DOI: 10.31357/fmesphd.2013.00123

THE EFFECTS OF LEAD AND CADMIUM ON SPERM PARAMETERS INCLUDING SPERM DNA FRAGMENTATION OF MEN INVESTIGATED FOR INFERTILITY

by

GAMAGE UDAYA SHANTHA WIJESEKARA

Ph.D 2013

THE EFFECTS OF LEAD AND CADMIUM ON SPERM PARAMETERS INCLUDING SPERM DNA FRAGMENTATION OF MEN INVESTIGATED FOR INFERTILITY

by

GAMAGE UDAYA SHANTHA WIJESEKARA

Thesis submitted to University of Sri Jayewardenepura for the award of the Degree of Doctor of Philosophy in Medical Laboratory Science on 15.07.2013.

We certify that the candidate has incorporated all corrections, amendments and additions recommended by the examiners.

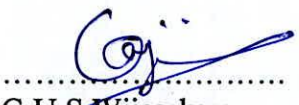
Professor D.M.S.Fernando — Date: 12/05/2014

Professor S.Wijeratne — Date: 12/05/2014

Professor N.Bandara — Date: 12/05/2014

DECLARATION BY THE CANDIDATE

I certify that the work presented in this thesis was carried out by me under the supervision of Professor D.M.S. Fernando, Professor S.Wijeratne and Prof. N.Bandara and a report of this work has not been submitted in whole or part to any other university or any other institution for another Degree or Diploma.



G.U.S.Wijesekara

12/05/2014

Date

DECLARATION BY THE SUPERVISORS

We certify that above statement made by the candidate is true and thesis is suitable for submission to the University of Sri Jayewardenepura for the purpose of evaluation.

.....Fernando..... ...12/05/2014.....

Professor D.M.S. Fernando Date

..... 12/05/2014.....

Professor S.Wijeratne Date

..... 12/05/2014.....

Professor N. Bandara Date

Dedicated to My Mother, beloved Father to whom I owe everything,

Wife, Apsara and son, Sanila.

TABLE OF CONTENTS

LIST OF TABLES ..... **VI**

LIST OF FIGURES ..... **VII**

ABBREVIATIONS ..... **VIII**

ACKNOWLEDGEMENT ..... **XIII**

ABSTRACT ..... **XV**

1 INTRODUCTION **1**

1.1 Background 1

1.2 Justification 5

1.3 Objectives 8

1.3.1 General Objective *8*

1.3.2 Specific objectives *8*

2 LITERATURE REVIEW **9**

2.1 Human reproduction and male reproductive system 9

2.1.1 Male reproductive system *9*

2.2 Semen and seminal fluid analysis 17

2.2.1 Semen *17*

2.2.2 Semen analysis *17*

2.2.3 Nomenclature related to semen quality *21*

2.3 Quality of semen and infertility 23

2.3.1 Genetic abnormalities, testicular cancer and semen quality *24*

2.3.2 Occupational and environmental exposure on semen quality *25*

2.3.3 Physical exposure and life style factors *26*

2.3.4 Effects of environmental chemicals on spermatogenesis *30*

2.3.5 Occupational exposure to various chemicals and pollutants 33

2.4 Exposure to lead 37

2.4.1 Main sources and routes of lead exposure 38

2.4.2 Other sources of Lead exposure 39

2.4.3 Absorption and metabolism of lead 40

2.5 Reproductive effects of lead on males 42

2.5.1 Effect of lead on reproductive hormones in men 42

2.5.2 Effects of lead on semen quality 46

2.5.3 Effects of lead on sperm chromatin structure 60

2.6 Exposure to cadmium 72

2.6.1 Sources of cadmium exposure 73

2.6.2 Routes of entry of cadmium 73

2.6.3 Metabolism of cadmium 75

2.7 Reproductive effects of Cadmium 76

2.7.1 Mechanism of the action of cadmium on male reproductive system 76

2.7.2 Effect of Cd on reproductive hormones and sperm parameters 79

2.8 Sperm DNA structure and DNA damage induced infertility 100

2.8.1 Sperm chromatin structure 100

2.8.2 Sperm DNA damage 103

2.8.3 Methods for assessment of sperm DNA fragmentation 107

2.8.4 Causes for sperm DNA damage 114

2.9 Oxidative stress induced DNA damage 119

2.9.1 Reactive Oxygen species and oxidative stress 119

2.9.2 Sources of oxidative stress 125

2.9.3 Relationship between ROS and sperm DNA damage 126

2.9.4 Mechanism of sperm DNA damage by oxidative stress 130

2.9.5 Lead and cadmium induced oxidative stress and DNA damage..... 136

3 METHODOLOGY 147

3.1 Phase 1 148

3.1.1 Study Design 148

3.1.2 Study Setting 148

3.1.3 Study population 148

3.1.4 Sample size 150

3.1.5 Data collection 150

3.1.6 Collection of semen 153

3.1.7 Seminal fluid analysis to determine sperm parameters 153

3.1.8 Analysis of lead and cadmium in seminal plasma 159

3.1.9 Data analysis 164

3.1.10 Statistical analysis 165

3.2 Phase 2 166

3.2.1 Study design 166

3.2.2 Study Setting 166

3.2.3 Study Population 166

3.2.4 Sample Size 167

3.2.5 Collection of Samples 168

3.2.6 Assessment of Sperm DNA Fragmentation 169

3.2.7 Data analysis 172

3.2.8 Statistical Analysis 173

3.3 Ethical Considerations 173

3.4 General Precautions 174

3.5 Quality control 174

4 RESULTS 176

4.1 Phase 1 176

4.1.1 Demographic characteristics of the study population *176*

4.1.2 Geographical Distribution *177*

4.1.3 Exposure to Toxicants *178*

4.1.4 Semen Characteristics of the Study Population *178*

4.1.5 Exposure to reproductive toxicants and sperm parameters *180*

4.1.6 Seminal plasma Pb and Cd in the study population *184*

4.1.7 Association between exposure to toxicants and Pb and Cd in seminal plasma *185*

4.1.8 The association between Sperm parameters and Pb and Cd *188*

4.1.9 Summary of results of Phase 1 ..... *189*

4.2 Phase 2 191

4.2.1 DNA fragmentation in spermatozoa *191*

4.2.2 Association between Pb and Cd levels and DNA Fragmentation *194*

4.2.3 DNA fragmentation and sperm parameters *195*

4.2.4 Summary of the results of phase 2 ..... *201*

5 DISCUSSION 202

5.1 Exposure to reproductive toxicants and sperm parameters 203

5.2 Lead and cadmium in seminal plasma 208

5.3 The association between exposure to toxicants and Pb and Cd in seminal plasma 210

5.4 Association between Pb and Cd in seminal plasma and semen parameters 212

5.5 The association between Pb and Cd in seminal plasma and sperm DNA fragmentation 217

5.6 Clinical Importance of detecting sperm DNA damage 224

5.7 Limitations of the study 225

6 CONCLUSION 226

7 RECOMMENDATIONS AND FUTURE DIRECTIONS 227

8 REFERENCES 228

9 LIST OF APPENDICES 261

9.1 Appendix. 1 Papanicolaou staining technique for sperm morphology 261

9.2 Appendix 2 Assessment of sperm DNA fragmentation 264

9.3 Appendix. 3 Publications and communications 266

9.4 Appendix. 4 Interviewer administered questionnaire 267

9.5 Appendix 5 Interviewer administered Questionnaire (Sinhala version). 274

9.6 Appendix 6 Ethicla Clearance Obtained from Ethical Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura 280

9.7 Appendix 7 Information sheet giving information on the nature of the study, samples required, the benefits and the importance of the study 281

9.8 Appendix 8 Information sheet (Sinhala Version) giving information on the nature of the study, samples required, the benefits and the importance of the study(Sinhala version) 283

9.9 Appendix 9 Volunteer consent form for participation in the study. 285

9.10 Appendix 10 Volunteer consent form for participation in the study(Sinhala version). 287

LIST OF TABLES

Table 2.1 WHO (2010) reference values for semen characteristics 21

Table 3.1 Lower reference limits of sperm parameters. 158

Table 4.1 Characteristics of the total population 177

Table 4.2 Characteristics of pathozoospermics and normozoospermics 177

Table 4.3 Frequency of exposure to different categories of toxicants (n=300) 178

Table 4.4 Comparison of mean values of sperm parameters in pathozoospermics and normozoospermics. 179

Table 4.5 Mean sperm parameters of pathozoospermics and normozoospermics exposed and not exposed to toxicants 181

Table 4.6 Sperm parameters of pathozoospermics and normozoospermics exposed and not exposed to toxicants 183

Table 4.7 Pb and Cd levels of Normozoospermics and Pathozoospermics 185

Table 4.8 Pb and Cd levels of different exposure categories 186

Table 4.9 Mean Pb and Cd levels of pathozoospermics and normozoospermics exposed to toxicants and not exposed to toxicants. 187

Table 4.10 Sperm parameters of Pb positive and negative men. 188

Table 4.11 Sperm parameters of Cd positive and negative men 189

Table 4.12 The relationship between Pb and Cd levels in seminal plasma and 194

Table 4.13 Sperm DNA fragmentation and Pb and Cd levels 195

Table 4.14 Sperm DNA fragmentation and sperm parameters 196

Table 4.15 Sperm parameters of Pb positive, Cd positive and control subjects 197

Table 4.16a Sperm parameters of Pb positive subjects 201

Table 4.16b Sperm parameters of Cd positive subjects 202

Table 4.16c Sperm parameters of control subjects 203

LIST OF FIGURES

Figure 2.1 Male Reproductive System 10

Figure 2.2 Structure of a spermatozoa 13

Figure 2.3 Hypothalamic-Pituitary-Testicular Axis. 15

Figure 2.4 Association of increasing reactive oxygen species (ROS) production with infertility (Cocuzza et al. 2007). 125

Figure 2.5 Mechanistic pathway showing sperm DNA damage due to oxidative stress. (Cocuzza et al. 2007) 130

Figure 3.1 The Organizational structure of methodology 147

Figure 3.2 Graphite Furnace for Atomic Absorption analysis and output signal. 162

Figure 3.3 Sperm chromatin dispersion patterns. 172

Figure 4.1 Distribution of different sperm abnormalities. 179

Figure 4.2 spermatozoa without fragmented DNA found in a subject in the study and positive control in control well. 192

Figure 4.3 spermatozoa with fragmented DNA found in a subject in the study and positive control in control well 193

ABBREVIATIONS

5-HIAA - 5-Hydroxyindoleacetic acid

5-HT - 5-hydroxytryptamine

8-OhdG - Oxo-2'-deoxyguanosine

AcE - *Allium Cepa*

ALA - δ-aminolevulinic acid

ALAD - δ-aminolevulinic acid dehydratase

ALAS - δ-aminolevulinic synthetase

ALT - Alanine aminotransferase

AMH - Anti-Müllerian hormone

AO - Acridine orange

AOT - Acridine orange test

AST - Aspartate aminotrasferase

ATP - Adenosine tri phosphate

ATPase -Adenosine tri phosphatese

ATSDR - Agency for Toxic Substances and Disease Registry

BMI - Body Mass Index

BP – Biochemical pregnancy

BSO - Bismuth silicon oxide

CAT – Catalase

CDC - Centre for Disease Control

CI - Confidence interval

CLP - Clinical pregnancy

CM - Circulate motile

CP – Ceruloplasmine

DAPI - 6-diamino-2-phenylindole

DBCP – Dibromochloroethane

DDT- Dichlorodiphenyltrichloroethane

DFI - Deoxyribonucleic acid fragmentation index

DHHS - United States Department of Health and Human Services

DMSO - Dimethyl sulfoxide

DNA -Deoxyribonucleic acid

DSBs - Double strand breaks

dUTP - Deoxyuridine triphosphate

EDTA - Ethylene diamine tetra acetic acid

EFT4 - Estimated free thyroxine

eGFR - Estimated Glomerular Filtration Rate

ELISA - Enzyme linked immunosorbent assay

EP - Erythrocyte protoporphyrin

EPA- Environmental Protection Agency

EPO - Serum erythropoietin

ESR - Erythrocyte sedimentation rate

EU - Endotoxin units

FDA- Food and Drug Administration

FCM - Flow cytometric

FISH - Fluorescence in situ hybridization

FP - Forward progressive

FR- Fecundity ratio

FSH- Follicle stimulating hormone

GnRH - Gonadotropin releasing hormone

GSH - Reduce glutathione

GSH-Px - Glutathione peroxidase

GSSG - Glutathione disulfide

GST - Glutathione-S-transferase

HDS - High Deoxyribonucleic acid Stainability

HPG - Hypothalamic- pituitary-gonadal

IARC- International Agency for Research on Cancer

ICSI - Intracytoplasmic sperm injection

IgA- Immunoglobulin A

IgG - Immunoglobulin G

IgM - Immunoglobulin M

IM- Immobility

IR – Implantation ratio

IUI - Intrauterine insemination

IVF - in vitro fertilization

LH - Luteinizing hormone

LI - Long Island

LIDPBC - Long Island database project for Breast cancer

MDA – Malondialdehyde

MEK - Methyl Ethyl Ketone

MM- Moderate motile

mRNA - Messenger ribonucleic acid

MT – Metallothionine

MTT - 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

NAG - N-acetyl-β-D-glucosaminidase

NCD- Nuclear chromatin de-condensation

NHANES - National Health and Nutrition Examination Survey

NIOSH- National Institute for Occupational Safety and Health

NK - Natural killer

NPR- Non progressive motility

NPV - Negative predictive value

OAT – Oligoasthenoteratozoospermic

OR - Odds ratio

OSHA - Occupational Safety and Health Administration

PbB - Average Blood lead level

PCB - polychlorinated biphenyls

PCR - Polymerase chain reactions

PCV – Packed cell volume

PHA – Phytohemagglutinin

PMN – Polymorphonuclear

PPV - Positive predictive value

PR - Progressive motility

PRL – Prolactin

RLU - Relative Luminescence Units

ROS - Reactive oxygen species

SBG - Steroid binding globulin

SCD - Sperm chromatin dispersion test

SCSA - Sperm chromatin structure assay

SD- Standard deviation

SM - Stationary motile

SMR - Standardizes mortality ratio

SOD - Superoxide dismutase

T3 - Tri-iodothyronine

T4 – Thyroxin

TAC - total antioxidant capacity

TBARS - Thiobarbituric acid reactive substances

TBG - Thyroid binding globulin

TCA - 1, 1,1-trichloroethane

TEC - Total erythrocyte count

TLC - Total leukocyte count

TNF - Tumor necrosis factor

TRH - Thyrotrophin releasing hormone

TSH - Thyroid stimulating hormone

TTP - Time to pregnancy

TUNEL - Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

UV - Ultra violet

WHO - World Health Organization

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to all those who contributed to complete my PhD study, particularly I wish to express my thanks to:

Prof. D.M.S.Fernando, my supervisor for her valuable guidance, inspiration and support given to me to complete the thesis.

Prof. Sumeda Wijeratne, my supervisor for sharing her expertise on the subject, and invaluable advice and guidance to complete the research.

Prof. Nilanthi Bandara, my supervisor for her invaluable advice and guidance.

I am deeply grateful to Dr. Nalinda Silva, Dr. Chandana Hewage and other staff of Department of Physiology for the support extended to me throughout the study.

I owe a special thank you to Mr. Darshana of the Medical Laboratory Science unit for the support given to me in numerous ways at a time when it was most needed.

I also wish to acknowledge the staff of the Vindana Reproductive Health Centre for the assistance rendered throughout the study and the laboratory staff of the Coconut Development Authority for the technical assistance given to me in analysing heavy metals.

The assistance of Prof.Wijesiri, Prof.Sivayogan and Prof. Rajitha Wickramasinghe in data analysis and applying statistical methods was very valuable. I greately acknowledge their services.

I would like to thank the coordinator Dr.Inoka Uluwaduge and other staff of the MLS unit for their assistance in numerous ways.

I wish to acknowledge my friends Mr. Pathmasiri, Mr.Asoka Bandara and Mr.Sarath Kamburapolage and for their assistance provided in numerous ways.

This study was funded by the Research grant of University of Sri Jayewardenepura (ASP/06/P/SCI/2011/12) and UGC grant(UGC/ICD/RG 2011/05). This study could not have been completed without the financial support of these two funding agencies.

Finally I would like to thank my parents and my wife for the support and encouragement extended to me throughout the study and my son for their tolerance, patience and for providing much needed relaxation during difficulties.

The effects of lead and Cadmium on sperm parameters including sperm DNA fragmentation of men investigated for infertility.

Gamage Udaya Shantha Wijesekara

ABSTRACT

The effects of Pb and Cd on sperm parameters have not been studied in Sri Lanka. The objective of this study was to describe the correlation between Pb and Cd in seminal plasma and sperm parameters including sperm DNA fragmentation of men investigated for infertility.

The study was conducted from August 2010 to March 2012. In the phase 1, the association between sperm parameters of pathozoospermics (99) and normozoospermics (201) and Pb and Cd concentrations in seminal plasma was described. For the phase 2, Pb positive, Cd positive and men negative for both metals (controls) were randomly selected with 20 men in each group to determine the sperm DNA fragmentation (DFI) by Halosperm method.

Of the men investigated, 54.6% (n=164) were exposed to toxicants through environmental or occupational sources while 26.6% (n=80) lived in areas with possible environmental toxicity and 37.3% (n=112) were exposed to toxicants through occupational sources.

In both normozoospermics and pathozoospermics, the sperm concentration, progressive motility, normal morphology and viability were lower in the exposed group (through environmental and occupational sources) when compared to the non-exposed. Petroleum products was the most exposed toxicant with both pathozoospermic and normozoospermic men having lower sperm parameters in comparison to the non-

exposed. Industrial chemicals came next followed by agrochemicals with lower sperm concentration, progressive motility and normal morphological forms in the pathozoospermic group and all sperm parameters being lower in normozoospermic men exposed to agrochemicals.

Proportionately higher number of normozoospermic men had detectable levels of Pb and Cd in their seminal plasma but quantitatively the pathozoospermics had higher concentration of Pb and Cd the mean (SEM) being 17.25(3.02) vs 15.04(1.70) for Pb and 1.25 (0.34) vs 1.15 (0.21) for Cd. The men with a positive exposure had higher Pb and Cd concentrations when compared to the non-exposed.

In the Pb positive group, all sperm parameters were lower while the normal morphology and viability percentage was less in the Cd positive group ($p>0.05$). A negative correlation was found between lead in seminal plasma and sperm count ($p=0.52$, $r=0.06$), viability ($p=0.41$, $r=0.07$), progressive motility ($p=0.25$, $r=0.1$) and normal morphology ($p=0.24$, $r=0.11$) and between Cd in seminal plasma and sperm count ($p=0.91$, $r=0.01$), viability ($p=0.61$, $r=0.06$), progressive motility ($p=0.14$, $r=0.18$) and normal morphology ($p=0.20$, $r=0.15$).

The mean DFI of Pb and Cd positives were higher than the controls with a highly significant difference between the DFI of Pb positives and the controls. The subjects with high DFI (30% or more) had significantly higher Pb concentration when compared to subjects with low DFI ($p<0.05$). A significant positive correlation ($p=0.006$, $r = 0.35$) was found between seminal plasma Pb level and Sperm DNA fragmentation. There was no correlation ($p=0.19$, $r=0.17$) between seminal plasma Cd level and DFI.

The means of all the sperm parameters of subjects with a DFI of 30% or more were lower than the sperm parameters of subjects with a DFI less than 30% with a significant

difference in progressive motility, normal morphology and viability. There was a significant negative correlation between sperm DNA fragmentation and sperm concentration ($p=0.032$, $r=0.23$), progressive motility ($p=0.008$, $r=0.34$), normal morphology ($p=0.025$, $r= 0.29$) and viability ($p=0.01$, $r=0.32$).

In conclusion environmental and occupational exposures to reproductive toxicants seem to have a negative effect on semen parameters in this population with a significant effect from petroleum products. The pathozoospermic men had a higher Pb and Cd concentration in seminal plasma. There was an inverse relationship between seminal plasma Pb and Cd concentrations and the sperm parameters with all sperm parameters being lower in the Pb positive men and the sperm normal morphology and viability being lower in the Cd positive men. The sperm DNA fragmentation was significantly higher in Pb positive men and the DNA fragmentation showed an inverse relationship with all sperm parameters in this group of men investigated for infertility.