

ZINC AND GENOMIC STABILITY

A thesis submitted to the University of Adelaide
for the degree of Doctor of Philosophy

Razinah Sharif

**School of Medicine,
Faculty of Health Sciences, University of Adelaide
and
CSIRO Food and Nutritional Sciences, Adelaide**

June 2012

TABLE OF CONTENTS

ABSTRACT	viii
DECLARATION	ix
ACKNOWLEDGEMENTS	x
PRESENTATIONS AND PUBLICATIONS ARISING FROM THE THESIS	xii
LIST OF ABBREVIATIONS	xv
Chapter 1: The Role of Zinc in Genomic Stability	1
1.1 Abstract	3
1.2 Introduction	3
1.2.1 Genomic stability and cancer; the role of nutrition	3
1.2.2 Zinc functions	5
1.3 Zinc deficiency, DNA damage and chromosomal instability	13
1.4 Zinc excess, DNA damage and toxicity	20
1.5 Zinc and telomeres	27
1.6 Knowledge gaps and future directions	26
Chapter 2: Aims, Hypotheses and Models	30
2.1 Aims and hypotheses	31
2.2 Experimental models	31
2.2.1 <i>In vitro</i> model	31
2.2.2 <i>In vivo</i> model	34
Chapter 3: The Effect of Zinc Sulphate and Zinc Carnosine on Genome Stability and Cytotoxicity in WIL2-NS Lymphoblastoid Cell Line	35
3.1 Abstract	38
3.2 Introduction	39
3.3 Materials and methods	42

3.3.1 WIL2-NS cell culture	42
3.3.2 Cell counting using the Coulter Counter	42
3.3.3 Culture medium	42
3.3.4 9-day WIL2-NS culture in 24 well plates	44
3.3.5 Inductively Coupled Plasma Optical Emission Spectrometry (ICPOES)	45
3.3.6 MTT assay	46
3.3.7 Alkaline comet assay	46
3.3.8 CBMN-Cyt assay	47
3.3.8.1 Scoring criteria	48
3.3.9 Gamma-ray-irradiation of cells	52
3.3.10 H ₂ O ₂ treatment of cells	52
3.3.11 Western blotting	53
3.3.12 Statistical analysis	55
3.3.13 Optimization of cell growth for long term culture	56
3.4 Results	57
3.4.1 Cellular Zinc concentrations	57
3.4.2 MTT assay	59
3.4.3 Alkaline comet assay	62
3.4.4 Effect of Zinc concentration on baseline levels of cytotoxicity and chromosome damage as measured by the CBMN-Cyt assay	63
3.4.5 Effect of Zinc concentration on γ -radiation induced cytotoxicity and chromosome damage as measured by the CBMN-Cyt assay	67
3.4.6 Effect of Zinc concentration on H ₂ O ₂ induced cytotoxicity and chromosome damage as measured by the CBMN-Cyt assay	70
3.4.7 Western blot analysis	74
3.5 Discussion	80

Chapter 4: Zinc Deficiency or Excess within the Physiological Range Increases Genome Instability, Cytotoxicity, respectively, in Human Oral Keratinocytes 86

4.1 Abstract	89
4.2 Introduction	90
4.3 Materials and methods	92
4.3.1 HOK cell culture and study design	92
4.3.2 Cell counting using the Coulter Counter	93
4.3.3 Culture medium	93
4.3.4 10-day HOK culture in 24 well plates	95
4.3.5 Inductively coupled plasma optical emission spectrometry (ICPOES)	96
4.3.6 MTT cell growth and viability assay	97
4.3.7 Alkaline comet assay	97
4.3.8 CBMN-Cyt assay	98
4.3.8.1 Scoring criteria	100
4.3.9 Gamma-ray-irradiation of cells	104
4.3.10 H ₂ O ₂ treatment of cells	104
4.3.11 Western blotting	104
4.3.12 Statistical analysis	107
4.3.13 Optimization of cell growth for long term culture	108
4.3.14 Optimization of Cytochalasin B (Cyto B) concentration	109
4.4 Results	111
4.4.1 Cellular Zinc concentrations	111
4.4.2 Effect of Zinc concentration on cell viability as measured via the MTT assay	114
4.4.3 Effects of Zinc concentration on DNA strand breaks as measured via the comet assay	116
4.4.4 Effect of Zinc concentration on baseline levels of cytotoxicity and chromosome damage as measured by the CBMN-Cyt assay	118
4.4.5 Effect of Zinc concentration on γ -radiation induced cytotoxicity and chromosome damage as measured by the CBMN-Cyt assay	121
4.4.6 Effect of Zinc concentration on H ₂ O ₂ induced cytotoxicity	

and chromosome damage as measured by the CBMN-Cyt assay	124
4.4.7 Western blot analysis	127
4.4.8 Cytotoxicity and genotoxicity effect of HOK cells in optimal medium	131
4.5 Discussion	134
Chapter 5: Zinc Deficiency Increases Telomere Length and is Associated with Increased Telomere Base Damage, DNA Strand Breaks and Chromosomal Instability	141
5.1 Abstract	142
5.2 Introduction	143
5.3 Materials and methods	144
5.3.1 WIL2-NS lymphoblastoid cell culture	144
5.3.2 HOK cell culture	144
5.3.3 Isolation of genomic DNA	145
5.3.4 Telomere length assay	145
5.3.4.1 qPCR of DNA for telomere length assay	146
5.3.5 Telomere base damage assay	147
5.3.5.1 Excision of 8oxodG and incision of oligomers at 8oxodG sites using FPG	147
5.3.5.2 qPCR of synthetic oligomers and genomic DNA	148
5.3.6 Zinc content of the cells, comet assay and CBMN-Cyt assay	149
5.3.7 Experimental design and statistical analysis	149
5.4 Results	150
5.4.1 Cellular Zinc content	150
5.4.2 Impact of Zinc on telomere length (TL) in WIL2-NS and HOK cells	150
5.4.3 Impact of Zinc on telomere base damage in WIL2-NS and HOK cells	153
5.4.4 Correlation between telomere length, telomere base damage with DNA damage biomarkers (tail moment, tail intensity, micronuclei, nucleoplasmic bridges and nuclear buds)	154
5.5 Discussion	158

Chapter 6: Genome Health Effect of Zinc Supplement in an Elderly South Australian Population with Low Zinc Status **160**

6.1 Abstract	162
6.2 Introduction	163
6.3 Materials and methods	165
6.3.1 Screening and recruitment of volunteers	165
6.3.2 Intervention design	166
6.3.3 Nutritional assessment	167
6.3.4 Blood collection and sample preparation	167
6.3.5 Plasma analysis	169
6.3.5.1 Plasma mineral, B12, Folate and Homocysteine analysis	169
6.3.5.2 FRAP analysis	171
6.3.5.3 eSOD assay	173
6.3.6 DNA damage assay	175
6.3.6.1 Cytokinesis Block Micronucleus Cytome (CBMN-Cyt) assay	175
6.3.6.2 Alkaline comet assay	185
6.3.6.3 Isolation of DNA/RNA	186
6.3.6.4 Telomere length	188
6.3.6.5 Telomere base damage	189
6.3.7 Gene expression	191
6.3.7.1 MT1A and ZIP expression	191
6.3.8 Statistical analysis	193
6.4 Results	193
6.4.1 Screening results	193
6.4.2 Characteristics of volunteers	196
6.4.3 Plasma micronutrients: Zinc, Carnosine, Mineral, B12, Folate and Homocysteine	196
6.4.4 Antioxidant activity (FRAP and eSOD)	197
6.4.5 DNA damage assay: CBMN-Cyt assay and alkaline comet assay	202
6.4.6 Telomere integrity: Telomere length and telomere base damage	208
6.4.7 Zinc transporter genes: MT1A and ZIP1	210

6.4.8 Correlation between plasma Zinc and other biomarkers measured in this study	212
6.4.9 Correlation between other measured biomarkers	212
6.5 Discussion	217
Chapter 7: Conclusions, Knowledge Gaps and Future Directions	224
7.1 Introduction	225
7.2 Zinc and genomic stability: <i>in vitro</i> (WIL2-NS and HOK cells)	225
7.3 Zinc and genomic stability: <i>in vivo</i> (Genome health effect of Zinc supplementation in an elderly South Australian population with low Zinc status)	227
References	229
APPENDIX: PAPER REPRINTS	241

Abstract

Zinc (Zn) is an essential trace element required for both optimal human health and maintaining genomic stability. The main aim of this thesis was to address important knowledge gaps regarding the possible impact of Zn status on genomic stability events in both lymphocytes and epithelial cells using both *in vitro* and *in vivo* models. The project also aimed to study the differential impact of Zn Carnosine (ZnC) and Zn Sulphate (ZnSO_4) on genome stability as the former is a newly emerging commercially available supplement renowned for its antioxidant capacity. The *in vitro* studies investigated the effects of ZnSO_4 and ZnC on cell proliferation via MTT assay and DNA damage rates and was measured using both the comet assay and the Cytokinesis-block micronucleus cytome (CBMN-Cyt) assay in the WIL2-NS human lymphoblastoid cell line and HOK cell line. This study also investigated the impact of Zn status on both telomere length and telomere base damage *in vitro*. An *in vivo* study was designed to further investigate the effect of Zn supplementation in minimising genome instability events in lymphocytes. An increased intake of Zn may reduce the risk of degenerative diseases but may be toxic if taken in excess. This study aimed to investigate whether taking daily supplements of 20 mg of Zn as Zn Carnosine can improve Zn status, genome stability events and Zn transporter genes in an elderly South Australian cohort characterised by having low plasma Zn levels. In conclusion, the *in vitro* studies suggest that 1) Zn deficiency (0 μM) and high Zn concentrations increase DNA damage; 2) Zn at 4-16 μM is optimal in maintaining genome stability events; 3) Zn at 16-32 μM is optimal in protecting the cell against DNA damage induced by irradiation and hydrogen peroxide challenges; and 4) Zn may play an important role in telomere maintenance. The *in vivo* study suggests that Zn supplementation may be beneficial in an elderly population with marginal lowered Zn status by raising plasma Zn levels, lowering DNA damage events and modifies Zn transporter gene expression.

Declarations

I, Razinah Sharif certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent for a copy of my thesis when deposited in the University Library, to be made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis (as listed on page xv) resides with the copyright holders of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research depository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

CSIRO Food and Nutritional Sciences retain the copyright of any subsequent publications arising from this thesis.

Signature:

Date:

Acknowledgements

First and foremost, I would like to praise God for everything whilst travelling through this PhD journey. It has been a roller coaster ride and whenever I became stuck or felt unmotivated, God always listened to me and things worked out fine eventually.

Secondly, I would like to thank my amazing supervisors (Prof Michael Fenech, Dr Philip Thomas and Dr Peter Zalewski) for giving me the opportunity to undertake this PhD project and for their guidance through out the study. I would also like to thank Prof Robin Graham and Prof Ross Butler who were initially involved with the project design.

I'm also grateful to all the staff and students at CSIRO Nutrigenomics lab and also to Kylie Lange (CSIRO), Erin Symonds (IMVS), Steve Henderson (CSIRO Waite), Eugene Roscioli (QEH), Rhys Hamon (QEH), Teresa Fowles (Waite campus), Lyndon Palmer (Waite campus), and Nathan O'Callaghan (CSIRO) who have always listened and helped me with some of the experiments and making it a complete story line. I would also like to acknowledge the group of PhD students who shared the pain, sweat and tears (Arnida, Carly, Eva, Sau Lai, Ann, Penny, Kacie, Mansi), you guys are the best bunch! I would really appreciate all the advice, the conversations and all the help. Thanks a million!

In order to complete the biggest part of my PhD project which was the *in vivo* study, I needed to conduct a human trial and I would like to express my gratitude to the staff at the CSIRO clinical trial unit (Julia Weaver, Lyndi Lawson, Rosemary McArthur, Vanessa Courage and Peter Royle) who helped me in completing this study. Thank you so much! On this occasion, I would also like to acknowledge Metagenics Company who provided the pills for the Zinc study without any charges at all. I would also like to thank all the volunteers who completed the study without any provided remuneration. They were willing to participate for the sake of science only. I'm really thankful to them!

I would also like to acknowledge my parents, my housemates (Maisara, Fauziah, and Norhalisa) and my other Malaysian communities for their friendship, support and prayers during the course of my study.

A PhD is always a stressful journey and for this matter, I am really thankful to Fernwood Gym Adelaide City, a place where I can go and ease my stress and a place that I can go whenever I'm having breakdown moments. Special credit to Abby, Rachel, Lou, Tam, Eman, Sandy, Sophie and Katrina for being the best gym buddies and also to Tracey for being my personal trainer.

Last but not least, I would like to acknowledge CSIRO Food and Nutritional Sciences for the funding provided to support all the chemicals needed in my study and also to my employer (Universiti Kebangsaan Malaysia) and Ministry of Higher Education, Malaysia who provided the scholarship (tuition fees and living allowances).

Thank you everyone for all the help and support. This thesis wouldn't be a thesis without all of your support and prayers.

Thank you!

Presentations and Publications arising from the thesis

Abstract/Poster Presentations

1. **Sharif, R.**, Thomas, P., Zalewski, P., Graham, R. & Fenech, M. The effect of Zinc Sulphate and Zinc Carnosine on cytotoxicity and genotoxicity in the WIL2-NS lymphoblastoid cell line. 19th International Conference on Nutrition. 4-9th October 2009, Bangkok, Thailand.

2. **Sharif, R.**, Thomas, P., Zalewski, P., Graham, R. & Fenech, M. The effect of Zinc Sulphate and Zinc Carnosine on cytotoxicity and genotoxicity in the WIL2-NS lymphoblastoid cell line. Australian Science Medical Research 2010. 9-10th June 2010, Adelaide, Australia.

3. **Sharif, R.**, Thomas, P., Zalewski, P., Graham, R. & Fenech, M. The effect of Zinc Sulphate and Zinc Carnosine on cytotoxicity and genotoxicity in the WIL2-NS lymphoblastoid cell line. Nutrigenomics Symposium, CSIRO. 30th July 2010, Adelaide, Australia.

4. **Sharif, R.** Thomas, P. Zalewski, P. and Fenech, M. Zinc deficiency increases genome instability in Human Oral Keratinocytes (HOK). Nutrition in Medicine Conference. 13–15th May 2011, Bondi, Australia.

5. **Sharif, R.** Thomas, P. Zalewski, P. and Fenech, M. Zinc deficiency increases genome instability in Human Oral Keratinocytes (HOK). Australian Science Medical Research 2011. 9-10th June 2011, Adelaide, Australia.

6. **Sharif, R.** Thomas, P. Zalewski, P. and Fenech, M. Zinc deficiency increases genome instability in Human Oral Keratinocytes (HOK). XI Asian Congress on Nutrition 2011. 13-16th July 2011, Singapore.

7. **Sharif, R.** Thomas, P. Zalewski, P. and Fenech, M. Zinc deficiency increases genome instability in Human Oral Keratinocytes (HOK). Postgraduate Research Conference, Faculty of Health Science, University of Adelaide. 25th August 2011, Adelaide, Australia.

8. **Sharif, R.** Thomas, P. Zalewski, P. and Fenech, M. The effect of Zinc Sulphate and Zinc Carnosine on cytotoxicity and genotoxicity in the WIL2-NS lymphoblastoid cell line. NSNZ & NSA Joint Annual Scientific Meeting. 30th November-2nd December 2011, Queenstown, New Zealand.

9. **Sharif, R.** Thomas, P. Zalewski, P. and Fenech, M. Zinc supplementation influences genomic instability biomarkers, antioxidant activity and Zn transporter genes in an elderly Australian population with low Zn status. International Society for Zinc Biology 2012 Conference. 15-19th January 2012, Melbourne, Australia.

Oral Presentations

1. Zinc and Genomic Stability. Wednesday Wrap. School of Medicine, University of Adelaide. 16th September 2009.

2. Zinc and Genomic Stability. Wednesday Wrap. School of Medicine, University of Adelaide. 14th December 2011.

3. Zinc and Genomic Stability. Special Seminar. Genome Stability Laboratory. Yong Loo Lin School of Medicine. National University of Singapore. 11th July 2011.

4. The effect of Zinc Sulphate and Zinc Carnosine on genome stability and cytotoxicity in the WIL2-NS lymphoblastoid cell line. International Society for Zinc Biology 2012 Conference. 15-19th January 2012. Melbourne, Australia.

Publications

1. **Sharif, R.**, Thomas, P., Zalewski, P., Graham, R. & Fenech, M. (2011) The effect of Zinc Sulphate and Zinc Carnosine on cytotoxicity and genotoxicity in the WIL2-NS lymphoblastoid cell line. *Mutation Research*. **720(1-2)**: 22-33.
2. **Sharif, R.**, Thomas, P., Zalewski, P. & Fenech, M. (2011) Zinc deficiency or excess within the physiological range increases genome instability and cytotoxicity, respectively, in human oral keratinocyte cells. *Genes and Nutrition*. In press.
3. **Sharif, R.**, Thomas, P., Zalewski, P. & Fenech, M. (2011) The role of zinc in genomic stability. *Mutation Research*. In press.
4. O'Callaghan, N., Baack, N., **Sharif, R.**, and Fenech, M. (2011) A qPCR-based assay to quantify oxidized guanine and other FPG-sensitive base lesions within telomeric DNA. *Biotechniques*. Vol. 51 (6): 403–412.

List of Abbreviations

ACCV	Anti Cancer Council of Victoria
AOA	Antioxidant Activity
ANOVA	Analysis of Variance
AP1	Activator Protein 1
APE	Apyrimidinic Endonuclease
ATCC	American Type Culture Collection
aTL	Absolute Telomere Length
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3 Related
ATRIP	Ataxia Telangiectasia and Rad3 Related Interacting Protein
AU	Arbitrary Unit
BCA	Bicinchoninic Acid
BER	Base Excision Repair
BN	Binucleate
BNed	Binucleated
BHMT	Betaine-homocysteine-S-methyltransferase
BSA	Bovine Serum Albumin
Ca	Calcium
CBMN Cyt assay	Cytokinesis Block Micronucleus Cytome assay
cDNA	Complementary Deoxyribonucleic Acid
CRP	C-Reactive Protein
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C _T	Cycle Threshold
Cu	Copper
CuSO ₄	Copper Sulphate
Cu/ZnSOD	Copper Zinc Superoxide Dismutase
CV	Coefficient of Variation
Cyto-B	Cytochalasin B
DCF	2'7'-dichlorofluorescein
DCFH	2'7'-dichlorofluorescein hydrochloride
dH ₂ O	Distilled Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	Deoxyribonucleic Acid Methyltransferase
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
eSOD	Erythrocyte Superoxide Dismutase
FapyGua	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FapyAde	4,6-diamino-5-formamidopyrimidine
FBS	Foetal Bovine Serum
Fe	Iron
FeCl ₃ .6H ₂ O	Iron Chloride
FFQ	Food Frequency Questionnaire
Fpg	Formamidopyrimidine-DNA Glycosylase
FRAP	Ferric reducing Ability of Plasma
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gDNA	Genomic Deoxyribonucleic Acid
H ₂ O ₂	Hydrogen Peroxide
HBSS	Hanks Balanced Salt Solution
HCy	Homocysteine
HCl	Hydrochloric Acid
HOK	Human Oral Keratinocyte
HUMN	HUman MicroNucleus/ The International Collaborative Project on Micronucleus Frequency in Human Populations
H ₂ O	Water
ICPOES	Inductively Coupled Plasma Optical Emission Spectrometry
IL-6	Interleukin-6
IMVS	Institute of Medical and Veterinary Science
IR	Irradiated
K	Potassium
Kb	Kilobases
MDA	Malondialdehyde
Mg	Magnesium
MgCl ₂	Magnesium Chloride
MNi	Micronuclei
MNed	Micronucleated
MnSOD	Manganese Superoxide Dismutase
mRNA	Messenger Ribonucleic Acid
MT	Metallothionein
MT1A	Metallothionein-1A
MTR	Methionine Synthase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
MZnD	Marginal Zinc Deficiency

Na	Sodium
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NaOH	Sodium Hydroxide
NBud	Nuclear Bud
NDI	Nuclear Division Index
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NI	Non Irradiated
NK	Natural Killer
NPB	Nucleoplasmic Bridge
NO	Nitric Oxide
Na ₄ P ₂ O ₇ ·10H ₂ O	Sodium Pyrophosphate
Na ₃ VO ₄	Sodium Orthovanadate
8-OHdG	8-Hydroxy-2-deoxyguanosine
8-oxoG	8-Oxoguanine
8-oxodG	8-Oxo-2'-deoxyguanosine
OGG1	8-Oxoguanine DNA glycosylase
OKM	Oral Keratinocyte Medium
OKGS	Oral Keratinocyte Growth Supplement
P	Phosphorus
p53	p53 Tumor Suppressor genes
PARP	Poly (ADP-ribose) Polymerase
PBL	Peripheral Blood Lymphocyte
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
PMSF	Phenylmethanesulfonylfluoride
Q-FISH	Quantitative Fluorescent In Situ Hybridization
RDA	Recommended Daily Allowance
RDI	Recommended Daily Intake
Ref1	Redox Factor-1
RPMI	Roswell Park Memorial Institute
ROS	Reactive Oxygen Species
RT	Real Time
RT	Room Temperature
RTPCR	Real Time Polymerase Chain Reaction
S	Sulphur
SAM	S-adenosyl Methionine
SE	Standard Error
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
TANK1	Human Tankyrase 1
TBAR	Thiobarbituric Acid Reaction
TBD	Telomere Base Damage
TI	Tail Intensity
TL	Telomere Length
TM	Tail Moment
TPEN	N,N,N'N'-tetrakis(-)[2-pyridylmethyl]-ethylenediamine
TPTZ	Tripyridyl Triazine
WAS	Waite Analytical Service
WHO	World Health Organization
WIL2-NS	WIL2-NS Lymphoblastoid Cell Line
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt
Zn	Zinc
ZnC	Zinc Carnosine
ZnD	Zinc Deficiency
ZnAD	Zinc Adequate
ZnSO ₄	Zinc Sulphate
ZIP1	ZIP1 human Zinc transporter gene
γ-H2AX	genes coding for Histone 2A (phosphorylated)