PCBs OR = 2.74,95% CI (1.92 – 3.90)		p,p′-DDE, OR = 1.86,95% CI (1.17 – 2.95); p,p′-DDT OR = 2.48,95% CI (1.77–3.48)	
[24]	Finland	Helsinki Birth Cohort Study representing a general	63.5	fasting plasma glucose ≥7.0 mmol/L or 2-h plasma	Sex, age, WC and mean arterial pressure	1,988	308	46.3	oxy-chlordane, trans nonachlor, p,p′ -DDE, PCB 153, BDE 47, and BDE153	PCB 153 OR = 1.64 (0.92 – 2.93) P = 0.050		Oxy chlordane OR = 2.08, 95% CI (1.18 – 3.69); trans nonachlor	

Table 1 Cross sectional studies of the association of POP with diabetes risk *(Continued)*

		adult urban Finnish population		glucose ≥11.1 mmol/L or on anti-diabetic medication.							OR = 2.24, 95% CI (1.25 – 4.03); DDE OR = 1.75, 95% CI (0.96 – 3.19)
[25]	Japan	Participants in the Saku Control Obesity Program.	52	HbA1c level ≥ 6.9% ,taking medication for diabetes, fasting plasma glucose ≥ 126 mg/dL, or a history of doctor-diagnosed diabetes	Sex, age, BMI and total lipids.	117	32	50.4	PCB 74, 99, 118, 138, 146, 153, 156, 163/164, 170, 180 and 182/187	PCB 146, OR = 2.46, 95% CI (1.09-5.59) PCB 180 OR = 1.39, 95% CI (1.10-1.76)	
[34]	Denmark	The Gentofte population and Odense population	52.2	FPG ≥ 7.0 mmol/liter on two occasions, absence of anti-glutamic acid decarboxylase 65 antibodies, and no need for insulin therapy	Age, sex, and percent body fat, study site	148	31	64.3	HCB, HCH, p'p'-DDE, op'-DDE, p'p'-DDT, PCB-105, 118 and 156, PCB-101, 138, 153 and 180	PCB-105 OR = 3.8, 95% CI (3.0–4.9) PCB 118 OR = 14.2, 95% CI (10.2–21.0)	organochlorine HCB, OR = 28.0, 95% CI (20.0–39.7);p',p'-DDE OR = 139.4,95% CI (73.6–288.4)
[63]	Spain	Participants of the Catalan Health Interview Survey (CHIS)	46	FPG ≥ 126 mg/dL, reported physician diagnosed diabetes and current use of insulin or antidiabetic medication	age, sex, and BMI,total cholesterol and triglycerides	886	143	42.9	op-'-DDT, p,p ' -DDT, o,p' -DDE, p,p ' -DDE, o,p' -DDD, p,p ' -DDD; PCB28, 52, 101, 118, 138, 153, and 180, P**eCB**, HCB, α –HCH, β -HCH, γ -HCH, and δ -HCH	Non dioxin like PCBs (PCB28, 52, 101, 118, 138, 153, and 180,) OR = 1.8, 95% CI (1.2 – 2.7)	op-'-DDT, o,p ' -DDE, o,p ' -DDD, p,p ' -DDD; HCB, α –HCH,, γ -HCH, and δ –HCH OR = 1.8, 95% CI (1.0 – 3.2)
[6]	USA	Employed at the La Salle Electrical Utilities Company in illinios and local residents not previously employed	57	self-reported diagnosed diabetes, and/or fasting glucose ≥ 126 mg/dL	age, BMI, alcohol use, smoking, high blood pressure, total lipids, medication use	63	7	100	6 dioxin-like PCBs and 33 non dioxin like PCBs	All PCBs OR = 3.0, 95% CI (1.3-7.2)	
[18]	USA	Anniston, Alabama general population	55.5	fasting glucose values > 125 mg/dL, self-reported or physician diagnosed diabetes	Age, sex, race/ethnicity, or BMI	774	207	30.0	35 PCB ortho-substituted congeners, DDE	All PCBs 2.78,95% CI(1.00-7.73)	DDE OR = 1.22, 95% CI(0.85-1.46)

Table 1 Cross sectional studies of the association of POP with diabetes risk *(Continued)*

[64]	Spain	San Cecilio University Hospital in thecity of Granada and Santa Ana Hospital in the town of Motril	55	self-reported information and clinical records showing a fasting glucose level Z 126 mg/dL in the routine analyses	Adipose tissue origin , sex, age, and BMI	386	34	51.0	PCB-138, PCB-153, PCB-180, HCB, b-HCH, p,p′-DDE,		p,p′-DDE OR = 4.4, 95% CI (1.0–21.0)
[65]	Canada	Wapekeka and Kasabonika two of the five Shibogama communities in northern Ontario	44.5	resting plasma glucose >7.0 mmol/L and/or a post-prandial level (2 hours after glucose inges-tion) > 11.0 mmol/L	Age, BMI and smoking status	72	26	43.1	Aroclor 1260, PCB28, 52, 99, 101, 105, 118, 128, 138,153, 156, 163, 170, 180,183,187,aldrin, – αchlordane, β-chlordane, – β-HCH, cis-nonachlor, trans-nonachlor, DDE, DDT, HCB,mirex, oxychlor dane, PBB153, PBDE47, 99, 100,153, Parlar26, and Parlar50.	PCB28, 52, 99, 101, 118, 128, 138, 153, 156, 163, 170, 180, 183,187,	Trans nonachlor, DDE, oxychlordane, all $p < 0.05$
[66]	South Korea	NA	NA	FPG ≥ 126 mg/dL, reported history of physician-diagnosed diabetes	age , sex, alcohol consumption , cigarette smoking and BMI	50	25	48.0	14 OC pesticides, 22 PCB congeners		DDTs in VAT, OR = 9.0,95% CI(1.3–62.9)
	South Korea	Community-based health survey	55.6	FPG ≥ 126 mg/dL, taking antidiabetic medication	age, sex, BMI, alcohol consumption, and cigarette smoking.	80	40	52.5	β-hexachlorocyclohexane, HCB, heptachlor epoxide, p,p′-DDE, p,p′-DDD, p,p′-DDT, o,p′-DDT, oxychlordane, trans-nonachlor, and mirex		p,p′-DDE OR = 12.7, 95% CI (1.9–83.7); **p,p′-**DDT OR = 10.6, 95% CI (1.3–84.9); o,p′-DDT OR = 12.3, 95% CI (1.3–113.2);oxychlordane, OR = 26.0, 95% CI (1.3–517.4); trans-nonachlor OR = 8.1, 95% CI (1.2–53.5) and heptachlor epoxide OR = 3.1, 95% CI (0.8–12.1)

2,2,4,4,5,5-hexabromophenyl (PBB-153), heptachlorodibenzo-p-dioxin (HpCDD), octachlorodibenzo-p-dioxin (OCDD), dichlorodiphenyldichloroethylene (DDE), 2,2′-bis(4-chlorophenyl)-1,1-dichloroethylene (p,p′-DDE), hexachlorobenzene (HCB), hexa-chlorocyclohexane (HCH), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), organochlorine (OC), 2,2',4,4'-tetrabromodiphenyl ether (BDE 47), 2,2',4,4',5,5'–hexabromodiphenyl ether (BDE 153), 2,2,4,4,5,5-hexabromophenyl (PBB-153), 2,4,4-tribromodiphenyl ether (PBDE-28), 2,2,4,4 tetrabromodiphenyl ether (PBDE-47), 2,2,4,4 ,5-pentabromodiphenylether (PBDE-99), 2,2,4,4,6-pentabromodiphenyl ether (PBDE-100), 2,2,4,4,5,5-hexabromodiphenyl ether (PBDE-153), 2,2 ′ -bis(4-chlorophenyl)-1,1,1-trichloro-ethane (p,p ′ -DDT), 2,2′-bis(4-chlorophenyl)-1,1-dichloroethylene (p,p′-DDE), di-chlorodiphenyl-dichloro-ethane (o,p′-DDD), pentachlorobenzene (PeCB), body mass index (BMI), confidence interval (CI), fasting plasma glucose (FPG), oral glucose tolerance test (OGTT), odd ratio (OR),waist circumference (WC).

Table 2 Longitudinal studies of the association of POP with diabetes risk

Reference	Location	Population/ setting	Mean Age (year)	Diagnostic criteria of diabetes	Confounders	Number of participants	Number of diabetic participants	Proportion of men	Duration of follow-up (years)	Compounds measured	Compounds with statistical significant difference			
											PCBs	Dioxins	OC pesticides	Others
[15]	USA	Michigan PBB cohort	≥ 20	Self-reported physician-diagnosed diabetes, anti-diabetic medication	Age, body mass index, smoking, and alcohol consumption	1384	180	49.7	25	PBB, PCB	PCB in women Incidence Density Ratio [IDR = 2.33; (95% CI 1.25– 4.34)]			
[17]	USA	Veterans of Operation Ranch Hand		medical diagnosis or 2-hour post-prandial glucose ≥200 mg/dL	BMI, smoking history, family history of diabetes	3049	439 men	100	10	TCDD		TCDDRR = 1.32, p = 0.04		
[10]	Sweden	WHILA cohort (1995 – 2000)	54.5	Baseline OGTT	Age, calendar year, BMI, heredity, country of birth, education, smoking, alcohol intake, hormone Replacement therapy, physical activity	742	371 women	0.0	10	PCB-153, p,p ′ -DDE	CB-153 OR = 1.6 (95% 0.61-4.0)		p,p ′ -DDE OR = 5.5 (95% CI 1.2-25)	
[12]	USA	Cross-section of Great Lakes sport fish consumers (1992, follow-up 2004 – 2005)	50.5	Self-reported physician-diagnosed diabetes,	Age, BMI, sex, serum lipids, smoking, alcohol use, all fish meals in the last year	471	36	59.2	10	PCB 74, 99, 118, 146, 180, 194, 201, 206, 132/153, 138/163, 170/190, 182/187, 196/20, DDE			DDE RR = 2.01 (95% CI 1.20–3.66)	
[31]	USA	CARDIA cohort study (1985 – 1986; follow up: 1987 – 2006)	24	Use of glucose-lowering medications	Age, sex, BMI, race	180	90	38.4	18	OC pesticides (9), PCBs (35), PBDEs (1), PBB (1)	Highly chlorinated PCBs OR = 2.8 (95% CI1.1–7.0)		Trans Nonachlor OR = 4.8 (95% CI1.7–13.7)	PBB-153 OR = 3.0 (95% CI1.1–8.1)
[11]	Sweden	General population of Uppsala	70	fasting blood glucose ≥6.2 mmol/L or the use of insulin or oral hypoglycemic agents	sex, BMI, cigarette smoking, alcohol consumption, and total lipids	725	36	48.3	5	PCB74, 99,105, 118, 138, 153, 156, 157, 170, 180, 189, 194, 206, 209, p,p'-DDE, Trans – nonachlor, Hexa-chloro benzene, brominated	PCB74, 99,105, 118, 138, 153, 156, 157, 170, 180, 189, 194, 206, 209 OR = 7.5 (95% CI1.4 – 38.8)		DDE, Trans nonachlor and Hexa-chloro benzene, OR = 3.4 (95% CI1.0 – 11.7)	

Table 2 Longitudinal studies of the association of POP with diabetes risk *(Continued)*

										diphenyl ether 47, dioxin	
[14]	USA	Nurses' Health Study.	42.5	Self-reported diagnosed diabetes, and/or fasting glucose ≥ 126 mg/dL	Age, BMI, smoking status, alcohol intake, physical activity and family history of diabetes	1,095 women	48	0.0	18	PCBs, DDT, DDE and HCB	HCB OR = 3.59 (95% CI 1.49-8.64)

2,2,4,4,5,5-hexachlorobiphenyl (CB-153), 2,2′-bis(4-chlorophenyl)-1,1-dichloroethylene (p,p′-DDE), polychlorinated biphenyls (PCBs), pentabromo-diphenylether (PBDEs), hexabromophenyl (PBB) tetrachlorodibenzo para dioxine (TCDD), di-chlorodiphenyl-trichloro-ethane (DDT), dichlorodiphenyldichloroethylene (DDE), hexachlorobenzene (HCB); body mass index (BMI), oral glucose tolerance test (OGTT).

≥200 mg/dl, self-reported physician-diagnosed diabetes, taking prescribed glucose-lowering medication, non-diagnosed participants with plasma HbA1c >6.1%, 2-h plasma glucose ≥ 199.8 mg/dL, and HbA1c level ≥6.9%. Over 58 POPs compound were investigated in those studies with a minimum of two and a maximum of 36 per study. Measures of associations used to assess the effect of these compounds on diabetes risk across studies included differences in the prevalence of diabetes amongst groups, odd ratios which were often adjusted for common diabetes risk factors like age, sex, body mass index, race/ethnicity, alcohol use, smoking, high blood (BP) pressure or BP indices, lipids, medication use and waist circumference. Exposure was expressed as categorized variables ranging from two groups to quintiles with the group having low plasma POP constituting the control group. The odd ratio in the fifth quintile compared to the reference group ranged from 1.9 for PBDE-153 Lim et al. [7]) to 37.7 [8]. Stratification in tertiles gave an OR of 3.9 (95% confidence interval, 1.5–10.6) for total PCBs [9]. PCB-153,pp'-DDT, and pp'-DDE were associated with type 2 diabetes in all studies while non dioxin like PCBs, HCB, BDE-47, BDE-153 and HCH were associated with diabetes only in one study (Table 1).

Evidence from longitudinal and time-series studies

Since 2005, at least 7 longitudinal studies were published from about 3 countries reporting on the association of POPs with diabetes risk. The number of participants across studies ranged from 180 to 3049. The proportion of men ranged from 38.4% to 59.2% and the proportion of participants with diabetes from 4.4% to 19.3%. Two studies were considered to be case control since the prevalence of diabetes was very high compared to that of the normal population. The prevalence was 50% in both studies [10,11]. In the study conducted by Rignell-hydbom et al., [10], the controls were randomly selected from the same cohort with the cases (Women's Health In the Lund Area cohort) and matched for age, calendar year, BMI and presence or absence of metabolic syndrome at baseline. The second study also included all participants from the same cohort whereby participants who developed diabetes during the follow-up were considered cases and those who were diabetes free were taken as controls. Diagnosis of diabetes varied across studies and was based on self-reported physician-diagnosed diabetes, baseline OGTT, taking glucose-lowering medications, fasting blood glucose ≥111.6 mg/dL (≥6.2 mmol/L) non-diagnosed participants with HbA1c >6.1% (or >6.3%), verified history of diabetes by medical diagnosis, 2-hour postprandial glucose ≥200 mg/dL, or fasting 5 glucose ≥126 mg/dL. Over 51 POPs compound were tested in those studies with a minimum of two and a maximum of 46 compounds per study. The duration of follow-up in studies varied between 5 and 25 years (mean = 13.2). Measures of associations used to assess the effect of these compounds on diabetes risk across studies included differences in the incidence of diabetes amongst groups, odd ratios, least squares geometric means, hazard ratios, incidence density ratios (IDRs), and relative risk which were often adjusted for common diabetes risk factors like age, body mass index, cigarette smoking, country of birth, education, alcohol intake, hormone, physical activity, race, lipids and family history of diabetes. Hexachloro benzene (HCB), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2,2′-bis(4-chlorophenyl)-1,1-dichloroethylene (pp'-DDE) were associated with the risk of type 2 diabetes while the association was found only in women when PCBs were investigated. pentabromodiphenylethers (PBDEs), 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), dichloro-diphenyl-trichloro-ethane (DDT) and non-dioxin like PCB were associated with the risk of diabetes in none of the studies (Table 2).

In all the longitudinal studies included in this review, bio-monitoring was performed from baseline until the end of the study. This is shown by the fact that all participants included were diabetes free at baseline and only developed diabetes during the follow-up and participants with diabetes before baseline were excluded from the study. Bio-monitoring of POPs was carried out only in one of the seven studies because exposure was accidental. Exposure in the other studies was background and follow up was carried out to determine incident diabetes cases ([11,12]; Lee et al., [13,14]; Vasiliu et al., [15]; RignellHydbom et al., [10]). In the study conducted by RignellHydbon et al., [10] blood samples were stored for at least three years at −70°C before POPs analysis. Analysis of POPs was performed at the end of follow-up. Other studies analyzed POPs at enrolment with blood samples store at −20°C or −70°C (Lee et al., [16]; Michalek and Pavuk., [12,14,17]). Moreover, analysis of POPs was performed once throughout the follow-up in most of the studies ([11]; Lee et al., [14,16]; Vasiliu et al., [15]; RignellHydbon et al., [10]). Even though multiple analyses of POPs was carried out by [17], only one point measurement was used in the analysis since veterans who participated in at least one of the last five examinations were included in the study. Reverse causality can be a possible association between type 2 diabetes and POPs where individuals with diabetes are unable to metabolise POPs resulting to a higher concentration compared to non diabeteic individuals. This can be evaluated in studies with repeated measurements POPs [12].

Limitations and strengths of the available epidemiological studies

Several epidemiological studies have evaluated the association between POPs and diabetes using accidental

exposure to PCBs [6,18] and high zone exposure to organo-chlorine (OC) pollutants [19]. These exposure rates are higher than those observed in the general population. Therefore, results from these studies can only evaluate the health risk of persistent organic pollutants but cannot be generalised in the total population more often affected by chronic low concentration exposure. Moreover most of the studies with background exposure to polychlorinated biphenyls (PCBs) and organochlorine pesticides ([9,20,21]; Lee et al. [22-25]) had a cross sectional design. As such, a causal relationship cannot be established considering for instance the possibility that diabetic individuals may have a reduced capacity to excrete or metabolise pollutants leading to a high concentration of POPs in diabetic individuals compared to those without. In-depth investigation of the effect of chemicals on the processes leading to the development of diabetes is thus required. The small sample size and prevalence of diabetes in some studies reduced to unstable estimates of the association. Moreover, Michalek and Pavuk did not observe any difference in diabetes prevalence between the exposed and the unexposed groups [17]. The study was aimed at assessing the association between 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and diabetes and TCDD and cancer in veterans involved in the spraying of Agent Orange and other TCDD contaminated herbicides in Vietnam from 1962 to 1971. Two groups were involved in the study; one responsible for the spraying of Agent Orange and a comparing group a cohort of other Air Force veterans who served in the Southeast Asia (SEA) region during the same period that the Ranch Hand unit was active but who did not spray herbicides. The test group was exposed to Agent Orange contaminated with TCDD during the Vietnam War. Despite the lack of association between the two groups, stratification by calendar period of service and days of spraying revealed a significant increase in the risk of diabetes among Ranch Hands who served during or before 1969 and who had at least 90 days of spraying [17]. This study suggested that the risk of type 2 diabetes might be associated with the duration of exposure which can better be evaluated in longitudinal studies

Longitudinal studies have been carried out to establish a causal relationship between type 2 diabetes and POPs (Vasiliu et al. [15], Rignell-Hydbom et al. [10-12], Lee et al., [14,16]) although exposure rates were higher than those observed in the general population. However, a study carried out among general inhabitants in Japan confirmed a high risk of diabetes in individuals with high level of Dioxin [26]. The most convincing evidence for a relationship between diabetes and persistent organic pollutants (POPs) in humans comes from accidental or occupational exposures to high levels of these compounds. This is documented by David O Carpenter in a review entitled "Environmental Contaminants as Risk Factors for Developing Diabetes". The studies carried out in individuals occupationally exposed to high level of this class of POPs showed an increase in the prevalence and incidence of diabetes compared to the general population. There was a significantly elevated deaths from diabetes among women exposed to dioxins at Seveso, Italy living in the zone with intermediate exposure (OR = 1.9, 95% CI: 1.1-3.2) [27]. Analysis of data reported from 36 international cohorts of workers in phenoxyacid herbicide and chloro-phenol plants, where dioxins were an un-intended by product reported a RR of 2.25 (95% CI: 0.53-9.50) for diabetes in these workers [28]. Moreover, 60% of workers in two US chemical plants had diabetes and workers with the highest extrapolated TCDD concentrations had a significantly increased mean serum glucose concentration [29]. All these studies substantiate the fact that high exposure to POPs from occupational and accidental setting increases the risk of type 2 diabetes.

Epidemiological associations between persistent organic pollutants and type 2 diabetes have been widely assessed in Europe, Asia and America. However, the intentional use has been restricted or banned in many developed countries. Studies on the association between these contaminants and diabetes in developing countries particularly Africa are lacking while the prevalence of diabetes is predicted to increase the highest in the region. Some studies [12,20,21,23,30] used only self-reported physician-diagnosed diabetes as a diagnostic criteria for diabetes. A false diagnosis will modify the prevalence or incidence rate and in turn dilute estimates of the association. Moreover, differences in the diagnosis criteria make it impossible to compare the magnitude of the association across studies.

Metabolic derangements associated with exposure to POP

Insulin resistance and defects in insulin secretion have been investigated in in-vitro and in-vivo experimental studies, as the possible metabolic derangements linking exposure to POPs and diabetes occurrence. Insulin plays a fundamental role in the uptake of glucose by the adipose tissue, liver and muscles. Insulin exerts its action by binding to its receptor and initiating a cascade of reactions culminating with the translocation of the glucose transporter from the endoplasmic reticulum to the plasma membrane for glucose uptake. Competition between insulin and other ligands for the receptor as well as modulation of the expression of genes involved in the signaling cascade will lead to an insulin resistance state. The overproduction of insulin by the pancreatic beta cell to compensate for the insulin resistance state might lead

to the exhaustion of the cells and future type 2 diabetes. POPs have been shown to affect these pathways both in human and animal studies.

Human studies: effect of POPs on insulin secretion and insulin resistance

Few studies in human subjects have reported that POPs increases the risk of insulin resistance both in diabetic and non-diabetic individuals [13,22,31,32]. In a study carried out in non-diabetic individuals with background exposure to TCDD, it was observed that individuals with level of TCDD greater than 15 ppb had high fasting and pros-prandial insulin levels after a glucose load compared to individuals with low level TCDD, suggesting that high blood TCDD may cause insulin resistance [31]. Another study focusing on low dose exposure to organo-chlorine (OC) pesticides and PCBs observed a non monotonic dose response (U shape) association between POPs and insulin resistance in non-diabetic individuals [16]. Toxicity is assumed to be directly proportional to the dose of the chemical. However, some substances especially endocrine disruptors may cause biological response at doses well below those levels previously tested and determined to be safe. A U-shape curve is therefore obtained between exposure and outcome when low exposure corresponding to high biological effect is considered to be the control group. However other studies have not found an association between POPs and insulin resistance defined by HOMA-IR [6,33]. Some have suggested an effect of POPs on insulin secretion instead of insulin resistance [33].We found only one published study exists on the association between POPs and insulin secretion in human subjects. Jørgensen and collaborators observed a significant negative association between POPs and indices of insulin secretion: stimulated insulin and HOMA-B indicating that POPs may affect basal and stimulated insulin secretion [33]. Studies with accurate measurement of insulin secretion and insulin action are warranted. In fact, few investigations used clamp studies or other dynamic testing. Two studies have evaluated the association between POPs and insulin action using euglycemic hyperinsulinaemic clamp as a measure of insulin action. Færch et al. used two different clamp procedures. In one study group, a euglycemic-hyperinsulinemic clamp at 40 mU/m^2.min for 4 h was performed after a 2-h basal period. In the second group, the 2-h basal period was followed by a 30-min IVGTT for assessment of first-phase insulin secretion [34]. Immediately after the IVGTT, a euglycemic-hyperinsulinemic clamp at 40 mU/m^2.min for 2 h was performed. In their study, individuals with prediabetes and diabetes had higher serum concentrations of several POPs (HCB, p,p'-DDE, p,p'-DDT, PCB-118 and PCB-156) compared with normoglycemic individuals. Moreover, in the non-diabetic population, higher POPs levels were associated with elevated fasting plasma glucose concentrations as well as reduced glucose oxidation, elevated lipid oxidation, and elevated serum concentrations of free fatty acids. However, after exclusion of diabetic patients, there were no differences between the two study populations with regard to fasting plasma glucose, serum insulin, HOMA-IR, or HOMA-B [34]. Gauthier et al. in their study measure insulin sensitivity using the 3-hour hyperinsulinemic-euglycemic clamp as described by Defronzo [35]. Despite the fact that an association between POPs and insulin resistance was not evaluated in this study, it could be deduced from the results that insulin sensitivity characterized by 3-hour hyperinsulinemic-euglycemic clamp was inversely associated with the concentration of POPs. This is because insulin sensitivity was higher in metabolically healthy but obese 8 individuals who had a lower concentration of POPs compared to metabolically abnormal obese individuals with a higher concentration of POPs [36].

Animal studies: effect of POPs on insulin secretion and insulin resistance

The effects of POPs on insulin secretion and insulin resistance have been evaluated in few animal studies. The first animal study to observe an effect of POPs on insulin resistance was carried out in Sprague–Dawley rats [37]. These rats were exposed for 28 days to lipopholic POPs through the consumption of high fat diet containing either refined or crude fish oil obtained from farmed Atlantic salmon. Insulin sensitivity was assessed in the soleus muscle and epididymal fat using the hyperinsulinemic-euglycemic clamp technique. Furthermore, cultured and differentiated 3 T3-L1 cells were used to assess insulin-stimulated glucose uptake in-vitro. There was a significant inhibition of insulin-dependent glucose uptake in skeletal muscle of animals fed on high fat diet containing POPs (in-vivo) as well as differentiated adipocytes treated with nano-molar concentrations of POP (in-vitro) [37]. Moreover, C57BL/6 J mice fed a very high-fat (VHF) diet containing farmed Atlantic salmon fillet (a source of POPs) for 8 weeks when compared with another group of very high fat diet without POPs or chow diet, had a significant high fasting and pros-prandial glucose level [38].There was also a reduced response to glucose clearance following insulin load relative to VHF- and Chow-fed mice all, features demonstrating a state of insulin resistance [38].Administration of PCB-77 or PCB-126 has also been shown to impair glucose homeostasis in lean mice and in obese mice [39].

Molecular mechanisms linking persistent organic pollutants exposure with metabolic derangements

Amongst the different classes of persistent organic pollutants (POPs), 2,3,7,8-tetrachlo-rodibenzo-p-dioxin (TCDD) has been extensively studied and the possible mechanism of disruption of beta cell function reviewed [40]. One prominent symptom of acute toxicity from TCDD is a loss of adipose tissue and body weight, a phenomenon known as the wasting syndrome [41]. This syndrome is characterised by hypophagia, hyper-lipidaemia and hypertriglyceridaemia suggesting that affected animals are unable to utilize the energy-rich nutritional compounds available in their blood. The main pathway of TCDD action is through the aryl hydrocarbon receptor. Binding of TCDD to aryl hydrocarbon receptor causes changes in translational and transcriptional mechanisms resulting in diminished GLUT expression (Enan et al., [42]). Inhibition of GLUT translocation from the cytoplasm to the plasma membrane as well as reduced glucose transporter availability limits the uptake of glucose which is a hallmark of insulin resistance. A decrease in the level of mRNA of the glucose transporter (GLUT) gene and protein levels as well as the glucose uptake was observed in TCDD treated P19 cells [43]. Moreover, treatment of 3 T3-L1 adipocytes with 10nM TCDD caused decreases in the expression levels of insulin 9 receptor beta (IRβ), insulin receptor substrate 1 (IRS1) and GLUT4 and insulin-stimulated glucose uptake activity, strongly suggesting that TCDD triggers dysfunction of the insulin signalling pathway in adipocytes [44]. Even low doses of TCDD (0.03 pg/kg) causes a significant reduction in the glucose transporting activity of guinea pig adipose tissue and pancreas [45]. Compared with TCDD, the effects of polychlorinated biphenyls (PCBs) on insulin secretion are less thoroughly studied. However, a more recent study investigated the in-vitro and in-vivo effects of coplanar PCBs on adipose expression of tumour necrosis factor α (TNF-α) and on glucose and insulin homeostasis in lean and obese mice [39]. In cultured adipocytes, PCB-77 promoted TNF-α expression through an AhR-dependent mechanism suggesting that PCBs could promote insulin resistance through adipose-specific increases in TNF-α [39].

Maintenance of blood glucose homeostasis by the endocrine system involves a series of complex gene-environment interactions between different tissues, including the liver, skeletal muscle, adipose tissue, brain and the pancreas. Altered glucose homeostasis leads to type 2 diabetes, which consists of defects in both insulin secretion and insulin action (insulin resistance) [46]. Since the beta cells of the pancreas are central to controlling glucose homeostasis, an endocrine disrupting chemical that can initiate, facilitate and/or accelerate the loss of beta cell function can play an important role in type 2 diabetes. Moreover, obesity the main predictor of type 2 diabetes is a complex endocrine-related disease caused by the interaction between genetic, behavioral, and environmental factors [47]. Therefore endocrine disruption could be the possible link between obesity and diabetes. Moreover, POPs have also been shown to possess endocrine disruption activities through interaction with estrogen receptors [48] and peroxisome proliferator receptor (PPAR) receptor [49]. Endocrine disruptors are chemicals that, at certain doses, alter the normal functioning of hormones and other signaling molecules in the body. The endocrine disrupting activities of these POPs include their effects on thyroid hormone. Thyroid hormone is essential for normal brain development, for the control of metabolism, and for many aspects of normal adult physiology. Therefore, changes in the functions of the thyroid gland or interference with the ability of thyroid hormones to exert their actions may produce effects on development, metabolism, or adult physiology. POPs interfere with the thyroid function by acting on different points of regulation of thyroid hormones synthesis, release, transport through the blood, metabolism of thyroid hormones, and thyroid hormones clearance [50]. The best example is PCB which can reduce circulating levels of thyroxine (T4) in animals (Bastomsky., [51,52]).

In-utero exposure to POPs and diabetes risk

In-utero exposure to several factors including persistent organic pollutants (POPs) has been shown to increase the risk of many diseases. In pregnant women, POPs have been shown to cross the placenta [53]. The developmental origin of health postulates that adverse foetal exposure may lead to permanent foetal adaptations in structure, physiology and metabolism. These adaptations might be beneficial for short term foetal survival, but may lead to foetal growth retardation, cardiovascular and metabolic diseases in adulthood. The association between intrauterine exposure to persistent organic pollutants and birth weight has been highly investigated. However findings from these studies are inconsistent and the association has been reviewed by Govarts et al. [54]. In the meta-analysis consisting of European birth cohorts they observed that decrease in birth weight independently of gestational age was associated with increasing foetal PCB-153 concentrations. [54]. Across all cohorts birth weight declined by 150 g for each 1-μg/L increase of PCB-153 in cord serum. However, no association was observed with the DDT metabolite p,p´-DDE. Due to inconsistent results in previous studies, other studies (summarised in Table 3) have been carried out to examine the nature of the association. It was observed that higher concentrations of PBDEs in maternal serum during pregnancy were associated with lower birth weight in a population of low-income women living in California [55]. Moreover a 10-fold increase in concentrations of

Table 3 In-utero exposure to persistent organic pollutants and birth weight

Reference	Sample size	Exposure type	Compound used	Direction of the association	Confounders
[67]	2246	Occupational	Not stated	No association	Maternal BMI, height, parity, smoking during pregnancy, infant sex
[56]	1322 singleton	Background	CB-153 and p,p' -DDE	Negative	Maternal age, pre-pregnancy BMI, education, marital status, smoking status, alcohol drinking, parity and newborn's sex
[55]	286 women	Background	PBDEs	Negative	Maternal age, education, marital status, parity, BMI, country of birth, alcohol and drug use during pregnancy and infant sex
[57]	494	Background	DDT, DDE, HCB and PCBs	Negative	Age, height, pregnancy weight gain, pre-pregnancy BMI, country of origin, residence, parity, education, employment during pregnancy, socioeconomic status
[68]	247 children	Occupational	Not stated	Negative	Maternal smoking, social class and gestational age
[59]	503 women	Background	Not stated	No association	Race, education, age, gestational age at delivery and Child's sex
[58]	413	Background	PCBs and PBDEs	Positive with PCB and negative with PBDEs	Maternal age, pre-pregnancy BMI, weight gain during pregnancy, education, smoking during pregnancy and sex of the child
[69]	325	Not stated	14 OC pesticides, 7 PCBs and 14 PBDEs	Negative	Age, pre-pregnancy BMI, educational level, and fish consumption

Organochlorine (OC) pesticides, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), 2,2,4,4,5,5-hexachlorobiphenyl (CB-153), 2, 2'-bis(4-chlorophenyl)-1,1-dichloroethylene (p,p'-DDE), di-chlorodiphenyl-trichloro-ethane (DDT), hexachlorobenzene (HCB), body mass index (BMI).

BDE-47,-99, and −100 was associated with an approximately 115-g decrease in birth weight. Another study found a significant reduction in birth weight and length of gestation associated with maternal CB-153 exposure among Inuits but not in the Kharkiv or War-saw cohorts [56]. A cohort study from Valencia revealed that prenatal exposure to OCs may exert adverse health effects on birth size, reducing the birth weight, length, and head circumference as shown by a significant decrease of 63 and 107 g in birth weight with each 10-fold increase in cord serum 4,4'-DDT and 4,4'-DDE concentrations, respectively [57]. A more recent study found a weak but significant positive association between background prenatal exposure to di-ortho PCBs and birth weight [58]. However, others studies showed that intrauterine exposure to some persistent organic pollutants was not associated with birth weight [59]. A large number of published studies have also reported negative associations between in-utero exposure to persistent organic pollutants and birth weight ([54], Table 3). A nested case control study carried out by Rignell-Hydbom and collaborators showed that POPs were not associated with type 1 diabetes [60]. Moreover, we found no published study on the association between in-utero exposures to POPs and type 2 diabetes risks. Inconsistency results across studies can be explained by the following reasons: some studies evaluated the association in background exposure while others used accidental or occupational settings. Moreover, the studies used different sampled sizes and evaluated different POPs. Nevertheless, a risk of type 2 diabetes can be attributed to intrauterine exposure to POPs because of the three way association diabetes, POPs and obesity. Therefore, we can speculate that low birth weight increases the risk of diabetes in adulthood either as a result of under nutrition, through exposure to POPs or both.

Conclusion

Type 2 diabetes is increasing at alarming rates in both developed and developing countries. Cross-sectional epidemiological studies have established an association between POPs and type 2 diabetes. Despite different levels of risk in prospective studies and inconsistent results, the causal effect of POPs on diabetes is supported by in-vitro and in-vivo experimental studies. However, detailed mechanistic information on how these pollutants interfere with insulin metabolism is lacking. Furthermore, experimental studies have focused more on the effect of POPs on insulin resistance. Therefore, more research efforts are needed on the interaction of compounds with beta-cell function and/or mass in animal models at human relevant concentrations to further evaluate the hypothesis that environmental pollutants can be additional risk factors for diabetes development. These studies should focus on both peri-natal and in-utero exposure which is thought to affect gene expression through epigenetic mechanisms. Simultaneously epidemiological studies focusing on low level background exposure should be carried out in the general population.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ES Substantially contributed in the conception and design, critical revision and final approval of the article. APK contributed in the design, data collection, critical revision and final approval of the article. BTA contributed in the critical revision of the article. ENN contributed in the design, data collection, and writing of the article. EPMM contributed in the data collection and writing of the article. All authors read and approved the final manuscript.

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Toxicological Research

Gestational Exposure to Pesticides Induces Oxidative Stress and Lipid Peroxidation in Offspring that Persist at Adult Age in an Animal Model

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Abstract

Pesticide exposure may induce biochemical alterations including oxidative stress and lipid peroxidation. However, in the context of developmental origin of health and disease, putative trans-generational effect of exposure to pesticides are insufficiently studied. We therefore aimed to evaluate the biochemical effect of gestational exposure to four pesticides on female Wistar rats and their offspring at adult age. We studied 30 female nulliparous Wistar rats divided into 5 equal groups. Group 1 served as the control group and received distilled water while group 2, 3, 4 and 5 received orally pesticide 1 (imidacloprid), pesticide 2 (chlorpyrifos), pesticide 3 (imidacloprid + lambda cyhalothrin) and pesticide 4 (oxamyl) respectively once daily throughout gestation at a dose equivalent to 1/10 lethal dose 50. The mothers were followed up until one month post gestation. The offspring were followed up from birth until adult age (12 weeks). In all animals at each time point we evaluated malondialdehyde (MDA), oxidative stress and liver function enzymes. There was similar variation of total body weight in all the groups during and after gestation. However, Female Wistar rats of the exposed groups had significant alterations in liver SOD (–30.8% to +64.1%), catalase (–38.8% to –85.7%) and GSH (–29.2% to –86.5%) and; kidney catalase (> 100%), GSH (> 100%). Moreover, MDA, alanine transaminase (ALT) and aspartate transaminase (AST) levels were significantly higher in pesticide exposed rats compared to the control group. Similar alterations in antioxidant enzymes, MDA and liver function enzymes were observed in offspring of treated rats evidenced at weaning and persisting until adult age. Exposure to

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Abbreviations: ALT, Alanine Transaminase; AST, Aspartate Transaminase; BSA, Bovine Serum Albumin; EC, Emulsifiable concentrate; EDTA, Ethylenediaminetetraacetic acid; GPx, Glutathione peroxidase; GR, Glutathione reductase; MDA, Malondialdehyde; NADP, Nicotinamide adenine dinucleotide phosphate; nAChR, Nicotinic acetylcholine receptors; OD, Optical Density; GSSG, Oxidized Glutathione; GSH, Reduced glutathione; SL, Soluble liquid; SEM, Standard error of mean; SOD, Superoxide dismutase.

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pesticides causes oxidative stress and lipid peroxidation in exposed female Wistar rats and their offspring. The persistence in offspring at adult age suggests transgenerational adverse effects.

***Key words*:** Foetal exposure, Insecticide, Biochemical alterations, Offspring, Adult age

INTRODUCTION

Environmental contaminants especially pesticides are now responsible for the development of diseases both in wildlife and humans (1,2). The recent classes of pesticides are organophosphate, carbamates, pyrethroids and neo-nicotinoids. Common pesticides belonging to these classes which are currently used are oxamyl, lambda cyhalothrin, chlorpyrifos and imidacloprid (3). The principal mechanisms of action of these pesticides include phosphorylation and inhibition of the action of acetylcholinesterase by organophosphates and carbamates, blocking of the open gates sodium channels by pyrethroids while neonicotinoids act by binding to the nicotinic acetylcholine receptors (nAChRs) and acting as nAChR agonists (4,5). In addition to their principal mode of action, lambda cyhalothrin, chlorpyrifos and imidacloprid have been reported to cause oxidative stress and lipid peroxidation both *in vitro* and *in vivo* (6-12). The degree of oxidative stress was enhanced with the combination of two pesticides (13,14).

The effect of gestational exposure to maternal pesticides are understudied meanwhile there is increasing evidence on the role of various exposures during early life (pre-natal and post-natal) on adult metabolism and biochemical status. Moreover, investigators like Tukhtaev and collaborators reported that long-term exposure to lambda-cyhalothrin led to the development of oxidative stress in pregnant female rats and their offspring during breastfeeding (15). Whether the effect of this exposure could persist at adult age is unknown. We therefore undertook this study aiming at investigating whether biochemical alteration resulting from exposure to pesticides during pregnancy would persist at adult age. We studied alone and in combination, the most commonly used agricultural pesticides in Africa.

MATERIALS AND METHODS

Chemicals and reagents. L-Drint 20 [(20% emulsifiable concentrate (EC) chlorpyrifos)], Confidor [(17.8% soluble liquid (SL) imidacloprid)], Parastar (2% imidacloprid + 2% lambda cyhalothrin) and Furaplant (10% oxamyl) were purchased from Bayer Company (New Delhi, India). Kits for ALT and AST were purchased from ThermoFisher Scientific (New Delhi, India), while antioxidant enzymes and MDA quantification was carried out with biochemical reagents of molecular grade.

Animals and handling. Nulliparous Wistar rats, aged 10-12 weeks and weighing on average 210 g, were used in this study. The animals were housed in the Animal House of Jamia Hamdard University in polypropylene cages under light controlled conditions with a 12/12-hr light-dark cycle and a temperature of 22 ± 2^{o}C and had free access to water and food. The study was approved by the Animal Ethics Committee of Jamia Hamdard University and the animals used in this study were treated humanely with regards for the alleviation of suffering.

Experimental design.

• ***Female rat sub-study:*** This was a prospective study with a follow-up period of 16 weeks. After acclimatization for a period of 7 days, the rats were fasted overnight and body weight was measured. Animals were then divided into 5 groups of six rats each (Group 1 to Group 5) matched with weight. In each group, animals were mated at a ratio of two females to each male in a cage. At gestation (vaginal swap smear and staining with methyl blue stain and examine for presence of sperms in the smear), the male rats were removed and, pesticide administration was initiated in all female rats. Female rats of Group 2, Group 3, Group 4 and Group 5 received pesticide 1 (44 mg imidacloprid per kg body weight/day), pesticide 2 (13.5 mg chlorpyrifos per kg body weight/day), pesticide 3 (5.6 mg imidacloprid + 5.6 mg lambda cyhalothrin per kg body weight/day) and pesticide 4 (0.4 mg oxamyl per kg body weight/day) respectively with gastro-esophageal probe throughout gestation for 22 days while Group 1 received distilled water. These doses were chosen as shown to induce biochemical alterations without causing mortality and considered to be smaller than the exposed doses in humans during pesticide application, accidental poisoning (water or food containing pesticides). Each solution was freshly prepared by diluting in water and administered between 9 and 10 AM at a dose volume of 10 mL/kg rat per day. Pesticide administration was discontinued at birth while animals were maintained on normal diet and rats were followed for a month. At the end of follow up blood was collected by retro orbital puncture into ethylenediaminetetraacetic acid (EDTA) tubes, plasma was prepared and stored at -20^{o}C for ALT and AST quantification. After collection of blood samples, the rats were sacrificed by decapitation after local anesthesia and dissected. Liver and kidney were removed, washed in ice cold normal saline solution and homogenates prepared and refrigerated at -80^{o}C for quantification of antioxidant enzymes (SOD, catalase, GPx, GSH and GR) and MDA.

• ***Offspring sub-study:*** At birth, offspring were followed up by weekly recording of body weight. The off-

spring were weaned at 4 weeks of age and were followed up until adult age (12 weeks). At adult age, 8 offspring (4 male and 4 female) from each group were randomly selected and sacrificed at the end of follow up. Blood was collected and plasma prepared for ALT and AST quantification while liver and kidney samples were collected for antioxidant enzymes and MDA quantification.

Biochemical assays.

• ***Super oxide dismutase quantification:*** Superoxide dismutase (SOD) was assayed using the method of Marklund and Marklund (16) with slight modifications. A single unit of enzyme was expressed as 50% inhibition of pyrogallol reduction/min/mg protein by superoxide is measured at 420 nm. Briefly, 0.1 mL of the sample solution (liver or kidney homogenate) was added to 2.8 mL of Tris-EDTA buffer solution in a cuvette, 0.1 mL of pyrogallol was added. The solution was mixed rapidly by inversion and the optical density was measured at 420 nm using the kinetic programme (30 sec interval for 3 min). A control tube was also prepared replacing the sample with 0.1 mL doubled distilled water. Enzyme activity was expressed as units/mg protein.

• ***Catalase quantification:*** Catalase was assayed using the method of Aebi (17). Briefly, samples were diluted in the ratio 1:50 with phosphate buffer. One mL of hydrogen peroxide (60 mM) was added into the diluted samples, mixed rapidly by inversion and the optical density read at 420 nm at 30 sec interval for 2 min.

Enzymatic activity was expressed as μmoles of H_2O_2 decomposed/min/mg protein using molar extinction coefficient of H_2O_2 (43.6 $M^{-1}cm^{-1}$).

• ***Glutathione peroxidase quantification:*** This assay was carried out using the method of Wendel (18). Briefly, the reaction mixture (7.0 mg of sodium azide, 33.2 mg reduced glutathione (GSH), and 1.08 mg reduced NADPH in 100 mL of phosphate buffer) was prepared and 3 mL added into the cuvette followed by 50 μL of hydrogen peroxide (0.042%). Fifty μL of sample was then added, rapidly mixed by inversion and the optical density read at 340 nm at 30 sec for 3 min. The results were expressed as nmoles NADPH oxidized/min/mg protein, using molar extinction coefficient of NADPH (6.22×10^6 M^{-1} cm^{-1}).

• ***Reduced glutathione quantification:*** This assay was carried out using the method of Moron (19). Briefly 200 μL of samples were diluted to 1,000 μL with phosphate buffer and reduced Glutathione standards were prepared. A blank tube was equally prepared containing 1 mL of double distilled water. Into each of the tubes (blank standard and samples) was added 1 mL of Ellman's Reagent solution. Each of the tube was incubated and the optical density (OD) read between 2 and 10 min at 412 nm in a spectrophotometer. A graph of OD = f (concentration) of standard was used to determine the concentration of GSH in samples by extrapolation and expressed in nmole.

• ***Glutathione reductase quantification:*** This assay was carried out using the method of Mavis and Stellwagen (20). Briefly, 2.9 mL of the reaction mixture (0.65 mL distilled water, 1.5 mL phosphate buffer 100 mM pH 7.0, 0.1 mL GSSG 30 mM, 0.35 mL β-NADPH 0.8 mM and 0.3 mL 1% w/v BSA) was introduced into the cuvette followed by 0.1 mL of sample and mixed rapidly by inversion. The optical density was then read at 340 nm using a kinetic program (30 sec for 3 min). The activity of the enzyme was expressed as nmoles NADPH oxidized/min/mg protein, using molar extinction coefficient of NADPH (6.22×10^6 M^{-1} cm^{-1}).

• ***Malondialdehyde quantification:*** Malondialdehyde (MDA) was quantified as thiobarbituric acid reactive substrate using the method modified from that of Ohkawa (21). One hundred microliters of each sample was added into a Pyrex test tube followed by 400 μL of the thiobarbituric acid substrate reagent mixture and the tubes were closed tightly. The mixture was heated at 100°C for 15 min. After heating the cork was removed and the tubes were placed in an ice bath for cooling to occur. After the cooling process, the mixture was centrifuged at 1,500 g for 5 min. The optical density of the supernatant was read at 532 nm. The concentration of MDA was then calculated using the molar extinction coefficient of MDA ($\varepsilon = 1.56 \times 10^5$ M^{-1} cm^{-1}) as follows:

$$\text{Concentration of MDA } (\mu M) = \frac{OD}{\varepsilon} \times 1{,}000{,}000 \times \text{dilution factor}$$

• ***Total protein quantification:*** Total protein content in tissue samples was determined by the method of Lowry (22), using bovine serum albumin as the standard.

• ***Alanine transaminase and aspartate transaminase quantification:*** Alanine transaminase (ALT) and aspartate transaminase (AST) were quantified with ALT and AST reagent kits according to the manufacturer's protocol (ThermoFisher Scientific) in the plasma.

• ***Statistical analysis:*** The results were analyzed using GraphPad Software (GraphPad Software, CA, USA). Unpaired *t*-test followed by Welch's correction was used to compare data between control and exposed groups. The data obtained were expressed as mean ± standard error of mean (SEM). All analyses were carried out at 95% confidence interval and $p < 0.05$ was considered to be statistically significant.

RESULTS

Reproductive outcome, weight variation and liver function enzymes level in parent rats. The weights of the dams were similar in all groups throughout the study (Fig. 1). The average gestation length was not signifi-

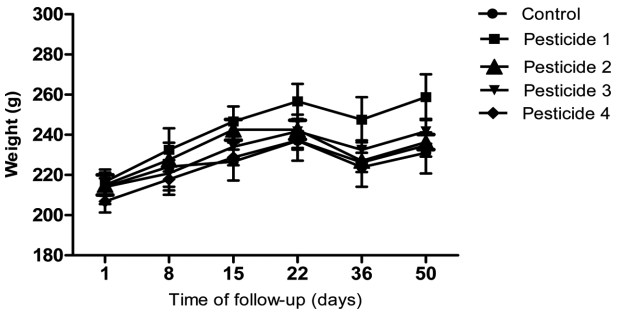

Fig. 1. Effect of pesticides exposure on body weight on female Wistar rats. Pesticide 1 = imidacloprid, pesticide 2 = chlorpyrifos, pesticide 3 = imidacloprid + lambda cyhalothrine, pesticide 4 = oxamyl. Data are reported as mean ± standard error of mean (SEM) for 6 samples.

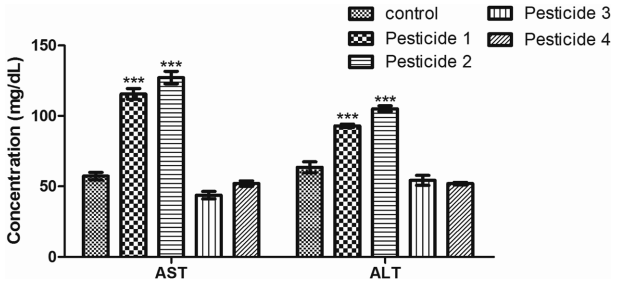

Fig. 2. Effect of pesticides on aspartate transaminase and alanine transaminase levels in female Wistar rats. Pesticide 1 = imidacloprid, pesticide 2 = chlorpyrifos, pesticide 3 = imidacloprid + lambda cyhalothrine, pesticide 4 = oxamyl, ***$p <$ 0.001 compared with normal control. Data are reported as mean ± standard error of mean (SEM) for 8 samples.

cantly different between the control group (22 ± 1 day) and imidacloprid (23 ± 2 days), chlorpyrifos (22 ± 1 day), lambda cyhalothrin + imidacloprid (22 ± 2 days) and oxamyl (24 ± 3 days) exposed groups. However, parent rats exposed to imidacloprid and chlorpyrifos had a significant increase in aspartate transaminase (> 100%) and alanine transaminase (46.4% for pesticide 1 and 65.5% for pesticide 2) levels compared to control (Fig. 2).

Antioxidant enzyme and malondialdehyde variation in liver and kidney of parent rats. Alterations in the level of antioxidant enzymes were recorded in the liver and kidney of parent rats exposed to pesticides when compared with the control group (Table 1). Liver SOD was significantly reduced in rats exposed to pesticide 1 (30.8%), pesticide 2 (38.5%) and 3 (51.3%) while a significantly higher value was recorded in rats exposed to pesticide 4 (64.1%) when compared with the control group. Exposure to all four pesticides led to a significant decrease in liver catalase and liver GSH of parent rats. Pesticide 1 and 2 significantly increase liver glutathione peroxidase (GPx) levels (50% and 64.9% respectively) while pesticide 3 and 4 significantly reduced liver GPx levels (81.1% and 83.9% respectively). Liver glutathione reductase (GR) was significantly reduced in rats exposed to pesticide 4 (64.5%) while liver MDA was significantly increased in rats exposed to pesticide 2 (> 100%). In the kidney, SOD was significantly increased in rats exposed to pesticide 1 (100%) while GPx was significantly increased in rats exposed to pesticide 4 (31.7%). Similarly, GSH was significantly increased in rats exposed to pesticide 1 (52.8%) and pesticide 2 (45.6%). Moreover, rats exposed to pesticide 1, 3 and 4 had a significantly higher MDA (> 100% in all cases) levels when compared to control. Kidney catalase levels were significantly higher in rats exposed to pesticide 3 and 4 (> 100% in both cases) while kidney GR levels were significantly lower in rats exposed to pesticide 3 (38.4%) and 4 (44.8%) compared to the control group.

Weight variation and liver function enzymes level in offspring. In offspring of pesticide exposed rats there was no significant different in body weight throughout the follow when compared with the control group (Fig. 3). However, offspring of rats exposed to all pesticides had a significant increase in ALT and AST levels when compared with the control group (Fig. 4).

Table 1. Effect of pesticides on antioxidant enzymes and malondialdehyde levels in liver and kidney of female Wistar rats

Sample	Liver					Kidney				
	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
SOD	3.9 ± 0.4	2.7 ± 0.3*	2.4 ± 0.2*	1.9 ± 0.4**	6.4 ± 0.4**	1.4 ± 0.3	2.8 ± 0.4*	2.0 ± 0.3	1.3 ± 0.2	1.2 ± 0.2
CAT	24.5 ± 1.9	14.7 ± 1.4**	15.0 ± 1.7**	3.5 ± 0.8***	12.7 ± 1.9**	14.9 ± 6.4	22.4 ± 0.9	31.7 ± 4.6	35.9 ± 1.9*	32.9 ± 2.5*
GPx	14.8 ± 1.2	22.2 ± 1.9*	24.4 ± 2.8*	2.8 ± 0.5***	2.4 ± 0.1***	20.8 ± 2.2	20.6 ± 1.4	21.1 ± 1.1	15.54 ± 2.6	14.2 ± 1.4*
GSH	26.7 ± 2.4	18.9 ± 1.9*	17.3 ± 2.4*	3.8 ± 0.2***	3.6 ± 0.3***	19.3 ± 1.4	29.5 ± 1.7**	28.1 ± 2.9*	15.0 ± 1.8	16.8 ± 1.6
GR	3.1 ± 0.5	1.7 ± 0.6	1.9 ± 0.5	2.2 ± 0.4	1.1 ± 0.3*	48.4 ± 3.4	50.1 ± 2.7	48.5 ± 3.0	29.8 ± 4.9*	26.7 ± 3.1**
MDA	0.7 ± 0.06	5.3 ± 0.3	5.9 ± 0.6***	0.6 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	2.4 ± 0.3*	1.6 ± 0.3	3.5 ± 0.6***	5.0 ± 0.3***

Pesticide 1 = imidacloprid, pesticide 2 = chlorpyrifos, pesticide 3 = imidacloprid + lambda cyhalothrine, pesticide 4 = oxamyl, SOD = super oxide dismutase, CAT = Catalase, GPx = Glutathione peroxidase, GSH = Reduced Glutathione, GR = Glutathione Reductase, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with normal control. Data are reported as mean ± standard error of mean (SEM) for 6 samples.

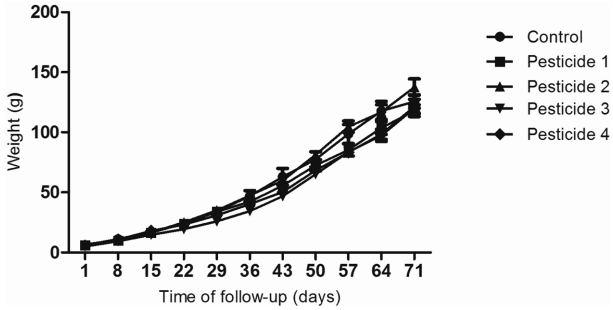

Fig. 3. Effect of pesticides on body weight in offspring. Pesticide 1 = imidacloprid, pesticide 2 = chlorpyrifos, pesticide 3 = imidacloprid + lambda cyhalothrine, pesticide 4 = oxamyl. Data are reported as mean ± standard error of mean (SEM) for 8 samples.

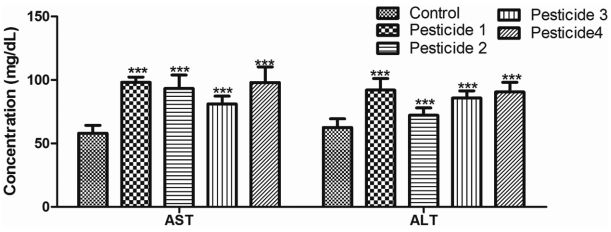

Fig. 4. Effect of pesticides on aspartate transaminase and alanine transaminase levels in offspring of female Wistar rats. Pesticide 1 = imidacloprid, pesticide 2 = chlorpyrifos, pesticide 3 = imidacloprid + lambda cyhalothrine, pesticide 4 = oxamyl, *** = $p < 0.0001$ compared with normal control. Data are reported as mean ± standard error of mean for 8 samples.

Antioxidant enzyme and malondialdehyde variation in liver and kidney of offspring. Similar alterations in liver and kidney antioxidants and MDA levels were recorded in offspring of pesticide exposed rats when compared to the control group at weaning (Table 2) and persisted until adulthood (Table 3). Liver SOD was significantly lower in offspring of rats exposed to pesticide 1 (87.6%) and pesticide 2 (84.9%). Liver catalase was significantly higher in offspring of rats exposed to pesticide 1 (30.3%) and pesticide 2 (36.9%) while a significantly lower catalase level (39.4%) was recorded in offspring of rats exposed to pesticide 4. GPx level was significantly reduced in offspring of rats exposed pesticide 1 (77.9%), pesticide 2 (78.1%) and pesticide 4 (30.3%) when compared to the control group. Similarly, liver GSH was significantly lower in offspring of rats exposed to pesticide 1 (30.4%), pesticide 3 (91.2%) and 4 (90.9%). Likewise, a significantly lower liver GR was recorded in offspring of parent rats exposed to pesticide 3 (41.4%) and 4 (34.5%). A one-fold increase in liver

Table 2. Effect of pesticides on antioxidant enzymes and malondialdehyde levels in liver and kidney of offspring Wistar rats sacrificed at aweaning

Sample	Liver					Kidney				
	Control	Pesti-cide 1	Pesti-cide 2	Pesti-cide 3	Pesti-cide 4	Control	Pesti-cide 1	Pesti-cide 2	Pesti-cide 3	Pesti-cide 4
SOD	1.9 ± 0.2	1.7 ± 0.2	1.9 ± 0.2	1.3 ± 0.2*	1.3 ± 0.2*	2.8 ± 0.6	5.0 ± 0.4*	5.7 ± 0.6**	4.6 ± 1.1	2.9 ± 0.5
CAT	9.2 ± 0.9	14.1 ± 1.8*	8.6 ± 0.7	11.9 ± 2.5	13.4 ± 5.8	10.7 ± 5.6	24.6 ± 13.7	48.9 ± 29.4	8.0 ± 1.0	8.3 ± 0.8
GPx	28.2 ± 2.3	29.8 ± 4.9	26.7 ± 3.1	27.2 ± 2.3	33.5 ± 1.4	4.2 ± 0.5	13.4 ± 5.8	11.9 ± 2.5*	7.0 ± 2.2	8.3 ± 0.9**
GSH	21.4 ± 3.3	15.0 ± 1.8	16.8 ± 1.6	15.5 ± 2.6	14.2 ± 1.4	33.5 ± 1.4	69.1 ± 6.5**	75.7 ± 4.9***	52.0 ± 5.9*	53.2 ± 7.0*
GR	7.1 ± 3.7	1.8 ± 0.3	1.4 ± 0.4	1.3 ± 0.2	1.2 ± 0.2	3.3 ± 0.4	11.1 ± 2.4*	6.9 ± 1.4	6.1 ± 1.2	6.8 ± 1.2*
MDA	3.5 ± 0.4	3.5 ± 0.6	5.0 ± 0.3*	4.4 ± 1.1	3.3 ± 0.4	0.3 ± 0.02	0.9 ± 0.2*	1.6 ± 0.1***	0.9 ± 0.1*	0.8 ± 0.1**

Pesticide 1 = imidacloprid, pesticide 2 = chlorpyrifos, pesticide 3 = imidacloprid + lambda cyhalothrine, pesticide 4 = oxamyl, SOD = super oxide dismutase, CAT = Catalase, GPx = Glutathione peroxidase, GSH = Reduced Glutathione, GR = Glutathione Reductase, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with normal control. Data are reported as mean ± standard error of mean (SEM) for 8 samples.

Table 3. 0Effect of pesticides on antioxidant enzymes and malondialdehyde levels in liver and kidney of offspring Wistar rats at adulthood

Sample	Liver					Kidney				
	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4	Control	Pesticide 1	Pesticide 2	Pesticide 3	Pesticide 4
SOD	23.3 ± 3.8	2.9 ± 0.3**	3.5 ± 0.4**	19.3 ± 1.4	16.6 ± 1.0	3.8 ± 0.3	3.1 ± 0.2	3.4 ± 0.3	5.0 ± 0.4*	5.7 ± 0.6*
CAT	16.5 ± 1.0	21.5 ± 1.2**	22.6 ± 0.7***	17.9 ± 2.5	10.0 ± 2.2*	17.4 ± 2.5	21.2 ± 1.5	26.9 ± 2.9*	24.6 ± 13.7	48.9 ± 29.4
GPx	70.9 ± 4.4	15.7 ± 1.9***	15.5 ± 1.3***	55.7 ± 8.7	49.4 ± 4.5**	7.5 ± 0.6	10.4 ± 0.5**	12.5 ± 0.8***	13.4 ± 5.8	11.9 ± 2.5
GSH	30.9 ± 3.3	21.5 ± 1.9*	24.3 ± 1.7	2.7 ± 0.2***	2.8 ± 0.2***	26.4 ± 3.3	21.5 ± 1.6	14.6 ± 3.3*	17.1 ± 4.7	20.7 ± 4.1
GR	2.9 ± 0.4	2.5 ± 0.6	1.9 ± 0.3	1.7 ± 0.3*	1.9 ± 0.2*	31.8 ± 1.2	34.3 ± 1.9	37.1 ± 1.4*	13.2 ± 1.6***	12.6 ± 1.4***
MDA	5.8 ± 1.4	10.0 ± 1.2*	10.3 ± 0.9*	5.6 ± 0.1	6.1 ± 0.2	2.2 ± 0.2	3.2 ± 0.3*	4.8 ± 0.8*	2.4 ± 0.5	2.6 ± 0.5

Pesticide 1 = imidacloprid, pesticide 2 = chlorpyrifos, pesticide 3 = imidacloprid + lambda cyhalothrine, pesticide 4 = oxamyl, SOD = super oxide dismutase, GPx = Glutathione peroxidase, GST = Glutathione S transferase, GSH = Reduced Glutathione, GR = Glutathione Reductase, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with normal control. Data are reported as mean ± standard error of mean (SEM) for 8 samples.

MDA was recorded in rats exposed to pesticide 1 (72.4%) and 2 (77.6%).

In kidney, SOD was significantly increased in offspring of rats exposed to pesticide 3 (31.6%) and pesticide 4 (50%). Kidney catalase was significantly higher (54.6%) while kidney GSH was significantly lower (44.7%) in offspring of parent rats exposed to pesticide 2. Moreover, kidney GPx and MDA levels were significantly higher in offspring of parent rats exposed to pesticide 1 (38.7% and 45.5% for GPx and MDA respectively) and pesticide 2 (66.7% and 118% for GPx and MDA respectively) when compared to the control group. GR was significantly higher in offspring of rats exposed to pesticide 2 (16.7%) and significantly reduced in offspring of rats exposed to pesticide 3 (58.5%) and pesticide 4 (60.4%).

DISCUSSION

Pesticide poisoning constitute a public health problem as 3,000,000 cases of poisoning and 220,000 deaths were recorded annually across the globe, the majority of which are reported from developing countries (23). Pesticide usage/hectare (ha) is less in Africa (1.23 kg a.i./ha) compared to Latin America and Asia (23). However, the concern in Africa is alarming due to poor status of legislation, insufficient education and training in pesticide usage amongst others. In Cameroon, pesticide exposure mainly occur in agricultural sector, practiced by local farmers or industrialized companies such as Cameroon Development Corporation (CDC), a major development partner in Cameroon which cultivates rubber, oil palm and banana (24). Lack of respect of pesticide application measures, high run off of pesticides into nearby water sources as a result of aircraft spraying and dumping of pesticide waste amongst others result to pesticide poisoning in Cameroon (25). This has as consequence, killing of aquatic organisms, intoxication of the applicators and local population which can result to dead.

Organophosphate, carbamates and neonicotinoids insecticides as well as fungicides are extensively used in agricultural settings in Cameroon with inhalation and ingestion being the main exposure routes (25-27). This study is therefore relevant as it investigated pesticides which are widely used especially for agricultural purposes as well as in public health disease control. Rats were exposed to a dose assumed to be same or inferior to human exposed dose in agricultural setting with similar mode of exposure in humans. Investigation of parameters in offspring was essential so as to depict possible transgenerational effect. The present study shows that all the pesticides tested induced oxidative stress in female Wistar rats, characterised by alterations in circulating levels of at least one antioxidant enzyme in liver and kidney tissues. The observed increase in lipid peroxidation as well as high plasma ALT and AST levels in rats exposed to imidacloprid and chlorpyrifos were confirmatory of tissue damage. Similar alterations observed in parents were equally recorded in offspring at adult age. Oxidative stress in liver and kidney were observed in offspring of rats exposed to pesticides when compared to the control group characterized by alterations in antioxidant levels. Moreover imidacloprid and chlorpyrifos were shown to induce lipid peroxidation in offspring of rats exposed to these two chemicals. In a similar manner, *in utero* exposure to all 4 pesticides induced liver injury as observed by a 3 fold increase in plasma ALT and AST when compared to the control group.

Our findings in directly exposed animals are in accordance with most previous studies. Lambda-cyhalothrin was observed to induced oxidative stress both *in-vivo* and *in-vitro* (6-9). It significantly increased liver and kidney malondialdehyde (MDA) of male adult Wistar rats (9,10). Similarly dysregulation in oxidative stress and lipid peroxidation biomarkers in rats exposed to imidacloprid was reported (11,28-30). Furthermore, chlorpyrifos was observed to induce oxidative stress as well as increase levels of lipid peroxides in the (12,31-35). Notwithstanding, ALT and/or AST levels were significantly increased in plasma of exposed rats and their offspring (36,37). However, the pesticide mixture (5.6 g/kg imidacloprid + 5.6 g/kg lambda cyhalothrine) did not affect the liver function enzymes which can be explained by the low dose.

The mechanism of generation of oxidative stress is related to pesticide biotransformation as in the case of chlorpyrifos. Biotransformation of chlorpyrifos by cytochrome P450 generate its metabolites chlorpyrifos oxon and 3,5,6-trichloro-2-pyridinol. These metabolites being more toxic than the parent compound directly or indirectly generate reactive oxygen species such as highly reactive superoxide radical and hydrogen peroxide (38). At the onset, there is an increase in the level of antioxidants to counteract the effect of these ROS. However, prolonged exposure leads to depletion of the antioxidant system leading to cellular damage (39) and oxidative stress. The end stage of oxidative stress is impairment of cellular functions and potential tissue damage which is characterized by lipid peroxidation (increased MDA levels) and leakage of cytosolic enzymes from hepatocytes (ALT and AST).

What is new in our findings is the effect on offspring with *in utero* exposure that can be evidenced at weaning (Table 3) persist with similar magnitude at adult age without additional exposure. Tukhtaev *et al*. (15) observed similar alterations in oxidative stress enzymes in offspring of rats with inutero exposure to pesticides. However, they reported that the alterations decreased both in exposed rats and their offspring with cessation of breast milk. The difference could be because of the dose used since that in our study was 10 times higher. Therefore, pesticide exposure at

certain doses could induce permanent alteration in exposed organisms. Even though pesticides were not quantified in body fluids of offspring, we can affirm that alterations in offspring resulted from foetal exposure since these chemicals have been shown to cross the placenta. Therefore, further studies looking at the epigenome to investigate the molecular mechanism as well as the second and third generation to characterize the trans-generational effect are warranted.

Commonly used pesticides in Africa (imidacloprid, chlorpyrifos, lambda cyhalothrin and oxamyl) induced oxidative stress, lipid peroxidation and caused liver injury in female Wistar rats and their offspring. These biochemical alterations on offspring remain until adult age suggesting that these chemicals persist in the offspring even after cessation of exposure or modify the epigenome through epigenetic mechanisms. Therefore, further studies looking at the epigenome to investigate the molecular mechanism are warranted.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest

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ORIGINAL ARTICLE

Metabolic effects of exposure to pesticides during gestation in female Wistar rats and their offspring: a risk factor for diabetes?

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Abstract

Some pesticides increase the risk of type 2 diabetes, but whether fetal exposure carries transgenerational risk remains unknown. We evaluated the metabolic effects of gestational exposure to chlorpyrifos and imidacloprid in female Wistar rats and their offspring. We studied female nulliparous Wistar rats, including six exposed to imidacloprid (IMI) and six to chlorpyrifos (CPF) once daily throughout gestation at 1/10 lethal dose 50, while six (control group) received distilled water. These were explored 1 month after the birth of the offspring, while their offspring were explored at weaning (4 weeks) and adult age (12 weeks). Blood glucose, insulin and lipid profile were determined at each stage, while glucose transporter 4 (GLUT4) and nuclear factor kappa beta (NFkβ) protein expression was measured in skeletal muscle at the end of follow up. Exposure to pesticides was associated with significantly higher fasting glucose (+25.4 to 30.9%) and insulin (> 100%) levels, with > 100% increased insulin resistance (HOMA-IR), − 18.3 to − 21.1% reduced HDL-cholesterol and + 60.9 to + 102.6% increased LDL-cholesterol in mothers. GLUT4 expression was reduced by 28.9–42.3% while NFkβ expression increased by 32.8–35.4% in mothers. In offspring, similar abnormalities were observed at weaning (+ 18.4 to 67.4% fasting glucose, + 57.1 to 72.2% LDL-cholesterol, + 72.3 to 78.2% fasting insulin), persisting at adult age with decreased expression of GLUT4 (− 52.8 to 54.5%) and increased expression of NFkβ (+ 30.5 to 30.7%). Gestational exposure to imidacloprid and chlorpyrifos induces hyperglycemia, insulin resistance and dyslipidemia in female Wistar rats and their offspring. The effects on offspring persist until adult age, suggesting intergenerational adverse effects.

Keywords Pesticide · Hyperglycemia · Dyslipidemia · GLUT4 · NFkβ

Abbreviations

ACh	Acetylcholine
ANOVA	Analysis of variance
CPF	Chloropyrifos
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immuno-sorbent assay
EC	Emulsifiable concentrate
FBG	Fasting blood glucose

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GLUT4	Glucose transporter-4
HDL	High density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
HOMA-β	Homeostasis model assessment of beta cell function
ICGEB	International Center for Genetic Engineering and Biotechnology
IMI	Imidacloprid
LDL	Low density lipoprotein
NFkβ	Nuclear factor kappa beta
PBST	Phosphate buffered saline tween
SEM	Standard error of mean
TC	Total cholesterol

Introduction

A pesticide is defined as any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feed stuffs [1]. They are classified as organochlorines, organophosphates, carbamates, pyrethroids and neonicotinoids according to their chemical composition. The effects of these pesticides on metabolic diseases such as type 2 diabetes have been reviewed [2]. Organochlorines were banned in the 1970s because of their resilience to environmental degradation through chemical, biological, and photolytic processes; their lipophilicity, environmental pollution, long-range transport, bioaccumulation in human and animal tissue, as well as bio-magnification in food chains, made them very toxic [3]. Currently, the most commonly used pesticides in agriculture and residential pest control throughout the world are chlorpyrifos and imidacloprid, belonging to the organophosphate and neonicotinoids classes respectively [4, 5]. Chlorpyrifos and imidacloprid directly or indirectly modulate the acetylcholine (ACh) pathway leading to excessive transmission of nerve impulse [6, 7]. The consequence is the involuntary twitching of muscles, paralysis and the death of the exposed organism. These two pesticides affect other biological pathways in ways that could be deleterious to non-target organisms, including humans. The main pathway involves alteration of the anti-oxidant system leading to the development of oxidative stress and lipid peroxidation. Chlorpyrifos and imidacloprid have been observed to induce oxidative stress and lipid peroxidation in the liver, kidney and other organs of rats [8–11]. These pesticides equally affect pathways associated with metabolic disorders such as diabetes and obesity. Acute oral exposure to chlorpyrifos causes hyperglycemia and hyperlipemia in rats [12]. Chronic oral administration of chlorpyrifos to male rats increased the blood glucose level while decreasing the serum insulin level [13]. Exposure of male rats to chlorpyrifos through subcutaneous injection caused hyperlipemia and hyper insulinaemia in adulthood [14]. In humans, fetal exposure to chlorpyrifos caused brain defects, as well as abnormalities of the eyes and other organ system in children [15–17], while acute exposure through poisoning induced drowsiness, dizziness, vomiting, disorientation, and fever [18, 19]. These signs and symptoms were recorded in a 69-year-old woman who ingested a formulated product containing 9.6% imidacloprid and who died 12 h after the exposure [20]. A 24-year-old man who accidentally inhaled a pesticide containing 17.8% imidacloprid while working on his farm was disoriented, agitated, incoherent, sweating and breathless following the exposure [21]. Likewise, direct exposure to imidacloprid may affect the insulin signaling cascade in cultured cell lines [22], and modulate adipocyte differentiation and lipogenesis [23]. Imidacloprid equally induces respiratory distress and neuropsychiatric features following accidental inhalational exposure in humans [24]. The potential for these pesticides to cause intergenerational alterations on oxidative stress and lipid peroxidation has been investigated. Chlorpyrifos, imidacloprid, lambda-cyhalothrin and oxamyl induced oxidative stress and lipid peroxidation in the offspring of exposed Wistar rats [11]. However, the effect on metabolic parameters such as glucose and lipid metabolism is unknown. In fact, the developmental origin of health and disease hypothesis stipulates that adverse fetal exposure may cause permanent fetal adaptations in structure, physiology and metabolism; these might be beneficial for short-term fetal survival, but may lead to fetal growth retardation, cardiovascular and metabolic diseases in adulthood [25]—as has been observed with several risk factors for type 2 diabetes. Possible programming factors investigated include diet and nutrition, environmental factors, maternal hormonal levels during pregnancy and the metabolic situation during pregnancy [26]. We thus investigated the metabolic effects of gestational exposure to the widely-used pesticides chlorpyrifos and imidacloprid in female Wistar rats and their offspring.

Materials and method

Chemicals and reagents

L-Drint 20 [(20% emulsifiable concentrate (EC) chlorpyrifos)] and Confidor [(17.8% soluble liquid (SL) imidacloprid)] were purchased from Bayer Company in New Delhi, India. Kits for insulin ELISA, lipid profile, plasma glucose quantification and western blotting analysis were purchased

from ThermoFisher Scientific in New Delhi, India. Both chemicals were stored at room temperature and diluted with distilled water to the required concentration before being administered to the rats.

Animals and handling

Nulliparous Wistar rats, aged 10–12 weeks and weighing on average 210 g, were used in this study. The animals were housed in the Animal House of Jamia Hamdard University in polypropylene cages under light controlled conditions with a 12/12-h light–dark cycle and a temperature of 22 ± 2 °C. They had free access to water and food. The study was approved by the Animal Ethics Committee of Jamia Hamdard University and the animals used in this study were treated humanely with regard to the alleviation of suffering.

Experimental design

Female rat sub-study

This was a prospective study with a follow-up period of 16 weeks. After acclimatization for a period of 7 days, the rats were subjected to an overnight fast (approximately 16 h). Fasting blood glucose levels and body weight were measured. Animals were then divided into three groups of six rats each (group 1, group 2 and group 3), matched with weight and fasting glycemia. In each group, animals were mated at a ratio of two females to each male in a cage. At gestation, the male rats were removed and pesticide administration was initiated. Female rats in group 1 served as the control group, receiving water; female rats in groups 2 and 3 received imidacloprid (44 mg/kg body weight/day) and chlorpyrifos (13.5 mg/kg body weight/day), respectively, with a gastro-esophageal probe throughout gestation. This dose was chosen as previously shown to induce biochemical alterations without causing behavioral signs of toxicity or mortality. Behavioral signs of toxicity that were checked included changes in skin and fur, eyes and mucous membranes, respiratory and behaviour patterns, with particular attention paid to possible tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Each solution was freshly prepared by diluting in water and administered between 9 and 10 AM at a dose volume of 10 mL/kg rat per day. Pesticide administration was discontinued at birth while animals were maintained on normal diet and parents were followed for a month. At the end of follow up, blood was collected by cardiac puncture into dry tubes and ethylenediaminetetraacetic acid (EDTA) tubes. Serum and plasma were prepared and stored at − 20 °C for glucose, lipid profile and insulin. After collection of the blood samples, the rats were killed by euthanasia after local anesthesia and dissected. Skeletal muscles were collected and refrigerated at − 80 °C for western blood analysis of GLUT4 and NFKβ.

Offspring sub-study

At birth, offspring were followed up by weekly recording of body weight and weaned at 4 weeks of age. At weaning, eight offspring (four male and four female) were randomly selected from each group and fasted overnight. Blood samples were collected into EDTA and dry tubes. Serum and plasma were prepared and stored at − 20 °C for glucose, lipid profile and insulin quantification. After collection of blood samples, the rats were killed by euthanasia after local anesthesia and dissected. The remaining offspring were followed up until 12 weeks and killed (four male and four female per group) after an overnight fast. Blood samples and skeletal muscles were collected for biochemical (blood glucose, insulin and lipid profile) measurements and western blotting analysis.

Biochemical assays

Plasma glucose concentration was measured with a glucose oxidase kit according to the TRINDER method [27]. Serum total cholesterol, High Density Lipoprotein (HDL) cholesterol and triglycerides were measured using standard enzymatic techniques [28–30]. Low Density Lipoprotein (LDL) cholesterol was calculated using Friedwald's formula [31]. Plasma insulin was measured with the ALPCO Rat Insulin ELISA kit as per the manufacturer's protocol (Biorbyt Ltd, UK).

Western blotting analysis

A muscle sample was chopped and homogenized on ice with a buffer solution four times its volume. It was kept on ice for 15 min, and 2.5 µL of Nonidet P-40 was added. The mixture was centrifuged at 20,000 g for 15 min at 4 °C and the supernatant collected as protein extract. A portion of the extract was used for protein quantification via the Lowry method, using BSA as standard [32]. Each sample was diluted to 5 mg/mL with the extraction buffer, mixed with the loading buffer in the ratio 4:1 and heated at 80 °C for 7 min. After cooling to r.t.p., 10 µL was loaded into the wells of a 10% separating and 5% stacking gel. The gel was run for 30 min at 90 volts and then 2 h at 120 volts. A nitrocellulose membrane and the gel were equilibrated in the transfer buffer for 2 min. The transfer assembly was set (negative electrode/sponge/three Whatmann filter paper/gel/membrane/three Whatmann filter paper/sponge/positive electrode) while rolling to remove bubbles. The assembly was then fitted into the gel tank and filled with the transfer buffer. The current was switched on and the transfer was effected at 95 volts

for 2 h. The membrane was removed and blocked with 5% bovine serum albumin (BSA) overnight at 4 °C. The next day, BSA was discarded and the membrane was incubated in the primary antibody (GLUT4 or NFkβ) or beta actin as the control protein at r.t.p. for 2 h, washed trice with 1× phosphate buffered saline containing 0.5% tween-20 (PBST). The membrane was again incubated with the secondary antibody (Goat Anti-Mouse IgG) for 90 min at room temperature and washed thrice with PBST. The developing solution (luminol) was spread on the surface of the membrane and the bands visualized and captured. The band of interest was then quantified using the image J software.

Calculations

Insulin resistance was determined by the homeostasis model assessment (HOMA-IR) using the formula: $\text{HOMA-IR} = \frac{\text{Fasting Glucose(mg/dL)} \times \text{Fasting Insulin(mU/L)}}{405}$. Beta cell function was determined by HOMA-β using the formula: $\text{HOMA-}\beta = \frac{360 \times \text{Fasting Insulin(mU/L)}}{\text{Fasting Glucose(mg/dL)} - 63}$.

Statistical analysis

The results were analyzed using GraphPad Prism software. The one way ANOVA test and the Bonferroni Multiple Comparison Test were used to compare data between the different groups. The data obtained were expressed as mean ± SEM. All analysis were carried out at 95% confidence interval and $p < 0.05$ was statistically significant.

Results

Reproductive outcome of dams and pups

The weights of the dams in the pesticides-exposed groups were similar to those in the control group throughout the study. The average length of gestation was 25 days, 23 days and 27 days in the control, imidacloprid-exposed and chlorpyrifos-exposed groups, respectively. Pregnancy was recorded in four rats of the control group, five rats of the imidacloprid-exposed group and two rats of the chlorpyrifos-exposed group. The total number of pups was 31 (15 males), 32 (17 males) and 17 (11 males) for the control, imidacloprid-exposed and chlorpyrifos-exposed groups, respectively. There was no significant difference in the weight at birth of the pups of imidacloprid (5.9 ± 0.4) and chlorpyrifos (6.7 ± 0.4) exposed rats when compared to the control group (6.2 ± 0.2). No sign of toxicity was recorded in dams and their pups upon observation throughout the study.

Metabolic parameters in female Wistar rats

The metabolic parameters investigated were blood glucose, insulin, and lipid profile. Insulin sensitivity and insulin secretion were assessed as HOMA-IR and HOMA-β respectively, calculated from fasting glucose and fasting insulin values. In parent rats, the baseline fasting blood glucose (FBG) was similar in the three groups (79.6–82.0 mg/dL). At the end of the follow up, FBG and insulin were significantly higher in rats exposed to imidacloprid (25.4% and 123.9% for FBG and insulin, respectively) and chlorpyrifos (30.9% and 97.8% for FBG and insulin, respectively) compared to the control group. HOMA-IR values were significantly higher in rats exposed to imidacloprid and chlorpyrifos (Fig. 1), while no difference in HOMA-β values were recorded (103.9 ± 23.9–141.8 ± 63.1). Similarly, alterations in lipid profile parameters were recorded in pesticide-exposed rats (Fig. 2). Rats exposed to imidacloprid had a significant higher total cholesterol (51.9%) and LDL-cholesterol (> 100%) levels, while rats exposed to chlorpyrifos had a significant higher LDL-cholesterol (60.9%) level. Equally, HDL-cholesterol was significantly lower in rats exposed to both pesticides (18.3% IMI and 21.1% CPF) than in rats in the control group (Tables 1, 2).

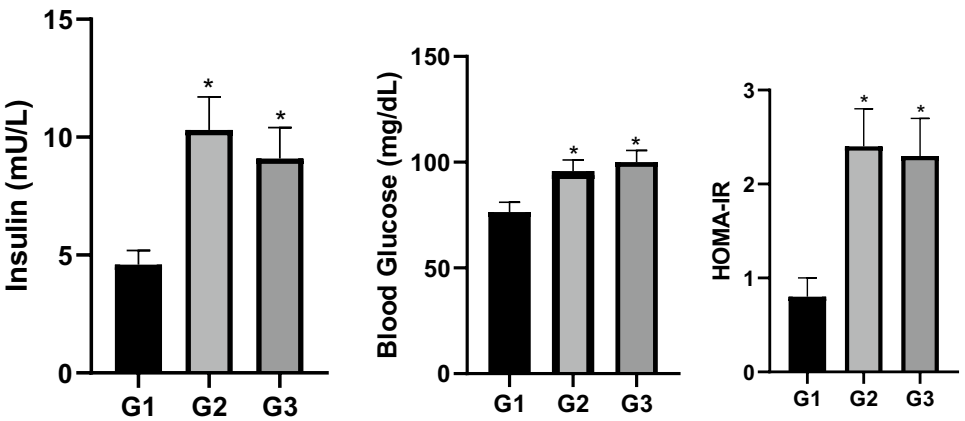

Fig. 1 Effect of pesticide exposure on fasting blood glucose, insulin, HOMA-IR and HOMA-β in female Wistar rats. G1 = normal control, G2 = imidacloprid, G3 = chlorpyrifos, HOMA-IR = homeostasis model assessment of insulin resistance, *$p < 0.05$, Values are given as mean ± SEM (n = 6)

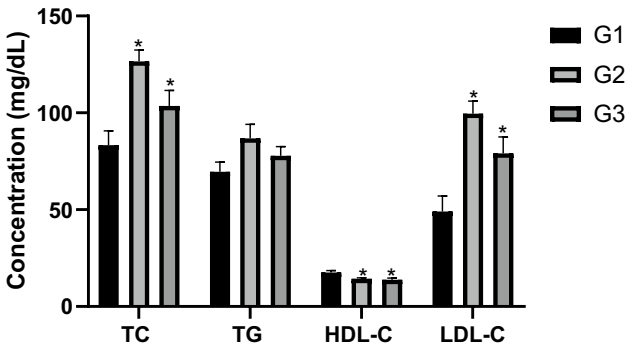

Fig. 2 Effect of pesticide exposure on lipid profile in female Wistar rats. G1 = normal control, G2 = imidacloprid, G3 = chlorpyrifos, TC = total cholesterol, TG = triglycerides, HDL–C = high density lipoprotein cholesterol, LDL–C = low density lipoprotein cholesterol, *$p < 0.05$, Values are given as mean ± SEM (n = 6)

Table 1 Effect of pesticide exposure on fasting blood glucose, insulin, HOMA-IR and HOMA-β

Variables	Parents		
	Control group	Imidacloprid group	Chlorpyrifos group
FBG 1 (mg/dL)	80.4 ± 5.4[a]	79.6 ± 6.6[a]	82.0 ± 4.2[a]
FBG 2 (mg/dL)	76.4 ± 4.7[b]	95.8 ± 5.3[a]	100.0 ± 5.6[a]
Insulin (mU/L)	4.6 ± 0.6[b]	10.3 ± 1.4[a]	9.1 ± 1.3[a]
HOMA-IR	0.8 ± 0.2[b]	2.4 ± 0.4[a]	2.3 ± 0.4[a]
HOMA-β	141.8 ± 63.1[a]	133.4 ± 32.5[a]	103.9 ± 23.9[a]

Group 1= normal control, Group 2= imidacloprid, Group 3 = chlorpyrifos, FBG 1 = fasting blood glucose before treatment, FBG 2 = fasting blood glucose after treatment, HOMA-IR = homeostasis model assessment of insulin resistance, HOMA-β = homeostasis model assessment of beta cell function. Values are given as mean± SEM (n = 6). Means with common lower case letter in column do not differ significantly at 5 % ($p < 0.05$) level of significance

Table 2 Effect of pesticide exposure on lipid profile

Variables	Parents		
	Control group	Imidacloprid group	Chlorpyrifos group
TC (mg/dL)	83.3 ± 7.4[b]	126.5 ± 5.9[a]	103.5 ± 8.1[a,b]
TG (mg/dL)	69.50 ± 5.0[a]	86.8 ± 7.2[a]	77.8 ± 4.7[a]
HDL-C (mg/dL)	17.5 ± 1.0[a]	14.3 ± 0.5[b]	13.8 ± 0.9[b]
LDL-C (mg/dL)	49.1 ± 7.9[b]	99.5 ± 6.6[a]	79.0 ± 8.5[a]

Group 1= normal control, Group 2= imidacloprid, Group 3 = chlorpyrifos, TC = total cholesterol, TG = triglycerides, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol. Values are given as mean± SEM (n =6). Means with common lower case letter in column do not differ significantly at 5 % ($p < 0.05$) level of significance

Metabolic parameters in offspring of female Wistar rats

The results of the evaluation of metabolic parameters in offspring after weaning (4 weeks of age) and at adult age (12 weeks) are presented in Fig. 3. Fasting blood glucose, insulin and HOMA-IR values were significantly higher in the 4 weeks-old offspring of rats exposed to imidacloprid (67.4% FBG, 78.2% insulin and > 100% HOMA-IR), while rats exposed to chlorpyrifos had a significant increase in insulin (72.3%) and HOMA-IR (> 100%) levels when compared to rats in the control group. This increase was observed to persist until adult age, despite a reduction in magnitude in rats exposed to imidacloprid. However, HOMA-β values were not significantly different between the offspring of exposed and control groups sacrificed at weaning (152.3 ± 23.4–315.5 ± 65.4), as well as those sacrificed at adult age (128.1 ± 27.4–150.4 ± 87.9). Alteration in lipid profile parameters was equally recorded in the offspring of exposed rats sacrificed at weaning (22.1% and 16.9% increase in TC for IMI and CPF, 72.2% and 57.1% increase in LDL-cholesterol for IMI and CPF, respectively) and those sacrificed at adult age, compared to the control group (Fig. 4).

Glucose transporter 4 and nuclear factor kappa beta protein expression

In dams, the expression of GLUT4 was significantly lower in the imidacloprid (28.9%) and chlorpyrifos (42.3%) exposed groups compared to the control group (Fig. 5A), while NFKβ expression was significantly higher in the imidacloprid (35.4%) and chlorpyrifos (32.8%) groups compared to the control group (Fig. 5B). Similarly, in pups sacrificed at adult age, the expression of GLUT4 was significantly lower in imidacloprid exposed (52.8%) and chlorpyrifos exposed (54.5%) groups compared to the control group (Fig. 6A), while NFKβ expression was significantly higher in imidacloprid exposed (30.5%) and chlorpyrifos exposed (30.7%) groups compared to the control group (Fig. 6B).

Discussion

Pesticide poisoning is presently considered a public health problem in Africa. This is largely due to misuse of pesticides resulting from, among other factors, a lack of training, the discharge of remains into nearby rivers, and the absence of personal protection equipment during pesticide applications [33]. Chlorpyrifos, imidacloprid and other insecticides and fungicides are extensively used, with inhalation and ingestion being the main exposure routes [33–35]. This study is highly relevant as it investigates

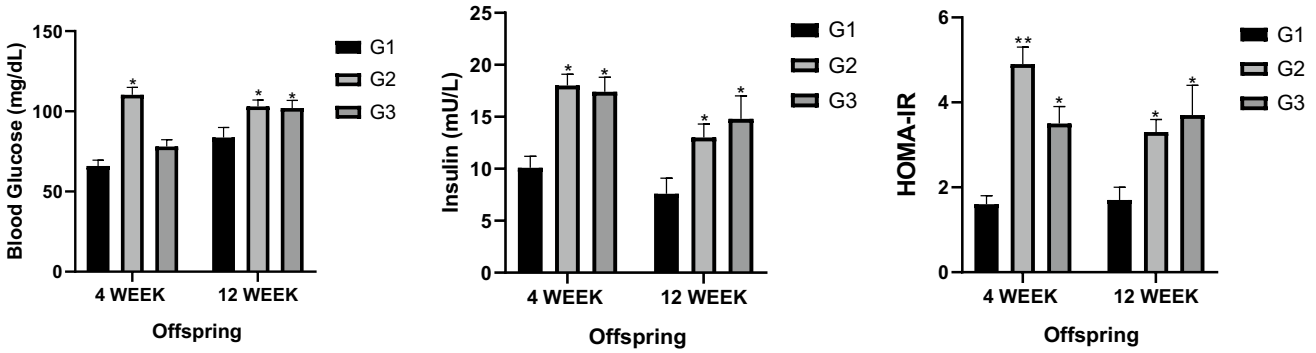

Fig. 3 Effect of pesticide exposure on fasting blood glucose, insulin, HOMA-IR and HOMA-β in offspring. G1 = normal control, G2 = imidacloprid, G3 = chlorpyrifos, HOMA-IR = homeostasis model assessment of insulin resistance, $*p < 0.05$, $**p < 0.01$, Values are given as mean ± SEM (n = 8)

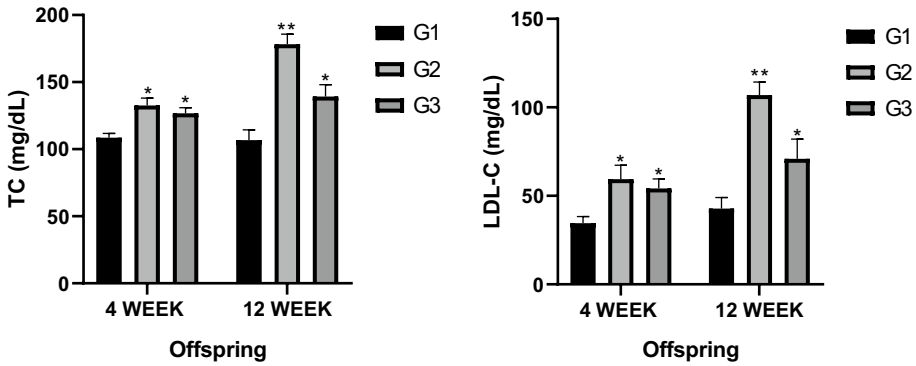

Fig. 4 Effect of pesticide exposure on lipid profile in offspring. G1 = normal control, G2 = imidacloprid, G3 = chlorpyrifos, TC = total cholesterol, LDL–C = low density lipoprotein cholesterol, $*p < 0.05$, $**p < 0.01$, values are given as mean ± SEM (n = 8)

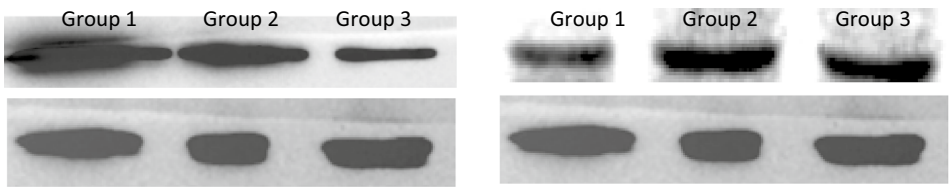

Fig. 5 Effect of imidacloprid on GLUT4 (left) and NFkβ (right) expression in exposed rats (n = 6). The blots below are beta actin control. Group 1 = normal control, Group 2= imidacloprid, Group 3 = chloropyrifos. Data are reported as mean ± standard error of mean (SEM)

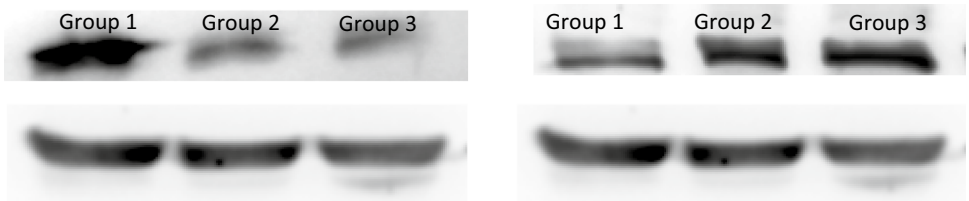

Fig. 6 Effect of imidacloprid on GLUT4 (left) and NFkβ (right) expression in offspring at adult age (n = 8). The blots below are beta actin control. Group 1 = normal control, Group 2 = imidacloprid, Group 3 = chloropyrifos. Data are reported as mean ± standard error of mean (SEM)

pesticides that are widely used for agricultural purposes as well as in public health disease control. We showed in previous studies that these pesticides were associated with the development of oxidative stress and lipid peroxidation [11], considered to be responsible for defects in glucose and lipid metabolism. Rats were exposed to a dose assumed to be the same as or inferior to a human exposure dose in an agricultural setting, with a similar mode of exposure. Given that chronic long-term exposure to pesticides has been reported to affect metabolic pathways, protein expression analysis was effected to confirm the possible mechanistic pathway. Investigations were also carried out on offspring exposed throughout gestation, to explore the intergenerational effects. The present study shows that exposure to imidacloprid and chlorpyrifos throughout gestation caused hyperglycemia, hyper insulinaemia and dyslipidemia in female Wistar rats. Our findings are in conformity with other studies which have reported hyperglycemic and hyperlipemic effects for chlorpyrifos and imidacloprid exposure [12–14].

Hyperglycemia and oxidative stress are well-known complications/outcomes of pesticide exposure [11, 36]. Moreover, the oxidative stress caused by pesticide exposure may exacerbate hyperglycemia and vice versa. Both conditions (oxidative stress and hyperglycemia) contribute to an oxidative environment that may alter insulin sensitivity, either by increasing insulin resistance or impairing glucose tolerance. A significantly high HOMA-IR value characteristic of insulin resistance in pesticide-exposed rats confirms the insulin resistant effect of imidacloprid and chlorpyrifos through the oxidative stress and/or hyperglycemic pathway. The possible link between oxidative stress and insulin resistance include ROS impaired insulin signaling caused by inducing IRS serine/threonine phosphorylation, disturbing cellular redistribution of insulin signaling components, decreasing GLUT4 gene transcription, or altering mitochondrial activity [37, 38]. Our study reported low expression levels of GLUT4 proteins in pesticide exposed rats, which should result from a decrease in gene transcription. Chronic oxidative stress has also been reported to induce a number of stress-sensitive signaling pathways, such as NF-κB JNK/SAPK, and p38 MAPK. Our study confirms such findings, as oxidative stress resulting from pesticide exposure significantly increased NF-κB protein levels.

Metabolic alterations in exposed parents were equally observed in their offspring, persisting until adult age despite no postnatal exposure. Our study therefore provides new findings as it is the first to report metabolic alterations such as hyperglycemia, dyslipidemia, insulin resistance as well as alterations in GLUT4 and NFkβ protein expression in offspring with in utero exposure. These were in evidence at weaning and persisted with similar magnitude at adult age without additional exposure.

This suggests that chronic exposure to pesticides induces permanent changes affecting the metabolic pathway directly or through the cholinergic system. Given that the pesticides were neither quantified in the biological fluids of parents nor offspring, we cannot affirm that the adverse effects observed in offspring were mainly due to fetal exposure, as alterations in the parents could be transmitted to offspring through fetal programming. Another limitation is the fact that we did not study the second generation necessary to establish putative trans-generational effect, or conduct methylation analysis to confirm the effect on the epigenome as well as the expression of other proteins involved in the insulin signaling pathway such as pPKB, p-mTOR, and p-IRS-1.

The 7th edition of the International Diabetes Federation Atlas shows that Africa will be faced with the highest increment in the prevalence of diabetes by 2040 [39]. It is also a continent that relies heavily on pesticide applications for the control of agricultural and household pests. With the possible metabolic alterations observed in this study, especially in offspring that persist until adult age, it seems likely that pesticide is a major contributor to the global burden of diabetes. The entire human population should be therefore be sensitized on the adverse effects of these chemicals, while a search is conducted for a biological replacement, since pesticides are essential to agriculture. Additionally, there is a need for further study of the epigenome to investigate the molecular mechanisms, with emphases on the insulin signaling pathway and on the second and third generations, to ascertain the trans-generational effects. Studies in humans are equally needed to create awareness and enable policy makers to regulate the use of these pesticides.

Imidacloprid and chlorpyrifos induce hyperglycemia, insulin resistance and dyslipidemia in female nulliparous Wistar rats and their offspring with in utero exposure throughout gestation. The biochemical alterations in offspring remain until adult age, suggesting that the chemicals responsible persist in the offspring even after the cessation of exposure, or modify the epigenome through epigenetic mechanisms. Further research on the epigenome is therefore required to investigate these molecular mechanisms.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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