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Zinc supplementation influences genomic stability biomarkers, antioxidant activity and zinc transporter genes in an elderly Australian population with low zinc status.

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Abstract

Scope:

An increased intake of Zinc (Zn) may reduce the risk of degenerative diseases but may prove to be toxic if taken in excess. This study aimed to investigate whether Zinc carnosine supplement can improve Zn status, genome stability events and Zn transporter gene expression in an elderly (65-85y) South Australian cohort with low plasma Zn levels.

Methods and results:

A 12 week placebo-controlled intervention trial was performed with 84 volunteers completing the study, (Placebo, n=42) and (Zn group, n=42). Plasma Zn was significantly increased ($p<0.05$) by 5.69% in the Zn supplemented group after 12 weeks. A significant ($p<0.05$) decrease in the micronucleus frequency (-24.18%) was observed for the Zn supplemented cohort relative to baseline compared to the placebo group. Reductions of -7.09% for tail moment and -8.76% for tail intensity were observed for the Zn group (relative to baseline) ($p<0.05$). Telomere base damage was found to be also significantly decreased in the Zn group ($p<0.05$). Both MT1A and ZIP1 expression showed a significant increase in the Zn supplemented group ($p<0.05$).

Conclusion:

Zn supplementation may have a beneficial effect in an elderly population with low Zn levels by improving Zn status, antioxidant profile and lowering DNA damage.

1.0   Introduction

It is known that more than 100 specific enzymes require Zn for their catalytic function emphasising the critical role played by Zn in cellular processes including genomic stability events [1]. Previous evidence has shown genomic instability events as being associated with increased cancer risk [2, 3]. The fact that Zn is required as a cofactor in DNA metabolism, is suggestive that deficiency in this micronutrient may induce important chromosomal mutations that increase cancer risk. Several studies show that Zn deficiency causes DNA oxidation, DNA breaks and chromosome damage [4-6].

Mild Zn deficiency has been reported in apparently normal ageing, older individuals with gastrointestinal problems, individuals with compromised immune function and in vegetarians [7]. Ageing populations are at risk of Zn deficiency because of numerous factors such as those reported by the International Zn Nutrition Consultative Steering Committee (2004) [8]. Zn deficiency has been shown to be prevalent in a number of studies investigating Zn status in elderly populations [9-11]. Deficiency of Zn may cause impaired immune function [12-14], delayed wound healing [15], depression [16], impaired cognitive function [17] and increased oxidative stress [18, 19]. As Zn deficiency may have serious implications for the health of ageing individuals, it is important to maintain adequate Zn nutriture within this population.

In 2004, the ZnAge project investigated various health-related impacts of Zn supplementation among the elderly in European countries [16, 18, 20-24]. Putics *et al* (2008) concluded that proper dietary Zn may have an important role in anti-aging mechanisms involving the immune system [24]. A further report showed that Zn supplementation reduces spontaneous inflammatory cytokine release and restores T cell function [25]. Marcellini *et al* (2008) showed that Zn supplementation given to the elderly in the ZnAge project affected

psychological dimensions dependent on IL-6-174G/C polymorphism [20]. Despite these findings knowledge gaps remain regarding the effect of Zn supplementation in relation to genomic stability and integrity in an elderly population.

The aim of this study was to investigate whether taking a daily supplement of 20 mg of Zn can improve Zn status, genome stability events and Zn transporter gene expression in an elderly (65-85y) South Australian cohort characterised by having low plasma Zn levels (<0.77 mg/L;11.7 µM).

2.0 Materials and methods

2.1 Screening and recruitment of volunteers

Volunteers were recruited through the CSIRO Clinical Research Unit database and by advertising in local newspapers. The volunteers did not receive any remuneration for their participation. In this study individuals were excluded if 1) supplementing with more than 25% of the recommended dietary intake of Zn, 2) had a habitual dietary Zn intake > 20 mg/day, 3) were undergoing radiotherapy/chemotherapy treatment for cancer and d) had plasma Zn levels > 0.77 mg/L. A total of 208 volunteers aged 65-85 years old met the selection criteria and were screened for plasma Zn status using a non-fasted blood sample collected in the morning in a lithium heparin tube. Those with plasma Zn concentration ≤ 10.0 µM (≤ 0.65 mg/L - cut-off point for Zn deficiency [26]) were given priority for inclusion in the study. As there were too few individuals with plasma Zn ≤ 0.65 mg/L (10.0 µM), we eventually selected all those with plasma Zn status < 0.77 mg/L (11.77 µM) (n=90) and the volunteers were consented to participate in the study. Body Mass Index was measured and estimated dietary Zn intake using Food Frequency Questionnaire was collected during their visit.

2.2 Intervention design

The study was designed as a randomised, placebo-controlled intervention in a free-living elderly, healthy population. This study was approved by the Human Research Ethics Committee of the CSIRO Food and Nutrition (09/25), University of Adelaide Ethics Committee and Australian New Zealand Clinical Trial Registry (ACTRN: ACTRN12610000059066). Volunteers with low plasma Zn (< 0.77 mg/L;11.7 µM) were randomised into two groups; placebo and Zn supplemented group.

The Zn supplement tablet used in this trial is a commercially available Zn carnosine chelate supplement, which was supplied and prepared by Metagenics (Queensland, Australia). Each Zn carnosine supplement tablet contained 86.9 mg of Zn carnosine with maltodextrin as excipient. The placebo tablet was 100% pure maltodextrin. The Zn dose in each supplement tablet was 20 mg/day which is within the safe physiological range [8]. The placebo group received an identical tablet, which only contained the excipient maltodextrin. The volunteers consumed one tablet (Placebo or Zn carnosine) per day.

2.3 Nutritional assessment

A nutrition survey was carried out to investigate the estimated dietary intake of Zn in the study cohort. The volunteers who participated in the study were required to complete the validated Anti-Cancer Council of Victoria (ACCV) Food Frequency Questionnaire at the start of the study [27] and analysed with NUTTAB95 nutrient composition data [27].

2.4 Blood collection and sample preparation

For screening, one tube (5 ml) of non-fasted blood per volunteer was collected in a lithium-heparin vacutainer (Vacuette, Austria). During the intervention, at baseline and 12 weeks, three tubes of blood (6-8 ml for each) were collected in the morning after an overnight fast into a potassium-EDTA tube for homocysteine (Hcy) measurement, and another two lithium-heparin tubes for all other assays.

a) Plasma Isolation

Blood was spun at 3000 rpm for 20 minutes at 4°C and plasma was transferred into new tubes according to the assays to be investigated. Spare plasma was labelled and kept at -80°C.

b) Isolation and storage of lymphocytes

Lymphocytes were isolated using Ficoll-Paque™ (Amersham Pharmacia Biotech). For the Cytokinesis block micronucleus cytome (CBMN Cyt) assay, a total cell count was performed and 750 µl cultures were set up at 1×10^6 /ml, in duplicate. Cultures were placed in an incubator at 37°C with 5% CO_2 until ready for phytohaemagglutinin (PHA) addition. For the alkaline comet assay, 500 µl of 1×10^5 cells/ml suspension was prepared for each volunteer. Spare cells were spun down and resuspended in 500 µl PBS with 10% DMSO (Sigma, Australia), then stored in -80°C prior to DNA/RNA isolation. The remaining blood was collected and transferred into new tubes and kept at -20 °C prior to erythrocyte superoxide dismutase measurements.

2.4.1 Plasma analysis

a) Plasma mineral, B12, Folate, Homocysteine and Carnosine analysis

Plasma samples were sent to the Waite Analytical Services (WAS, Adelaide University) for plasma Zn and other mineral analysis. A further 300 µl of plasma was transferred to new tubes and kept at -80°C before being sent to the Institute of Medical and Veterinary Science (IMVS) for vitamin B12 and folate analysis. The concentration of plasma Zn and other minerals (Iron, Copper, Calcium, Magnesium, Sodium, Potassium, Phosphorus, Sulphur) was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICPOES, Agilent Technologies 7500c, Japan), following digestion with nitric acid and hydrochloric acid. Plasma Carnosine was measured using an ELISA antibody kit (USCN Life Science, USA).

b) *Ferric Reducing Ability of Plasma (FRAP) analysis*

The FRAP assay was used to measure the combined antioxidant effect of the non-enzymatic defences in plasma samples [28]. The absorbance of the products of the FRAP reaction was measured on an ELISA microplate reader (SpectraMax 250, Molecular Devices, CA, USA) at a wavelength of 593 nm. 96 well plates were used in this assay for multiple readings as only 300 µl solution was used for absorption readings.

c) *eSOD assay*

For this analysis, we used the SOD assay kit (Trevigen Inc, USA). A standard curve to verify that the assay is working predictably and that concentration dose-response is linear within the relevant range was established prior to analysis of samples

2.4.2   DNA Damage assays

a) *CBMN-Cyt assay*

Following Cyto-B treatment, cells are harvested following a well validated protocol onto slides by cytocentrifugation, stained and scored for chromosomal damage biomarkers using light microscopy [29].   500 cells were scored to determine the frequency of necrotic, apoptotic, mono-, bi- and multi-nucleated cells and nuclear division index (NDI). 1000 binucleated cells were scored to determine the frequency of chromosomal DNA damage biomarkers including micronuclei (MNi), nucleoplasmic bridges (NPB) and nuclear buds (NBUDs).

b) *Alkaline comet assay*

Single cell gel electrophoresis (comet assay) was used to measure DNA strand breaks and alkaline labile sites in lymphocytes. The assay was conducted under alkaline conditions as previously described [30, 31] with slight modification for use with a high throughput CometSlide HT (Trevigen Inc. Cat 4252-02K-01). 100 cells were randomly selected from each spot and scored with online software (Tritek - http://autocomet.com/main_home.php) for tail moment and tail intensity. Tail moment (tail length X DNA density) and tail intensity (% DNA in tail) were used as indicators of DNA damage.

2.3.3 Isolation of DNA/RNA

DNA and RNA were isolated from lymphocytes using TRIzol (Ambion). A 0.5 ml volume of trizol was used to homogenise each sample. The only alteration from the manufacturer's directions was the use of 2 µg/ml glycogen (Ambion) and 5 mM sodium acetate to aid RNA precipitation.

a) *Telomere length*

A quantitative real-time polymerase chain reaction (qPCR) based method was used to measure absolute telomere length (aTL). This method is based on the Cawthon method for relative measurement of telomere length (TL) but modified by introducing an oligomer standard to measure aTL and was performed as previously described [32-34].

b) *Telomere base damage*

Telomere base damage was measured using a qPCR based method [35] to measure the amount of oxidised residues as well as other base lesions within telomeric DNA that are recognised and excised by the bacterial enzyme, formamidopyrimidine DNA-glycosylase (FPG). This method is based on differences in PCR kinetics between the DNA template exhaustively digested by FPG and undigested DNA i.e. ΔC_T (C_T treated- C_T untreated) and was performed as previously decribed [35].

c) *MT1A and Zip1 expression*

Gene expression of two different types of Zn transporters, MT1A and Zip1 was measured using Taqman quantitative Real-Time PCR (ABI 7500 Fast Sequence Detection system, Applied Biosystems) in the isolated RNA from lymphocytes. Inventoried Taqman gene expression assays and one custom-designed assay were obtained for *Zip1(Hs00205358_m1) and MT1A (Hs00831826_s1)* mRNA. All samples were quantified in duplicate, using the comparative C_T method for relative quantification of gene expression, normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All samples were run on an Applied

Biosystem 7300 Real Time PCR System and ABI 7300 Sequence Detection System with the SDS Ver 1.9 software (Applied Biosystem, Foster City, CA). Cycling conditions were: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. The level of expression of a specific gene in each sample was quantified by measuring the level of fluorescence relative to that of baseline samples.

2.3.4 Statistical analysis

With 40 subjects per group, the study had 80% power to detect an increase in plasma Zn of 0.055 µg/ml and a decrease in micronucleus frequency of 8.30 per 1000 binucleated cells at P=0.05 (one-tailed). These power calculations are based on standard deviation values of 0.095 for plasma Zn and 14.8 for micronucleus frequency in lymphocytes obtained from our own database from previous studies on healthy individuals aged 65-85 years. The detectable effect size of micronucleus frequency is equivalent to the DNA damage level induced by 0.2 Gy of X-rays which is a carcinogenic dose [36].

Q-Q plots on standard residual of all outcome measures were performed to test the normality of the data sets. Comparisons between groups were performed using general linear models and having treatment and gender as factors. To compare the difference in response between groups after 12 weeks, the percentage difference relative to baseline values was calculated as recommended by Vickers and Altman (2001) [37]. Correlation analysis between two sets of data was performed using Pearson's test and Partial Correlation Test. A value of *p<0.05* was considered to be statistically significant. Prism 5.0 (GraphPad Inc, San Diego, CA, USA) and SPSS for Windows (version 17.0, SPSS Inc, Chicago) software were used to obtain these values.

3.0 Results

3.1 Characteristics of the volunteers

A total of 208 volunteers aged 65-85 years old met the selection criteria and were screened for plasma Zn status. Those with plasma Zn concentration ≤ 10.0 µM (≤ 0.65 mg/L - cut-off point for Zn deficiency [26]) were given priority for inclusion in the study. As there were too few individuals with plasma Zn ≤ 0.65 mg/L (10.0 µM), the cut-off point was increased and eventually selected all those with plasma Zn status < 0.77 mg/L (11.77 µM) (n=90) and the volunteers were then consented to participate in the study. The volunteers were then randomly distributed into two groups (Zn supplement vs placebo). 42 out of 45 participants from each group completed the intervention. Reasons for drop-out during the trial included overseas travel (n=2), noncompliance (n=2), adverse events (n=1) and voluntary withdrawal (n=1). Age (Placebo group: 73.12 ± 5.19 years; Zn group: 73.88 ± 4.99 years), BMI (Placebo group: 26.73 ± 4.29 kg/m^2; Zn group: 26.81 ± 4.07 kg/m^2) and the estimated dietary Zn intake (Placebo group: 10.58 ± 3.62; Zn group: 10.97 ± 3.91 mg/day) was not significantly different between placebo and Zn groups, respectively (Supp Table 1). However, plasma Zn values at screening were marginally but significantly lower in the placebo group (0.69 ± 0.06 mg/L) compared to the Zn group (0.71 ± 0.04 mg/L). Compliance rate was high (>98%) with respect to tablet consumption in both groups.

3.3 Plasma micronutrients: Zinc, Carnosine, Mineral, B12, Folate and Homocysteine

At baseline, no difference between groups was observed, but significant change was found at the end of 12 weeks intervention. In the placebo group, plasma Zn showed a significant drop of -4.48% after 12 weeks of intervention from 0.905 ± 0.099 mg/L (13.84 ± 1.51 µM) to 0.863 ± 0.080 mg/L (13.19 ± 1.22 µM). However, in the Zn group, plasma Zn was significantly increased by 5.69% after 12 weeks of intervention from 0.926 ± 0.096 mg/L (14.16 ± 1.46 µM) to 0.972 ± 0.135 mg/L (14.86 ± 2.06 µM) (Table 1). Despite the changes in plasma Zn concentration, there was no significant increase in the carnosine concentration in the group consuming the Zn carnosine supplement (Table 2).

No significant changes were observed between both groups following intervention for other minerals such as iron, copper, potassium, sodium, magnesium and phosphorus as well as vitamin B12, folate and homocysteine except for calcium, and sulphur levels (Table 1 and 2). However, within groups, a significant increase of 92.36 ± 3.77 to 95.56 ± 3.61 mg/L in the Placebo group and 92.86 ± 3.66 to 97.75 ± 3.69 mg/L in the Zn group were observed for calcium in plasma ($p<0.05$). For magnesium, an increase from 20.08 ± 1.61 to 20.58 ± 1.49 mg/L was observed in the Placebo group while a significant increase of 5.7% (relative to baseline) was observed in the Zn group (baseline: 19.49 ± 1.76; 12 weeks: 20.61 ± 1.51 mg/L). Plasma folate showed a significant increase in the Zn group from baseline levels at 33.50 ± 7.81 to 35.88 ± 8.81 nmol/L at the end of the 12 week intervention (Table 2).

3.4 Antioxidant activity (FRAP and eSOD)

Antioxidant activity measured via FRAP was significantly increased in the Zn group following 12 weeks of supplementation ($p<0.05$) from 1180.62 ± 315.40 to 1447.79 ± 471.58 μmol/L (Table 2). However, no significant difference between groups was observed following the intervention. eSOD which reflects Cu-Zn SOD activity in erythrocytes was also significantly increased in the Zn group following the 12 week intervention and the changes were significant between groups ($p<0.05$). eSOD showed a 33.07% increment (relative to baseline) in the Zn group, compared to the Placebo group which showed only 2.45% increment (relative to baseline) (Figure 2).

3.5 DNA damage assays: CBMN-Cyt assay and alkaline comet assay

The CBMN-Cyt assay results (Table 3), showed a significant decrease in the frequency of MNi for the Zn group following supplementation ($p<0.05$). The reduction of 24.18% (relative to baseline) was statistically significant compared to the placebo group which showed an increase of 1.77% (Fig 3). There were no significant changes in the frequency of NPB and NBUD for both groups. Both tended to decrease in the Zn supplemented group but the changes were not significant. Necrotic cells were higher in the placebo group following 12 weeks intervention with a significant increase of 44.28% (relative to baseline). However, no change was observed in the percentage of necrotic cells in the Zn supplemented group with only an 18.21% increase relative to baseline. Apoptotic cells were decreased in the Zn group ($p<0.05$) following the duration of intervention with a reduction of 7.53% relative to baseline. NDI was increased in both groups following the 12 weeks of intervention ($p<0.05$) (Table 3) but no significant change between groups was observed.

The comet assay results showed a significant effect of time and treatment of Zn supplementation for both tail moment and tail intensity ($p<0.05$) (Table 3). Tail moment for the placebo group increased from 0.499 ± 0.286 to 0.809 ± 0.354 after 12 weeks of intervention and tail intensity values were also increased from 4.301 ± 1.306 to 5.139 ± 1.953. For the Zn group, both tail moment and tail intensity values were decreased following 12 weeks intervention (Tail moment-baseline: 0.476 ± 0.210; 12 weeks: 0.376 ± 0.229; Tail intensity-baseline: 4.700 ± 1.365; 12 weeks: 2.985 ± 1.587). Reductions of 7.53% for tail moment and 8.76% for tail intensity were observed for the Zn group (relative to baseline) (Fig 4). Tail moment and tail intensity in the Placebo group showed a significant 141.6% and 28.95% increase respectively relative to baseline ($p<0.05$).

3.6 Telomere integrity: Telomere length and telomere base damage

Telomere length was found to be shorter in the Zn supplemented group following the 12 week intervention but the difference was not significant relative to the Placebo group. Both groups showed a significant increase in telomere length within groups following 12 weeks of intervention with an increment of 9.65% for the Zn supplemented group and 10.36% for the Placebo group (relative to baseline) (Fig 5). Telomere base damage was found to be significantly decreased in the Zn group ($p<0.05$) (Baseline: 13.88 ± 18.04, 12 weeks: 6.82 ± 15.18 [8 oxodG/kb telomere]), but no difference was observed between groups.

3.7 Zinc transporter genes: MT1A and ZIP1

MT1A and ZIP1 were measured as biomarkers for Zn status and Zn transportation. Both MT1A and ZIP1 expression showed a significant increase in the Zn supplemented group compared to the Placebo group ($p<0.05$) (Table 3). MT1A expression was significantly higher in the Zn supplemented group ($p<0.05$) following the 12 week intervention compared to the Placebo group (Placebo: 5.29 ± 5.86, Zn: 8.46 ± 4.69 arbitrary unit). ZIP1 expression showed a 19.09% increase relative to the baseline measure, compared to a 9.38% increase in the Placebo group (Fig 6).

3.8 Correlation between plasma Zinc and other measured biomarkers

Pearson correlation analysis of the relationship between plasma Zn (at the baseline and at the end of the study) with other biomarkers was performed (Supporting Information Table 2). At baseline, no correlation between plasma Zn and other plasma and DNA damage biomarkers was observed. However, at the end of 12 weeks of supplementation, plasma Zn was significantly correlated with eSOD (r=0.279, p=0.010), tail moment (r=-0.219, p=0.045) and micronuclei (r=-0.235, p=0.032) (Supporting InformationTable 2).

Partial correlation analysis of the relationships between plasma Zn (at the start and at the end of the trial), after controlling for age and gender, and all biomarkers in this study was also performed. At baseline, no correlation between plasma Zn and other plasma and DNA damage biomarkers was observed. At the end of 12 weeks of supplementation, plasma Zn was found to be correlated only with ZIP1 expression (r=0.576, p=0.039) (Supporting Information Table 3).

3.8 Correlation between other measured biomarkers

Based on the partial correlation, at baseline, vitamin B12 was correlated with folate (r=0.221, p=0.047) and Hcy was negatively correlated with B12 (r= -0.438, p=0.000) and folate (r= -0.221, p=0.048). FRAP values were negatively correlated with MT1A gene expression (r=-0.259, p=0.019) and carnosine concentration was negatively correlated with Hcy (r=-0.293, p=0.008). For DNA damage events, tail intensity values was positively correlated with telomere length (r=0.264, p=0.017) and negatively correlated with carnosine concentration r=-0.236, p=0.034). Necrosis was negatively correlated with folate concentration (r=-0.241, p=0.030) and significantly correlated with ZIP1 expression (r=0.322, p=0.003) and eSOD levels (r=0.222, p=0.046). Apoptosis was also correlated with ZIP1 expression (r=0.265, p=0.017) and necrosis (r=0.541, p=0.000). Nuclear Division Index was significantly correlated only with apoptosis (r=0.232, p=0.037). Nucleoplasmic bridges was significantly correlated with ZIP1 expression (r=0.334, p=0.002), necrosis (r=0.557, p=0.000) and apoptosis (r=0.530, p=0.000). There were more significant correlations observed for nuclear buds. Nuclear bud frequency was significantly correlated with ZIP1 expression (r=0.274, p=0.013), necrosis (r=0.451, p=0.000), apoptosis (r=0.401, p=0.000), micronuclei frequency (r=0.259, p=0.020) and nucleoplasmic bridges (r=0.615, p=0.000).

Following 12 weeks intervention, Hcy levels was found to be negatively correlated with B12 concentration (r=-0.627, p=0.022). MT1A expression was significantly correlated with folate levels (r=0.660, p=0.014). For antioxidant measures, there was no significant correlation observed for FRAP values with any of the biomarkers measured in the study while eSOD values were found to be negatively correlated with telomere base damage (r=-0.619, p=0.024). With regards to DNA damage events, tail moment was negatively correlated with eSOD values (r=-0.693, p=0.009), while nuclear division index was found to be correlated with tail moment (r=0.623, p=0.023) and negatively correlated with necrosis (r=-0.567, p=0.043). Micronuclei frequency was found to be negatively correlated with telomere length (r=-0.567, p=0.043) and carnosine concentration (r=-0.564, p=0.045) but positively correlated with nuclear division index (r=0.639, p=0.019). Only one positive correlation was found for nucleoplasmic bridges, which was with apoptosis (r=0.572, p=0.041). Nuclear buds were found to be negatively correlated with eSOD values (r=-0.588, p=0.035) but positively correlated with telomere base damage (r=0.564, p=0.045) and strongly correlated with apoptosis (r=0.725, p=0.005).

4.0   Discussion

The results of this study show that Zn carnosine supplementation in an elderly population is beneficial in reducing the micronucleus frequency measured by the CBMN Cytome assay and both tail moment and intensity as measured via the comet assay following 12 weeks of intervention. To the authors' knowledge, this is the first study conducted to investigate the impact of Zn supplementation on CBMN Cytome biomarkers and telomere integrity in an elderly population.

Plasma Zn at baseline was found to be higher than plasma Zn levels at screening. This may possibly be due to the fasting state and diurnal variation as both can affect plasma Zn concentrations [8]. Volunteers who were fasting tended to have higher plasma Zn compared to non-fasting volunteers [8]. This possibly explains why in our study, a lower plasma Zn was observed at screening but the value increased at baseline although they were from the same cohort. According to Brown (1998) [38], in a population aged 22-75 years old, the reference value for plasma Zn is 16.6 ± 6.2 μM. The lower limit of fasting plasma Zn levels has been recommended at 10.7 μM [39] and therefore the cut-off level below which a Zn-deficient status is possible [40]. The cut off point for Zn deficiency was set to 11.6 μM in order to obtain 90 people with the lowest Zn status at screening, which was slightly higher than the 10.7 μM value recommended.

An increase of 5.40% in plasma Zn after 12 weeks of supplementation in the Zn group indicates that daily intake of Zn at 20 mg/day improved Zn status. Savarino *et al* (2001) [41] showed that high plasma Zn in older individuals may contribute to their longevity. The increased plasma Zn levels is in agreement with the beneficial effect of Zn supplementation reported in an older institutionalized population presenting a large prevalence of biological Zn deficiency (< 10.7 μM) [42].

In this study, we also measure plasma levels of other minerals to see the changes and correlation with plasma Zn and other variables. Calcium and magnesium concentration appeared to increase in both the Placebo and Zn groups. This could be explained by the excipient maltodextrin in the zinc carnosine and placebo tablets. A recent study in rats showed that maltodextrin increased absorption of calcium, magnesium, iron and zinc from the diet possibly by increasing bacterial fermentation in caecum [43]. However, in our study iron and zinc concentration did not increase in plasma which suggests that the effects of maltodextrin on mineral absorption in humans may be limited to calcium and magnesium. Further studies in humans are required to test this hypothesis.

Concentration of plasma B12, folate and Hcy were also measured as these may affect DNA damage and telomere maintenance [44-46]. It was found that Zn supplementation did not alter plasma B12, folate and Hcy substantially although a trend for increased plasma folate in the Zn group was evident. These results are in agreement with a previous study by Ducros *et al* 2009 [47] that showed after six months of intervention with Zn at 15 mg/day and 30 mg/day, there was no effect on Hcy, B12 and folate levels. It is evident that the extent of Zn deficiency in our cohort was not sufficient to substantially alter plasma B12 and Hcy levels in plasma and Zn supplementation may have contributed marginally to improved folate concentration in plasma.

The impact of Zn supplementation in relation to the antioxidant profile (FRAP and eSOD) of the participants was also investigated. It was shown that FRAP and eSOD were increased following Zn supplementation. This result is similar to a previous study that reported Zn supplementation increased the eSOD activity following both 3 and 6 month interventions [48]. However, in the same study, an increase in FRAP was not found. This negative result following Zn supplementation may have been because only 4.8% were middle-aged and 5.6% were older subjects that were below the cut off level for biological Zn deficiency (10.7 μM) [48]. In another study, Song *et al* [49] found that FRAP activity was unaffected following Zn supplementation while eSOD tended to decrease after marginal Zn depletion and increased after Zn repletion, but the changes were not statistically significant.

To our knowledge, this is the first study to investigate the effect of Zn supplementation on genome stability in an elderly South Australian population. A reduction in the micronucleus frequency was observed together with both tail moment and tail intensity as measured via the alkaline comet assay following 12 weeks intervention with Zn at 20 mg/day. A previous study in rats has shown that severe Zn depletion caused an increase in DNA damage in peripheral blood cells compared to Zn replete controls and this was

associated with impairment in DNA repair, compromised p53 DNA binding, and upregulation of the base excision repair (BER) proteins, OGG1 and PARP [50]. A further study conducted in rats showed that marginal Zn deficiency (MZnD) caused an increase in oxidative DNA damage in the prostate after chronic exercise [51]. Dietary Zn restriction and repletion of Zn to its normal status was found to affect DNA integrity in healthy men aged 19-50 years old [49]. Plasma Zn concentrations showed a negative correlation with DNA damage measures as measured via the alkaline comet assay (*r=-0.47, p=0.014*) in agreement with our study [49].

Zinc carnosine at optimal concentration in cells was shown to have an impact on Zn status and to have lower DNA damage events as measured via CBMN Cytome assay as well as following induced damage [52-54]. Activation of MT and SOD was predicted to be key components in this effect as measured via protein expression, in addition to activation of PARP and OGG1 [54]. This is in line with our results that observed an increase in eSOD following Zn supplementation. In addition, Zn is an essential component for PARP-1, which binds via its Zn domain to DNA strand breaks and thereby assisting in the recruitment of DNA repair complexes [55]. Positive correlation between cellular poly(ADP-ribosyl)ation and Zn status in human peripheral blood mononuclear cells was reported previously [56], indicating that Zn plays an important role in maintaining genomic stability [5]. Besides, Zn is also part of OGG1 which is another DNA repair enzyme that is involved in base excision repair by removing of 8-hydroxyl-2'-deoxyguanosine, one of the more prevalent oxidative DNA damage events [57].

These studies suggest that Zn deficiency may lead to an increase in DNA damage and in our study it was shown that Zn supplementation may be beneficial in lowering DNA damage especially DNA strand breaks and the frequency of micronuclei. In this study, Zn carnosine was used and it has been shown that carnosine is a potent antioxidant [58] and may have an impact on DNA damage. The levels of carnosine were very low and did not change significantly in the treatment group versus the placebo. However, plasma carnosine was inversely correlated with micronuclei at week 12 (r=-0.564) but not at baseline, and inversely with comet tail intensity at baseline (r=-0.236) but not at week 12. This suggests that the possibility that significant changes in carnosine concentration in blood may affect genome stability in lymphocytes cannot be disregarded.

The relationship between Zn status and telomere biology is not clearly defined. Accelerated telomere shortening may result in a DNA damage response leading to chromosomal end-to-end fusions, cell arrest and apoptosis [44, 59, 60]. No difference in telomere length and telomere base damage between the placebo and Zn treatment group was observed in this current study. However, telomere length was observed to increase and telomere base damage decreased in the Zn supplemented group when week 12 data was compared to baseline. This suggests that the effects are small or a larger study is required to achieve statistically significant time and treatment interaction. There are actually very few studies that have investigated the impact of Zn on maintaining telomere integrity. Liu and colleagues (2004) found that Zn sulphate at 80 μM accelerated telomere loss in hepatoma cells (SMMC-7721) after 4 weeks of treatment [61]. Cells with short telomeres are associated with impaired Zn homeostasis in hypertensive patients [62].

In this study, we focused on the measurement of main Zn transporter for human which are MT1A and ZIP1. Gene expression analysis showed that Zn supplementation over 12 weeks of intervention increased both MT1A and ZIP1 expression significantly. These genes were selected because both are expected to be responsive to Zn concentration because of their important role in Zn transport and Zn storage [1, 63]. MT has been found to affect the release of Zn for the activity of PARP-1, which is involved in base excision DNA-repair [64]. Previous studies have shown that Zn and metallothionein are genome-protective against the insults associated with oxidative stress [65-67]. In our study, MT expression was increased in the Zn group which may explain the reductions in both tail moment and tail intensity observed in the comet assay and also the frequency of micronuclei.

In particular, polymorphisms of the metallothionein gene MT1A were found to influence the efficacy of Zn supplementation [21, 23]. As MT induction and expression is mediated by IL-6, a multifunctional cytokine which regulates the differentiation and activity of different cell types, stress reactions and inflammatory responses [21, 23, 68-70]. Several studies suggest that the common IL-6-174 G/C and MT1A +647 polymorphisms interactively affect Zn bioavailability and bioefficacy and are likely to be a useful indicator for the selection of elderly people who would benefit from Zn supplementation [21-23, 70-72]. It would be of interest for future studies to investigate whether polymorphisms in ZnT, ZIP, MT1A and IL6 genes modify the relationship between Zn status and genome stability.

5.0 Conclusion

The results from this study show that Zn supplementation in an elderly populations with low Zn status can (a) improve Zn status, (b) lower DNA damage events, hence improving genome stability, (c) increase antioxidant activity which may lower DNA damage risk [6], and (d) increase Zn storage and transporter gene expression (MT1A and ZIP1). Given that there is some evidence that the benefits and/or adverse effects of Zn supplementation may depend on genotype variations e.g. (MT1A) [18, 20, 23], it will be necessary to find out in future whether there are specific genotypes or polymorphisms in genes in high susceptibility groups, who are more likely to benefit, or be at risk of any toxic effect of Zn supplementation.

6.0 Acknowledgements

7.0 Conflict of interest

None declared.

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Figure 1: Percentage change in Plasma Zn at 12 weeks relative to baseline. Percentage change was adjusted for baseline values. Mean values with * were significantly different (*p<0.05*).
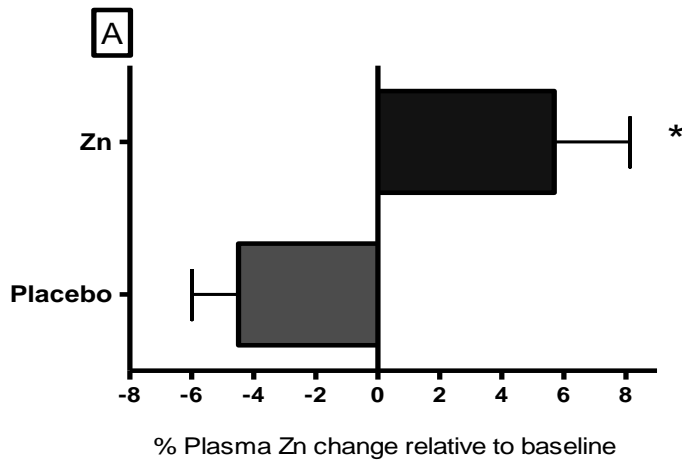
Figure 2: Percentage change in A) Plasma FRAP and B) eSOD, at 12 weeks relative to baseline. Percentage change was adjusted for baseline values. FRAP – Ferric Reducing Activity in Plasma; eSOD – erythrocytes Superoxide Dismutase.
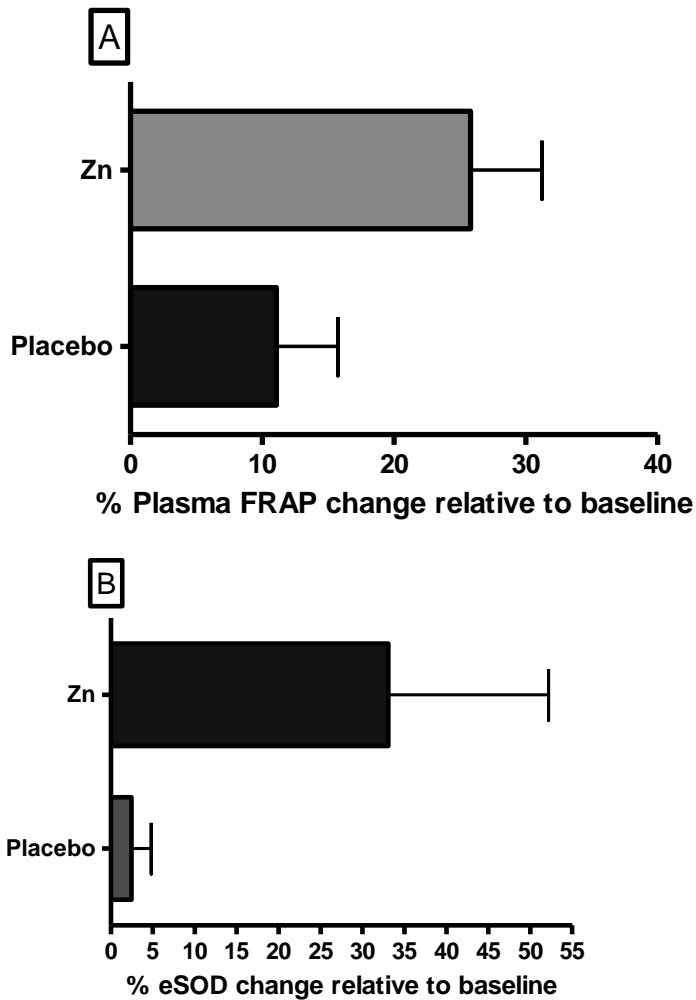
Figure 3: Percentage change in A) necrotic cells and B) micronuclei frequency at 12 weeks relative to baseline. Percentage change was adjusted for baseline values. Mean values with * were significantly different (*p<0.05*).
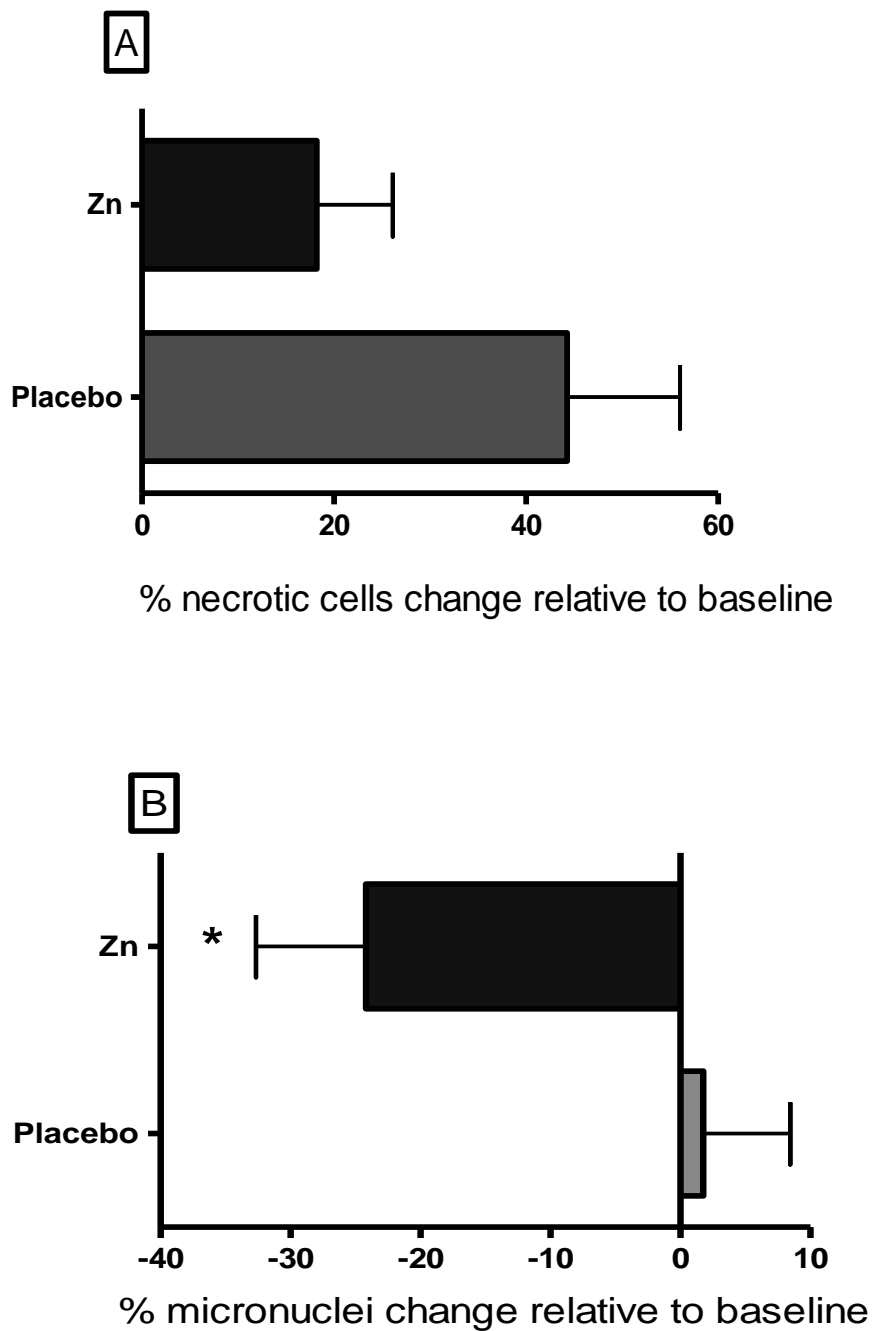
Figure 4: Percentage change in A) tail moment and B) tail intensity at 12 weeks relative to baseline. Percentage change was adjusted for baseline values. Zn and Mean values with * were significantly different (*p<0.05*).

Figure 5: Percentage change in A) telomere base damage and B) telomere length at 12 weeks relative to baseline. Percentage change was adjusted for baseline values.

% telomere base damage change relative to baseline

% telomere length change relative to baseline

Figure 6: Percentage change in A) MT1A and, B) ZIP1 gene expression at 12 weeks relative to baseline. Percentage change was adjusted for baseline values. Mean values with * were significantly different (*p<0.05*).
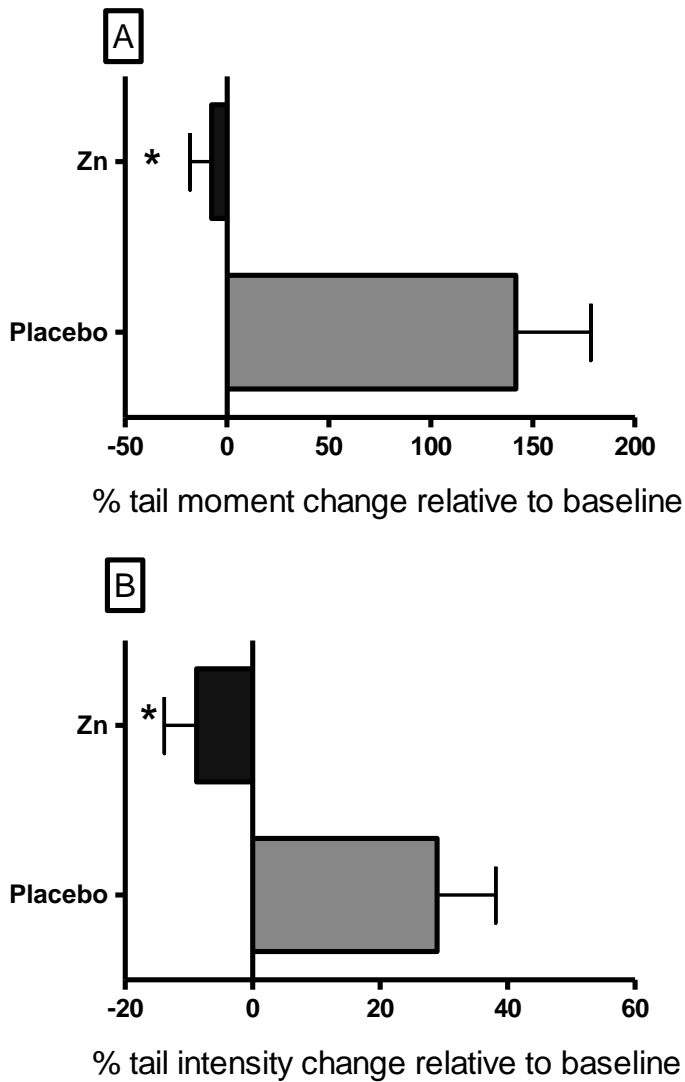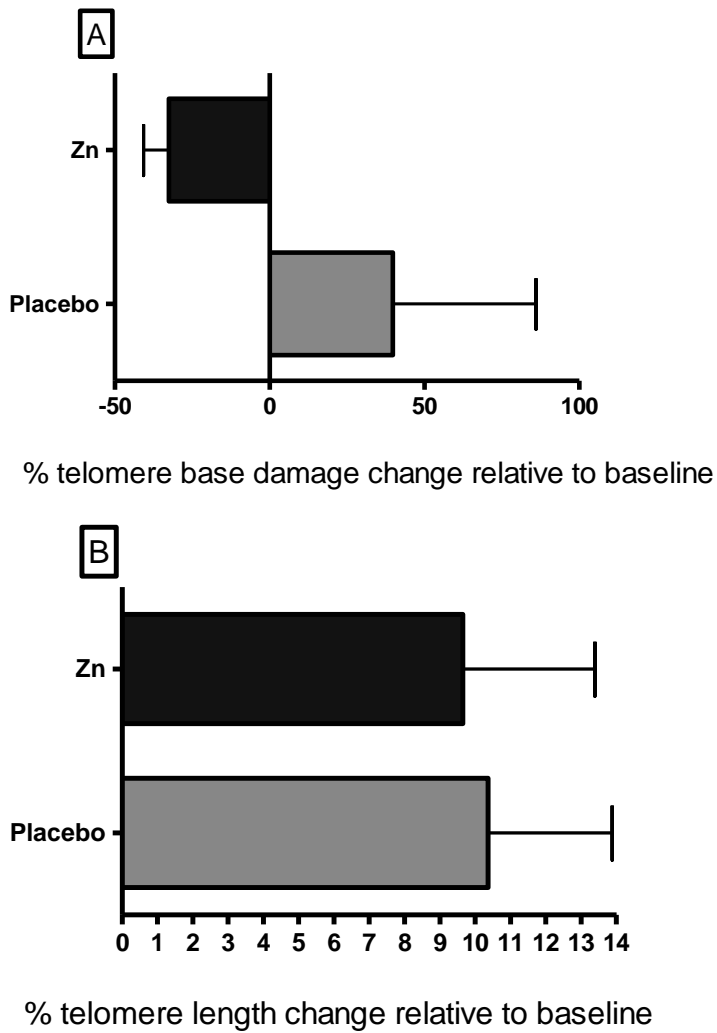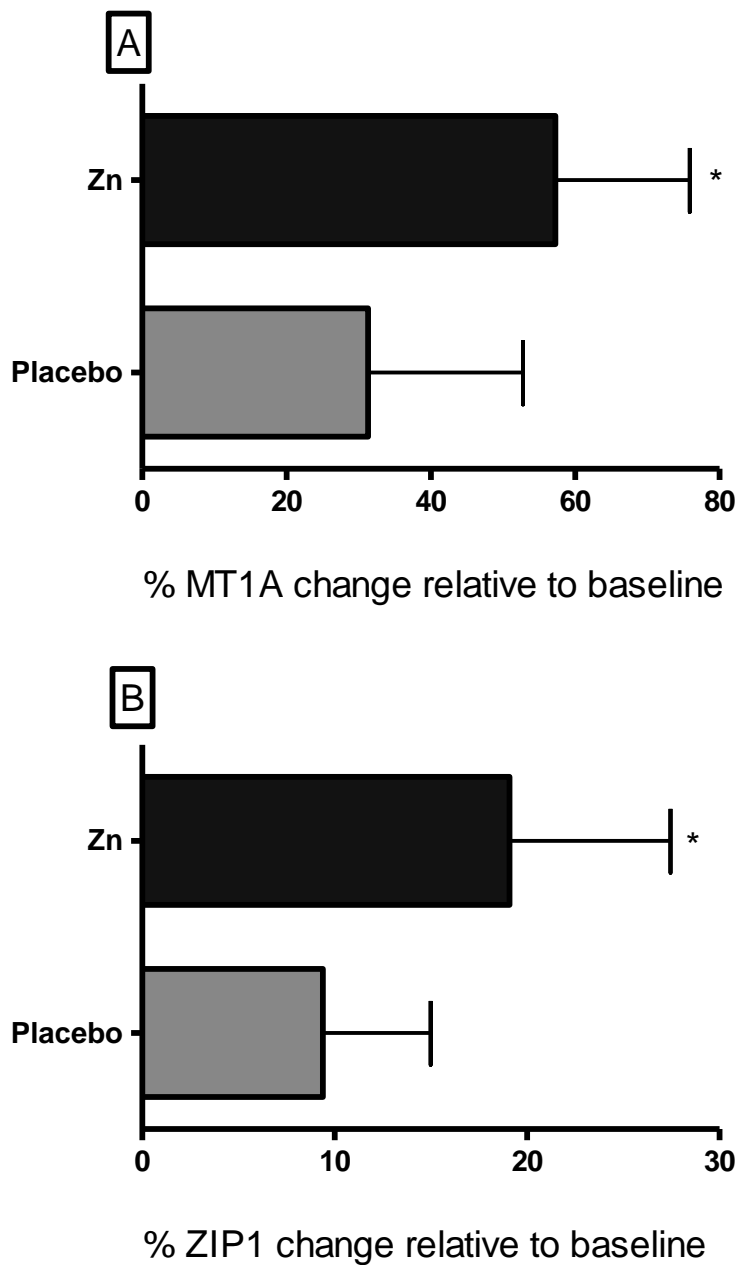
Table 1: Comparison of plasma mineral, B12, Folate, Homocysteine, plasma Carnosine, plasma FRAP and eSOD during the stages of the intervention within and between groups. Values are represented as mean ± SD. Mean values were significantly different for comparison *P<0.05, **P<0.01. *† p value for comparison between groups at baseline and at week 12 ;‡ p value for comparison between baseline and week 12 within the Placebo or Zn group.*

	Placebo (n=42)		Zn (n=42)		P value *†*
	Baseline	12 weeks	Baseline	12 weeks	
Plasma Mineral (mg/L)					
Zinc (Zn)	**0.905 ± 0.099**	**0.863 ± 0.088**	**0.926 ± 0.096**	**0.972 ± 0.135**	Baseline : 0.340 **Week 12 : 0.001****
P value ‡		**0.028***		**0.016***	
Iron (Fe)	1.276 ± 0.977	1.079 ± 0.412	1.231 ± 0.414	1.198 ± 0.487	Baseline : 0.838 Week 12 : 0.106
P value ‡		0.142		0.827	
Copper (Cu)	1.197 ± 0.237	1.195 ± 0.232	1.154 ± 0.191	1.160 ± 0.196	Baseline : 0.138 Week 12 : 0.208
P value ‡		0.842		0.614	
Calcium (Ca)	**92.363 ± 3.772**	**95.557 ± 3.608**	**92.859 ± 3.661**	**97.748 ± 3.690**	Baseline : 0.551 **Week 12 : 0.009****
P value ‡		**0.001****		**0.001****	
Magnesium (Mg)	**20.079 ± 1.611**	**20.575 ± 1.493**	**19.489 ± 1.762**	**20.613 ± 1.514**	Baseline : 0.120 Week 12 : 0.911
P value ‡		**0.005****		**0.001****	
Sodium (Na)	3256.250 ± 121.379	3225.529 ± 77.144	3260.641 ± 85.404	3254.611 ± 79.543	Baseline : 0.756 Week 12 : 0.050
P value ‡		0.097		0.659	
Potassium (K)	150.161 ± 16.860	152.894 ± 15.272	**150.196 ± 11.512**	**156.604 ± 13.614**	Baseline : 0.991 Week 12 : 0.234
P value ‡		0.186		**0.002****	
Phosphorus (P)	128.272 ± 18.892	128.744 ± 17.282	132.349 ± 19.108	133.181 ± 18.708	Baseline : 0.895 Week 12 : 0.765
P value ‡		0.889		0.645	
Sulphur (S)	**1165.337 ± 66.558**	**1148.990 ± 55.597**	1176.922 ± 61.243	1182.654 ± 58.724	Baseline : 0.369 **Week 12 : 0.004****
P value ‡		**0.049***		0.543	

Table 2: Comparison of B12, Folate, Homocysteine, plasma Carnosine, plasma FRAP and eSOD during the stages of the intervention within and between groups. Values are represented as mean ± SD. FRAP – Ferric Reducing Ability of Plasma, eSOD – erythrocyte Superoxide Dismutase. Mean values were significantly different for comparison *P<0.05, **P<0.01.

† p value for comparison between groups at baseline and at week 12

‡ p value for comparison between baseline and week 12 within the Placebo or Zn group

	Placebo (n=42)		Zn (n=42)		P value †
	Baseline	**12 weeks**	**Baseline**	**12 weeks**	
Plasma B12 (nmol/L)	383.065 ± 274.343	392.286 ± 283.529	305.906 ± 105.830	300.835 ± 102.408	Baseline : 0.084 Week 12 : 0.052
P value ‡	0.441		0.611		
Plasma Folate (nmol/L)	33.639 ± 7.628	33.821 ± 7.691	**33.500 ± 7.812**	**35.882 ± 8.818**	Baseline : 0.741 Week 12 : 0.244
P value ‡	0.905		**0.024***		
Plasma Homocysteine (µmol/L)	11.389 ± 3.014	11.476 ± 3.049	11.393 ± 2.801	11.623 ± 2.468	Baseline : 0.797 Week 12 : 0.582
P value ‡	0.851		0.237		
FRAP (µmol/L)	1216.589 ± 326.284	1311.345 ± 418.126	**1180.622 ± 315.404**	**1447.799 ± 471.582**	Baseline : 0.610 Week 12 : 0.167
P value ‡	0.155		**0.001****		
eSOD (U/g Hb)	2920.93 ± 330.2	2980.615 ± 275.645	**2807.957 ± 571.478**	**3190.228 ± 374.159**	Baseline : 0.780 **Week 12 : 0.005****
P value ‡	0.452		**0.001****		
Plasma carnosine (µmol/L)	0.664 ± 0.405	0.688 ± 0.382	0.682 ± 0.382	0.739 ± 0.444	Baseline : 0.972 Week 12 : 0.621
P value ‡	0.969		0.936		

Table 3: Comparison of DNA damage biomarkers, DNA stability biomarkers (CBMN-Cyt, Comet and Telomere assays and gene expression (MT1A and ZIP1) during the stages of the intervention within and between groups. Values are represented as mean ± SD. BN – binucleates, MNi – Micronuclei, NPB – Nucleoplasmic Bridges, NBuds – Nuclear Buds, kb – kilobase, 8-oxodG - 8-Oxo-2'-deoxyguanosine.
Mean values were significantly different for comparison *P<0.05, **P<0.01
† p value for comparison between groups for baseline and week 12
‡ p value for comparison between baseline and week 12 within the placebo and Zn group

	Placebo		Zn		P value †
	Baseline	**12 weeks**	**Baseline**	**12 weeks**	
DNA damage					
CBMN-Cyt assay					
MNi (per 1000 BN)	12.058 ± 5.310	11.125 ± 5.475	11.200 ± 5.034	6.930 ± 3.467	Baseline : 0.456 Week 12 : **0.001****
P value ‡	0.270		**0.001****		
NPB (per 1000 BN)	7.469 ± 4.741	7.523 ± 3.886	7.818 ± 3.972	7.486 ± 2.556	Baseline : 0.861 Week 12 : 0.839
P value ‡	0.911		0.555		
NBUD (per 1000 BN)	3.244 ± 2.080	3.805 ± 1.968	3.648 ± 2.066	3.405 ± 2.065	Baseline : 0.411 Week 12 : 0.452
P value ‡	0.294		0.553		
Necrosis (%)	8.655 ± 3.302	11.365 ± 4.245	8.755 ± 2.632	9.489 ± 2.835	Baseline : 0.878 Week 12 : **0.018**
P value ‡	**0.001****		0.275		
Apoptosis (%)	3.283 ± 1.216	3.371 ± 1.723	3.748 ± 1.397	2.951 ± 1.359	Baseline : 0.200
P value ‡	0.678		**0.012***		Week 12 : 0.216
NDI	1.862 ± 0.165	1.965 ± 0.198	1.877 ± 0.150	1.968 ± 0.222	Baseline : 0.650 Week 12 : 0.949
P value ‡	**0.028***		**0.030***		

Comet assay

Tail moment (arbitrary unit)	0.499 ± 0.286	0.809 ± 0.354	0.476 ± 0.210	0.376 ± 0.229	Baseline : 0.686 Week 12 **: 0.001****
P value ‡		**0.001****		0.100	
Tail intensity (% DNA in tail)	4.301 ± 1.306	5.139 ± 1.953	4.700 ± 1.365	2.985 ± 1.587	Baseline : 0.178 Week 12 : **0.001****
P value ‡		**0.011***		**0.001****	
Telomere Integrity					
Telomere length (kb/diploid genome)	2.878 ± 0.458	3.069 ± 0.463	2.725 ± 0.420	2.929 ± 0.449	Baseline : 0.122 Week 12 : 0.174
P value ‡		**0.054**		**0.035***	
Telomere base damage (8-oxodG/kb telomere)	14.503 ± 14.870	9.937 ± 10.671	13.879 ± 18.036	6.820 ± 15.181	Baseline : 0.867 Week 12 : 0.291
P value ‡		**0.096**		**0.009****	
Gene expression					
MT1A (arbitrary units)	4.981 ± 5.371	5.294 ± 5.863	4.287 ± 7.710	8.463 ± 4.697	Baseline : 0.643 Week 12 : **0.009****
P value ‡		0.658		**0.004***	
ZIP1 (arbitrary units)	2.693 ± 0.712	2.760 ± 0.663	2.894 ± 0.705	3.197 ± 0.598	Baseline : 0.294 Week 12 : **0.010***
P value ‡		0.543		**0.045***	