In Vitro Cellular & Developmental Biology - Animal

Genomic instability related to zinc deficiency and excess in an in vitro model: Is the upper estimate of the physiological requirements recommended for children safe?

--Manuscript Draft--

Manuscript Number:	IVAN-D-16-00390R1
Full Title:	Genomic instability related to zinc deficiency and excess in an in vitro model: Is the upper estimate of the physiological requirements recommended for children safe?
Article Type:	Original Article
Keywords:	Zinc suplementation; Children; Genomic stability; Recommended dietary allowances; Health
Corresponding Author:	Gisel Padula, PhD IGEVET ARGENTINA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	IGEVET
Corresponding Author's Secondary Institution:	
First Author:	Gisel Padula, PhD
First Author Secondary Information:	
Order of Authors:	Gisel Padula, PhD
	María Virginia Ponzinibbio
	Rocío Celeste Gambaro
	Analía Isabel Seoane
Order of Authors Secondary Information:	
Funding Information:	CONICET (0187) — Dr Gisel Padula
	UNLP (11/V212) — Dr Analía Isabel Seoane
Abstract:	Micronutrients are important for the prevention of degenerative diseases due to their role in maintaining genomic stability. Therefore, there is international concern about the need to redefine the optimal mineral and vitamin requirements to prevent DNA damage. We analyzed the cytostatic, cytotoxic and genotoxic effect of in vitro zinc supplementation to determine the effects of zinc deficiency and excess, and whether the upper estimate of the physiological requirement recommended for children is safe. To achieve zinc deficiency, DMEM/Ham´s F12 medium (HF12) was chelated (HF12Q). Lymphocytes were isolated from healthy female donors (age range, 5-10 years) and cultured for seven days as follows: negative control (HF12, 60 µg/dl $ZnSO_4$); deficient (HF12Q, 12 µg/dl $ZnSO_4$); lower level (HF12Q + 80 µg/dl $ZnSO_4$); average level (HF12Q + 180 µg/dl $ZnSO_4$); upper limit (HF12Q + 280 µg/dl $ZnSO_4$), and excess (HF12Q + 380 µg/dl $ZnSO_4$). The comet (quantitative analysis) and cytokinesis-block micronucleus cytome assays were used. Differences were evaluated with Kruskall-Wallis and ANOVA ($P < 0.05$). Olive tail moment, tail length, micronuclei frequency and apoptotic and necrotic percentages were significantly higher in the deficient, upper limit and excess cultures compared with the negative control, lower and average limit ones. In vitro zinc supplementation at the lower and average limit (80 and 180 µg/dl $ZnSO_4$) of the physiological requirement recommended for children proved to be the most beneficial in avoiding genomic instability, whereas the deficient, upper limit and excess (12, 280 and 380 µg/dl) cultures increased DNA and chromosomal damage and apoptotic and necrotic frequencies.

IGEVET
CONICET
INSTITUTO DE GENÉTICA VETERINARIA
"Ing. Fernando Noel Dulout"
CCT - LA PLATA | FACULTAD DE CIENCIAS VETERINARIAS | UNIVERSIDAD NACIONAL DE LA PLATA

La Plata, February 7, 2017.

Tetsuji Okamoto, D.D.S., Ph.D.
Editor-in-Chief
In Vitro Cellular & Developmental Biology - Animal

Dear Dr. Okamoto,

We are submitting for your consideration the revised version of our manuscript entitled "Genomic instability related to zinc deficiency and excess in an *in vitro* model: Is the upper limit of the normal physiological range recommended for children safe? (IVAN-D-16-00390). following the reviewers´ comments and suggestions, which are listed below.

We appreciate the opportunity to review and improve our manuscript. Hoping that in its present form the manuscript will be suitable for publication,

Sincerely,

Dr. Gisel Padula

Reviewer 1

1- We have reformulated the Introduction section (pages 3-4).
2- In the Study Design we have included a paragraph about the use of Chelex (page 5).
3- Table 1 has been replaced by Figure 1.
4- We have included a table with data of mineral level before and after supplementation with Zn sulfate (Table 1).
5- Unfortunately, we cannot perform Western Blot. Note that we conclude in the manuscript that "Similarly, future research on this subject should focus on the elucidation of the underlying mechanisms whereby Zn would modulate DNA repair". We are presently adjusting techniques related with oxidative stress assessment (T-Bars, Catalase and SOD) so as to continue our research on this subject and present the corresponding results in the near future.
6- We have rewritten the Discussion section (pages 8-10).

7- We have moved the first paragraph of the Discussion section to the Study design (page 5).
8- In the Sharif et al. 2011 and 2012 works, the authors expressed Zn sulfate concentration in μM, while we expressed it in μg/dl. Then, 32 μM would correspond to 517 μg/dl.
9- We know that Zn bioavailability depends on several factors but we performed an *in vitro* model, so we do not pretend to relate the concentration with physiological conditions as in children. In this sense, we conclude that "Although at first *in vitro* dose-response studies would allow to evaluate the effect of the micronutrient concentration on genetic damage and cytotoxicity, *in vivo* cross-sectional and interventional research are required to assess the association between nutrient intake and genetic damage." Future *in vivo* studies would be necessary to determine the adequate daily intake of this micronutrient and these should consider factors that could affect Zn bioavailability.

Reviewer 2

1- The title has been modified as suggested (page 1).
2- The ages of the healthy donors has been specified in the Abstract (page 2).
3- The spelling mistake has been corrected (height) (page 3).
4- The phrase "physiological range" has been replaced by "physiological requirement established for children" along the manuscript.
5- Transporter gene expression has been replaced by Zn uptake transporter gene expression (page 8).
6- We have added a table (Table 1) with information about mineral level before and after Zn sulfate supplementation.
7- P-values has been changed in the Results section (page 8).
8- In Table 2 P-values have been added.
9- The limitations of our study were clearly addressed (page 10).

Genomic instability related to zinc deficiency and excess in an *in vitro* model: Is the upper estimate of the physiological requirements recommended for children safe?

Gisel Padula, María Virginia Ponzinibbio, Rocío Celeste Gambaro, Analía Isabel Seoane

IGEVET – Instituto de Genética Veterinaria "Ing. Fernando Noel Dulout" (UNLP-CONICET LA PLATA), Facultad de Ciencias Veterinarias UNLP, 60 y 118 s/n, La Plata B-1900-AVW, Argentina. Tel./fax: +54 221 421 1799

Running head: Genomic damage and zinc supplementation

Corresponding author: G. Padula

giselpadula@conicet.com.ar; gpadula@igevet.gob.ar

Abstract

Micronutrients are important for the prevention of degenerative diseases due to their role in maintaining genomic stability. Therefore, there is international concern about the need to redefine the optimal mineral and vitamin requirements to prevent DNA damage. We analyzed the cytostatic, cytotoxic and genotoxic effect of *in vitro* zinc supplementation to determine the effects of zinc deficiency and excess, and whether the upper estimate of the physiological requirement recommended for children is safe. To achieve zinc deficiency, DMEM/Ham´s F12 medium (HF12) was chelated (HF12Q). Lymphocytes were isolated from healthy female donors (age range, 5-10 years) and cultured for seven days as follows: negative control (HF12, 60 µg/dl $ZnSO_4$); deficient (HF12Q, 12 µg/dl $ZnSO_4$); lower level (HF12Q + 80 µg/dl $ZnSO_4$); average level (HF12Q + 180 µg/dl $ZnSO_4$); upper limit (HF12Q + 280 µg/dl $ZnSO_4$), and excess (HF12Q + 380 µg/dl $ZnSO_4$). The comet (quantitative analysis) and cytokinesis-block micronucleus cytome assays were used. Differences were evaluated with Kruskall-Wallis and ANOVA ($P < 0.05$). Olive tail moment, tail length, micronuclei frequency and apoptotic and necrotic percentages were significantly higher in the deficient, upper limit and excess cultures compared with the negative control, lower and average limit ones. *In vitro* zinc supplementation at the lower and average limit (80 and 180 µg/dl $ZnSO_4$) of the physiological requirement recommended for children proved to be the most beneficial in avoiding genomic instability, whereas the deficient, upper limit and excess (12, 280 and 380 µg/dl) cultures increased DNA and chromosomal damage and apoptotic and necrotic frequencies.

Keywords

Zinc suplementation; Children; Genomic stability; Recommended dietary allowances; Health

Introduction

Argentina is presently undergoing the nutrition transition, characterized by a decrease of acute malnutrition and an increased prevalence of obesity, stunting and hidden hunger (Luchesse et al. 2016). The World Health Organization (WHO) and the United Nations Children´s Fund (UNICEF) define hidden hunger as specific vitamin and mineral deficiency. The main micronutrients required for child growth are iron, zinc (Zn), vitamin A, vitamin B (like riboflavin), folic acid, niacin and essential fatty acids. Micronutrient quantification is performed with the recommended dietary allowances (RDA) from the National Research Council (National Research Council Food and Nutrition Board 1989), which express the absolute value of the nutrient recommended per day.

Micronutrients are important for the prevention of degenerative diseases such as cancer, cardiovascular disease, Alzheimer and premature aging, due to their role in maintaining genomic stability (Fenech 2010; Fenech 2014). In human populations, genetic mutation and chromosomic aberrations may be increased by *in vivo* exposure to mutagenic and carcinogenic agents (Parry 1988) and by an imbalanced diet (Ames 1998). Considering that many minerals and vitamins act as substrate and/or cofactors in DNA maintenance reactions, their exact concentration in the cell is critical. Thus, non-optimal levels of these micronutrients would impair the activity of enzymes needed for genomic stability, since they produce DNA double or single strand breaks, oxidative damage, or both (Fenech 2001; Fenech 2005). Therefore, there is international concern about the need to redefine the optimal mineral and vitamin requirements to prevent DNA damage. This becomes especially important for early stages of life, since the unique nutritional requirements of children make them unusually susceptible to inadequate nutrient intake.

Although severe Zn deficiency is not common, moderate deficiencies are relatively frequent, particularly in infancy and childhood (Hambidge 1989). This deficiency would

cause a decrease in child growth and development (Roohani et al. 2013), mainly affecting height (Siklar et al. 2003; Varea et al. 2006; Grandy et al. 2010). Zn deficiency not only affects the immunological system by disturbing cell division and multiplication, but also partially suppresses thymus function and decreases T and B lymphocyte proliferation and function (Haase and Lothar Rink 2009). The induction of brain growth and development alterations that cause cognitive impairments have also been reported (Prasad 2013). In this regard, the role of Zn in DNA synthesis and cell proliferation would account for these Zn deficiency-associated effects.

Zn is an essential component of approximately 300 proteins, such as Cu/Zn superoxide dismutase, endonuclease IV, p53, and Zn-finger proteins, such as poly ADP-ribose polymerase (PARP). It is involved in the process of genomic stability and gene expression in different ways, including the formation of chromatin structures and the participation in DNA replication and RNA transcription through transcription factors and RNA and DNA polymerases (Shariff et al. 2011; Shariff et al. 2012). Likewise, Zn plays a key role in DNA repair (Dreosti 2001) and programmed cell death or apoptosis (Chung et al. 2005; Clegg et al. 2005; Bae et al. 2006; Chang et al. 2006).

Presently, knowledge about the optimal levels of Zn for genomic stability is scarce and disordered. However, there is evidence supporting the fact that marginal Zn deficiency impacts significantly on the percentage of spontaneous chromosome damage (Fenech 2001; Fenech 2002; Fenech and Ferguson 2001; Shariff et al. 2011; Shariff et al. 2012). On the other hand, *in vitro* studies have shown that excess of different Zn salts, such as chromate, citrate and sulfate, would produce a cytotoxic and genotoxic effect on different cell lines (Bae et al. 2007; Xie et al. 2009; Wise et al. 2010; Sharif et al. 2011; Shariff et al. 2012) and laboratory animals (Tapisso et al 2009).

Considering that Zn is an important micronutrient for children growth and that it is involved in the process of genomic stability, the purpose of this study was to analyze the cytostatic, cytotoxic and genotoxic damage of *in vitro* Zn supplementation to determine the effects of Zn deficiency and excess, and whether the upper estimate of the physiological requirement recommended for children is safe.

Materials and methods

Study design *In vitro* modeling of peripheral blood lymphocyte cultures was used to determine the effect of micronutrients on cytotoxicity and genomic damage (Kimura et al. 2004; Wu et al. 2009; Fenech 2010). This model helps to define the optimal concentration and the safe upper limit of micronutrients (Fenech 2010). Isolated peripheral blood lymphocytes obtained from six healthy female donors (age range, 5-10 years) were used. Written parental informed consent was obtained. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institutional Review Board of the Instituto de Desarrollo e Investigaciones Pediátricas (IDIP), La Plata Children Hospital, Buenos Aires, Argentina.

Lymphocytes were isolated with Ficoll (Histopaque®-1077 Sigma-Aldrich, St. Louis, MO, USA) according to Fenech´s protocol (2007) and cultured in 10 ml of DMEM/Ham's F12 medium (HF12) (Sigma-Aldrich, St. Louis, MO, USA) formulated for culture without fetal bovine serum, with antibiotics (50 IU penicillin and 50 μg/ml streptomycin) (Bagó Laboratories, Buenos Aires, Argentina), in a humidified atmosphere with 5% CO_2. Cells were cultured in Falcon T-25 (Nunc, Denmark).

For all experiments, lymphocytes were cultured in Zn-depleted medium (HF12Q). HF12Q was prepared with HF12 mixed with 10% Chelex-100 (95577 Sigma, St. Louis, MO, USA)

for 2 h; the cycle of depletion was repeated for another 4 h (Sharif et al. 2011; Shariff et al. 2012). Because other divalent cations (Cu, Fe, Mn and Ca) were also chelated, we replenished the same concentration as that indicated in the formulation of the culture medium used.

In this study, Zn sulfate ($ZnSO_4$) was used to supplement cultures because of its proven higher bioavailability (Sharif et al. 2011; Shariff et al. 2012). Three doses of $ZnSO_4$ (Sigma-Aldrich, St. Louis, MO, USA) within the physiological requirement recommended for children (80-280 µg/dl) (Feliu et al. 2005) were used. Two other doses outside of such range were used to evaluate the effect of both deficiency and excess. Thus, the resulting cultures were as follows: negative control (HF12Q, 60 µg/dl $ZnSO_4$); deficient (HF12Q, 12 µg/dl $ZnSO_4$); lower level (HF12Q + 80 µg/dl $ZnSO_4$); average level (HF12Q + 180 µg/dl $ZnSO_4$); upper limit (HF12Q + 280 µg/dl $ZnSO_4$); and excess (HF12Q + 380 µg/dl $ZnSO_4$).

Lymphocytes were cultured with 5% CO_2 at 37 ºC for seven days (Fenech 2010, 2014). Thereafter, 50 µl were transferred to conic tubes (Eppendorf) for quantitative comet assay, and the remaining cells were used for cytokinesis-block micronucleus cytome (CBMN-Cyt) assay.

Each experimental point was set up in duplicate. Experiments were repeated three times so as to allow an accurate estimation of inter-experimental variations.

Zn levels Atomic absorption spectrophotometry (AAS) was used to determine Zn levels in media before and after supplementation. Samples were diluted with trichloroacetic acid and analyzed by AAS at the Department of Physiology, National University of La Plata School of Veterinary Sciences.

Comet assay Single cell gel electrophoresis was performed using the alkaline version described by Singh and coworkers (1988) with some modifications (Tice and Strauss 1995).

142 Briefly, slides were covered with a first layer of 180 µl of 0.5% normal agarose (Carlsbad, Ca,
143 USA). An amount of 75 µl of 0.5% low melting point agarose (Carlsbad, Ca, USA) was
144 mixed with 25 µl blood and layered onto the slides, which were immediately covered with
145 coverslips. After agarose solidification at 4 °C for 10 min, coverslips were removed and slides
146 were immersed overnight at 4 °C in fresh lysis solution. The slides were equilibrated in
147 alkaline solution for 20 min.

148 Electrophoresis was carried out for 30 min at 25 V and 250 mA (1.25 V/cm). Afterwards,
149 slides were neutralized by washing them three times with Tris buffer (pH 7.5) every 5 min,
150 and subsequent washing in distilled water. Slides were stained with 1/1000 SYBR Green I
151 solution (Molecular Probes, Eugene, Oregon, USA) (Olive 1999). Scoring was performed at
152 400 x magnification using a fluorescent microscope (Olympus BX40 equipped with a 515-
153 560 nm excitation filter) connected to a Sony 3 CCD-IRIS Color Video Camera. DNA
154 migration was determined with the Comet Assay Software Project (CASP) (public domain)
155 (Końca 2003). DNA damage was expressed as olive tail moment (OTM, arbitrary units) and
156 tail length (TL, µm) (Tice et al. 2003). From each of the two slides made for one experimental
157 point, 50 randomly selected cells were measured, thus giving 100 cells per sample and 300
158 cells per dose in all the experiments (three determinations).
159
160 **CBMN-Cyt assay** Cytostatic and cytotoxic effects and chromosomal damage were assessed
161 by the CBMN-Cyt assay according to the method of Fenech (2007) with some modifications.
162 Briefly, after 6 days of culture, Cytochalasin-B (3 µg/ml final concentration) (Sigma-Aldrich,
163 St. Louis, MO, USA) was added for 28 h. Samples were centrifuged and the pellet was
164 resuspended in 5 ml fixative 1 (sodium chloride:methanol:acetic acid 6:5:1). Cells were
165 washed twice with fresh fixative 2 (methanol:acetic acid 5:1) and later resuspended, dropped
166 onto clean slides and finally stained with 5% Giemsa for 10 min. The chromosome damage

167 biomarkers scored were micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds
168 (NBuds). One thousand binucleated cells (BN) were analyzed per experimental point.
169 Cytostatic effects were analyzed through the nuclear division index (NDI), and estimated by
170 the ratio of mono-, bi-, and multinucleated cells. Five hundred viable cells were scored per
171 experimental point to determine the frequency of cells with one, two, three or four nuclei and
172 calculate the NDI using the formula $(M_1+2M_2+3M_3+4M_4)/N$, where M_{1-4} represents the
173 number of cells with 1-4 nuclei and N is the total number of viable cells scored (Fenech
174 2007). Cytotoxicity events were assessed by the percentage of apoptotic (AC) and necrotic
175 (NC) cells in 500 cells. Fenech's scoring criteria for MNi, NPBs, NBuds, apoptotic and
176 necrotic cells were used (Fenech 2007).
177
178 **Statistical analysis** Data are presented as means ± standard error and P values less than 0.05
179 were considered statistically significant. Kurtosis was estimated to determine the normal
180 distribution of data. Quantitative comet assay results were evaluated with the non-parametric
181 Kruskal-Wallis Contrast test, which analyzes the null hypothesis of equal medians of comet
182 parameters within each of the four treatments. Results of the CBMN-Cyt assay were
183 statistically analyzed by simple ANOVA and Multiple Range test. Statgraphics® 5.1 software
184 (Manugistics Inc., Rockville, MD) was used for all the analyses.
185
186 **Results**
187 The mineral level before and after $ZnSO_4$ supplementation is presented in Table 1.
188 DNA strand breaks and alkali labile sites were determined with the comet assay (Figure 1).
189 OTM and TL quantitative analysis results were statistically significant ($p < 0.001$). In the case
190 of OTM, the Kruskal-Wallis test showed statistically significant differences for almost all
191 comparisons, excepting negative control with 80 µg/dl $ZnSO_4$ and control with 180 µg/dl

192 $ZnSO_4$. OTM was significantly higher in the deficient, 280 and 380 µg/dl $ZnSO_4$
193 supplemented cultures with respect to the negative control, 80 and 180 µg/dl $ZnSO_4$
194 supplemented ones ($p < 0.001$). Cultures supplemented with 80 and 180 µg/dl $ZnSO_4$ had the
195 lowest OTM. Similar results were obtained for TL (negative control *vs.* deficient and negative
196 control *vs.* 380 µg/dl $ZnSO_4$ $p < 0.001$; negative control *vs.* 280 µg/dl $ZnSO_4$ $p < 0.01$).
197 Table 2 shows the results obtained with the CBMN-Cyt assay. The cytotoxic effect
198 evaluated with NDI decreased in deficient cultures as well as in those supplemented with 280
199 and 380 µg/dl $ZnSO_4$. The highest NDI was observed in 80 and 180 µg/dl $ZnSO_4$ cultures.
200 However, none of the differences observed resulted statistically significant. Assessment of
201 cytotoxic effects with AC and NC percentages in 500 cells by the analysis of variance showed
202 statistically significant differences among treatments ($p < 0.001$). No differences were found
203 among the negative control, 80 and 180 µg/dl $ZnSO_4$ cultures. The highest AC and NC
204 percentage was observed in the deficient and 380 µg/dl $ZnSO_4$ cultures. On the other hand,
205 the 280 µg/dl $ZnSO_4$ supplemented culture presented higher AC and NC percentages than the
206 negative control, 80 and 180 µg/dl $ZnSO_4$ supplemented ones. Chromosome damage
207 evaluation was determined by the frequency of MNi, NPBs and NBuds in 1,000 BN cells. The
208 analysis of variance showed statistically significant differences among treatments for MNi
209 frequency ($p < 0.001$). Regarding the negative control, the 80 and the 180 µg/dl $ZnSO_4$
210 cultures, no significant differences could be detected with the Multiple Range test. For this
211 marker, the highest MNi frequency was observed in the 380 µg/dl $ZnSO_4$ supplemented
212 culture, as compared with the rest of the cultures. On the other hand, the deficient and 280
213 µg/dl $ZnSO_4$ supplemented cultures presented the same level of damage, markedly higher than
214 that of the negative control, 80 and 180 µg/dl $ZnSO_4$ supplemented ones. Cultures
215 supplemented with 80 and 180 µg/dl $ZnSO_4$ had the lower chromosome damage. Results

obtained for NPBs and NBuds were mixed, showing higher but not significant values in cultures supplemented with 80 and 280 μg/dl $ZnSO_4$.

Discussion

In this study, we analyzed the cytostatic, cytotoxic and genotoxic effect of *in vitro* Zn supplementation in order to determine the effects of zinc deficiency and excess, and whether the upper estimate of the physiological requirement recommended for children is safe.

The effect of Zn deficiency and excess was analyzed with the comet and the CBMN-Cyt assays because they complement each other and provide extensive information about the possible damage induction to genetic material caused by an inadequate nutrient intake (Sharif et al. 2011).

Our results showed that Zn deficiency increased DNA damage. Other studies using the comet assay also reported high frequencies of DNA damage (Ho and Ames 2002; Yan et al. 2008; Song et al. 2009a, b, c). *In vitro* studies showed that Zn-deficient cells present high levels of damage and failure in the DNA repair mechanisms (Dreosti 2001; Ho and Ames 2002; Sharif et al. 2012; Yan et al. 2008). *In vivo* studies have reported a relationship between Zn nutritional status and DNA damage (Bae et al. 2007; Xie et al. 2009; Sliwinski et al. 2009; Wise et al. 2010; Sharif et al. 2015). In another study carried out in men between 19 and 50 years of age whose micronutrient intake was restricted and then reestablished, the authors recorded a significant increase of DNA breaks during the depletion period (Song et al. 2009c). Such increase reverted after Zn repletion, suggesting that the damage was related to the Zn level.

On the other hand, the increase in the frequency of DNA damage could be due to an increase in oxidative stress. There is evidence from experimental *in vitro* studies suggesting that addition of Zn protects sulfhydryl groups against oxidative damage (Bagchi et al. 1997;

241 Bray and Bettger 1990; Sunderman 1995; Szuster-Ciesielska 2000). The results from a recent
242 intervention study (Sharif et al. 2015) showed that Zn supplementation in an elderly
243 population with low Zn status could lower DNA damage events, hence improving genome
244 stability, increasing antioxidant activity which may lower DNA damage risk, and increasing
245 Zn storage and Zn uptake transporter gene expression (MT1A and ZIP1).

246 In the present study, supplementation with 80 and 180 μg/dl Zn sulfate reduced DNA
247 breaks with respect to the deficient culture, whereas a high concentration (380 μg/dl)
248 increased the damage, suggesting a potential genotoxic effect of excess Zn sulfate. Further,
249 the upper estimate of the physiological requirements recommended for children (280 μg/dl)
250 also increased DNA damage.

251 The results obtained with the CBMN-Cyt assay showed that both Zn deficiency and excess
252 increased MNi frequency; in this case, the excess was more marked. Interestingly, the upper
253 estimate of the physiological requirements recommended for children (280 μg/dl) was
254 responsible for the same magnitude of chromosome damage as the deficiency. In other
255 studies, increased MNi and NPBs frequencies in both deficiency (0 μM) and excess (32 μM)
256 were observed (Sharif et al. 2011, 2012). Those works reported that all the assessed
257 concentrations within the physiological requirements recommended for children (4-16 μM)
258 decreased such damage in both WIL2-NS and human oral keratinocyte cells. On the other
259 hand, the higher limit of the pharmacological range (100 μM) adversely affected some cell
260 parameters (Sharif et al. 2011; Shariff et al. 2012). By contrast, another study evaluating the
261 effect of supplementation with 100 μM Zn sulfate showed that human lymphocyte viability
262 was not affected (Sliwinski 2009). Elevated MNi frequency in lymphocytes has been shown
263 to be associated prospectively with an increased risk of cancer in a cohort study, with severe
264 adverse cardiovascular events in coronary artery disease patients and with mortality from
265 cancer or cardiovascular disease in a case-control study (Milne et al. 2015).

In our study, the highest percentage of NC was observed in cultures with Zn excess, where chromosome damage was also highest. A recent study suggests that excess Zn supplementation reduces cell viability in INS-1E rats due to increased necrosis (Nygaard et al. 2014). *In vitro* studies have shown that excess of other Zn salts, such as chromate and citrate, also caused a cytotoxic and genotoxic effect in different cell lines (Sharif et al 2011; Bae et al. 2007; Xie et al. 2009; Wise et al. 2010) and laboratory animals (Tapisso et al. 2009). In our study, deficient cultures presented a higher percentage of AC. In this sense, other reports showed that Zn deficiency induced apoptosis in different cell types such as fibroblasts, hepatocytes, T cell precursors, and glioma and testicular cells (Bao and Knoell 2006; Ho and Ames 2002; Ho et al. 2003; Yamaguchi et al. 2009; Yan et al. 2008).

Daily RDA provide a guide for the appropriate intake of nutrients for the prevention of diseases caused by deficiency or excess. Determining these extremes is important, but the biggest challenge in the prevention of developmental and degenerative diseases is defining the appropriate intakes of micronutrients to optimize cellular and organism performance at different life stages. Optimization of cellular function ultimately depends on the prevention of damage to the nuclear and mitochondrial genome (Fenech 2010). This becomes especially important in childhood since the unique nutritional requirements of children make them unusually susceptible to inadequate nutrient intake.

Animal source foods are the major Zn source. Red meat, seafood and fish make up the biggest contributions to Zn intake in the diets and present high bioavailability (Rosado 1998). In the last 30 years, Argentina has changed the food consumption pattern, with a decrease in meat intake (Aguirre 2005). Moreover, a late incorporation of animal source food during the dietary complementation stage has been recorded in the child population, thereby exposing young children to an inadequate intake of Zn and irreversibly compromising their growth potential.

Conclusions

The results obtained in the present study suggest that *in vitro* supplementation of cultures with 80 and 180 µg/dl Zn sulfate would avoid genomic instability. On the other hand, deficient cultures (12 µg/dl) and those presenting excess Zn (380 µg/dl) would induce increased AC and NC percentages and cause higher DNA and chromosome damage. Likewise, the upper estimate of the physiological requirements recommended for children (280 µg/dl) would increase the cytostatic and cytotoxic effect as well as DNA and chromosome damage. These findings are important since optimal mineral and vitamin levels are necessary to prevent DNA damage and, therefore, degenerative diseases such as cancer, cardiovascular disease, Alzheimer and premature aging.

We are aware of the limitations of our work, and future studies would be necessary to determine the adequate daily intake of this micronutrient. Although at first *in vitro* dose-response studies would allow to evaluate the effect of Zn concentration on genetic damage and cytotoxicity, *in vivo* cross-sectional and interventional research is required to assess the association between nutrient intake and genetic damage. Similarly, future research on this subject should focus on the elucidation of the underlying mechanisms whereby Zn would modulate DNA repair.

Acknowledgments This work was supported by the National Scientific and Technical Research Council - Argentina (Grant No. 0187) and La Plata National University (Grant No. 11/V212). The authors thank Adriana Di Maggio for careful manuscript correction and César E. Bianchi for technical assistance.

316 **References**

317 Ames BN (1998) Micronutrients prevent cancer and delay ageing. Toxicol Lett 102/103:5-18

318 Bae SN, Kim J, Lee YS, Kim JD, Kim MY, Park LO (2007) Cytotoxic effect of zinc citrate
319 compound on choriocarcinoma cell lines. Placenta 28:22-30

320 Bae SN, Lee YS, Kim MY, Kim JD, Park LO (2006) Antiproliferative and apoptotic effects
321 of zinc-citrate compound (CIZAR(R)) on human epithelial ovarian cancer cell line,
322 OVCAR-3. Gynecol Oncol 103:127-136

323 Bagchi D, Bagchi M, Stohs SJ (1997) Comparative in vitro oxygen radical scavenging ability
324 of zinc methionine and selected zinc salts and antioxidants. Gen Pharmac 28(1):85-91

325 Bao S, Knoell DL (2006) Zinc modulates airway epithelium susceptibility to death receptor-
326 mediated apoptosis. Am J Physiol Lung Cell Mol Physiol 290:L433–441

327 Bray TM, Bettger WJ (1990) The physiological role of zinc as an antioxidant. Free Rad Biol
328 Med 8:281-291

329 Chang KL, Hung TC, Hsieh BS, Chen YH, Chen TF, Cheng HL (2006) Zinc at
330 pharmacologic concentrations affects cytokine expression and induces apoptosis of human
331 peripheral blood mononuclear cells. Nutrition 22:465-474

332 Chung MJ, Walker PA, Brown RW, Hogstrand C (2005) ZINC-mediated gene expression
333 offers protection against H2O2-induced cytotoxicity. Toxicol Appl Pharmacol 205:225-
334 236

335 Clegg MS, Hanna LA, Niles BJ, Momma TY, Keen CL (2005) Zinc deficiency induced cell
336 death. IUBMB Life 57:661-669

337 Collins A (2004) The comet assay for DNA damage repair: principles, applications, and
338 limitations. Mol Biotechnol 26(3):249-261

339 Dreosti IE (2001) Zinc and the gene. Mutat Res 475:161-167

340 Feliu SM, Piñeiro A, López C, Slobodianik NH (2005) Valores de referencia de cobre, zinc y
341 selenio en niños. Acta bioquím clín latinoam 39:459-462
342 Fenech M (2001) Recommended dietary allowances (RDAs) for genomic stability. Mutat Res
343 480-481:51-54
344 Fenech M (2002) Micronutrients and genomic stability: a new paradigm for recommended
345 dietary allowances (RDAs). Food Chem Toxicol 40(8):1113-1117.
346 Fenech M (2005) The Genome Health Clinic and Genome Health Nutrigenomics concepts:
347 diagnosis and nutritional treatment of genome and epigenome damage on an individual
348 basis. Mutagenesis 20(4):255-269
349 Fenech M (2007) Cytokinesis-block micronucleus cytome assay. Nat Protoc 5:1084-1104
350 Fenech M (2010) Dietary reference values of individual micronutrients and nutriomes for
351 genome damage prevention: current status and a road map to the future. Am J Clin Nutr
352 91:1438S
353 Fenech M (2014) Nutriomes and Personalised Nutrition for DNA Damage Prevention,
354 Telomere Integrity Maintenance and Cancer Growth Control. Cancer Treat Res 159:427-
355 441
356 Fenech M, Ferguson LR (2001) Vitamins/minerals and genomic stability in humans. Mutat
357 Res 475:1-6
358 Grandy G, Weisstaub G, López de Romaña D (2010) Deficiencia de hierro y zinc en niños.
359 Rev Soc Bol Ped 49(1):25-31
360 Haase H, Rink L (2009) The immune system and the impact of zinc during aging. Immun
361 Ageing 6:9
362 Hambidge KM (1989) Mild zinc deficiency in human subjects. In: Mills C.F. (Ed.) Zinc in
363 Human Biology, Springer, London, pp. 281-296

364 Ho E, Ames BN (2002) Low intracellular zinc induces oxidative DNA damage, disrupts p53
365 NFkappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. Proc
366 Natl Acad Sci USA 99:16770-16775
367 Ho E, Courtemanche C, Ames BN (2003) Zinc deficiency induces oxidative DNA damage
368 and increases p53 expression in human lung fibroblasts. J Nutr 133:2543-2548
369 Kimura M, Umegaki K, Higuchi M, Thomas P, Fenech M (2004) Methylenetetrahydrofolate
370 reductase C677T polymorphism, folic acid and riboflavin are important determinants of
371 genome stability in cultured human lymphocytes. J Nutr 134:48-56
372 Końca K, Lankoff A, Banasik A, Lisowska H, Kuszewski T, Góźdź S, Koza Z, Wojcik A
373 (2003) A cross-platform public domain PC image-analysis program for the comet assay.
374 Mutat Res 53:415-420
375 Milne E, Greenop KR, Ramankutty P, Miller M, de Klerk NH, Armstrong BK, Almond T,
376 O'Callaghan NJ, Fenech M (2015) Blood micronutrients and DNA damage in children.
377 Mol Nutr Food Res 59:2057-2065
378 National Research Council (1989) Food and Nutrition Board: Recommended daily dietary
379 allowances, 10th ed. National Academy of Sciences, National Research Council,
380 Washington DC
381 Nygaard SB, Larsen A, Knuhtsen A, Rungby J, Smidt K (2014). Effects of zinc
382 supplementation and zinc chelation on in vitro β-cell function in INS-1E cells. BMC
383 Research Notes 7:84
384 Olive P (1999) DNA damage and repair in individual cells: applications of the comet assay in
385 radiobiology. Int J Radiat Biol 75:395
386 Parry JM (1988) Studies upon the genetic effects of environmental chemicals: the coordinated
387 research programme of the European Economic Community. Mutagenesis 3(2):105-136
388 Prasad AS (2013) Discovery of Human Zinc Deficiency: Its Impact on Human Health and

389 Disease. Adv Nutr 4:176-190
390 Roohani N, Hurrell R, Kelishadi R, Schulin R (2013) Zinc and its importance for human
391 health: An integrative review. J Res Med Sci 18 (2):144-157
392 Sharif R, Thomas P, Zalewski P, Graham RD, Fenech M (2011) The effect of zinc sulphate
393 and zinc carnosine on genome stability and cytotoxicity in the WIL2- NS human
394 lymphoblastoid cell line. Mutat Res 720:22-33
395 Sharif R, Thomas P, Zalewskib P, Fenech M (2012) The role of zinc in genomic stability.
396 Mutat Res 733:111-121
397 Sharif R, Thomas P, Zalewskib P, Fenech M (2015) Zinc supplementation influences
398 genomic stability biomarkers, antioxidant activity, and zinc transporter genes in an elderly
399 Australian population with low zinc status. Mol Nutr Food Res 59:1200-1212
400 Siklar Z, Tuna C, Dallar Y, Tanyer G (2003) Zinc deficiency: a contributing factor of short
401 stature in growth hormone deficient children. J Trop Pediatr 49(3):187-188
402 Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantification
403 of low levels of DNA damage in individual cells. Exp Cell Res 175:184-191
404 Sliwinski T, Czechowska A, Kolodziejczak M, Jajte J, Wisniewska-Jarosinska M, Blasiak J
405 (2009) Zinc salts differentially modulate DNA damage in normal and cancer cells. Cell
406 Biol Int 33:542-547
407 Song Y, Chung CS, Bruno RS, Traber MG, Brown KH, King JC, Ho E (2009) Dietary zinc
408 restriction and repletion affects DNA integrity in healthy men. Am J Clin Nutr 90:321–
409 328
410 Song Y, Elias V, Loban A, Scrimgeour AG, Ho E (2009) Marginal zinc deficiency increases
411 oxidative DNA damage in the prostate after chronic exercise. Free Radic Biol Med 48:82-
412 88

413 Song Y, Leonard SW, Traber MG, Ho E (2009) Zinc deficiency affects DNA damage,
414 oxidative stress, antioxidant defenses, and DNA repair in rats. J Nutr 139:1626-1631
415 Sunderman FW Jr (1995) The influence of zinc on apoptosis. Ann Clin Lab Sci 25:134-142
416 Szuster-Ciesielska A, Stachura A, Slotwinska M, Kaminska T, Sniezko R, Paduch R,
417 Abramczyk D, Filar J, Kandefer-Szerszen M (2000) The inhibitory effect of zinc on
418 cadmium-induced cell apoptosis and reactive oxygen species (ROS) production in cell
419 cultures. Toxicology 145:159-171
420 Tapisso JT, Marques CC, Mathias L, Ramalhinho G (2009) Induction of micronuclei and
421 sister chromatid exchange in bone-marrow cells and abnormalities in sperm of Algerian
422 mice (Musspretus) exposed to cadmium, lead and zinc. Mutat Res 678:59-64
423 Tice R, Strauss G (1995) The single cell gel electrophoresis/comet assay: a potential tool for
424 detecting radiation-induced DNA damage in humans. Stem Cells 13:207-214
425 Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas
426 E, Ryu J, Sasaki Y (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo
427 genetic toxicology testing. Environ Mol Mutagen 35:206-221
428 Varea A, Disalvo L, González H (2006) Repercusión de las deficiencias de micronutrientes en
429 salud pública. Ludovica Pediátrica VII:10-15
430 Wise SS, Holmes AL, Qin Q, Xie H, Katsifis SP, Thompson WD, Wise Sr. JP (2010)
431 Comparative genotoxicity and cytotoxicity of four hexavalent chromium compounds in
432 human bronchial cells. Chem Res Toxicol 23:365-372
433 Wu J, Lyons GH, Graham RD, Fenech M (2009) The effect of selenium, as selenomethionine,
434 on genome stability and cytotoxicity in human lymphocytes measured using the
435 cytokinesis-block micronucleus cytome assay. Mutagenesis 24:225-232
436 Xie H, Holmes AL, Young JL, Qin Q, Joyce K, Pelsue SC, Peng C, Wise SS, Jeevarajan AS,
437 Wallace WT, Hammond D, Wise Sr. JP (2009) Zinc chromate induces chromosome

instability and DNA double strand breaks in human lung cells. Toxicol Appl Pharmacol 234:293-299

Yamaguchi S, Miura C, Kikuchi K, Celino FT, Agusa T, Tanabe S, Miura T (2009) Zinc is an essential trace element for spermatogenesis. Proc Natl Acad Sci USA 106:10859-10864

Yan M, Song Y, Wong CP, Hardin K, Ho E (2008) Zinc deficiency alters DNA damage response genes in normal human prostate epithelial cells. J Nutr 138:667-673

Table 1. Mineral level before and after supplementation with $ZnSO_4$.

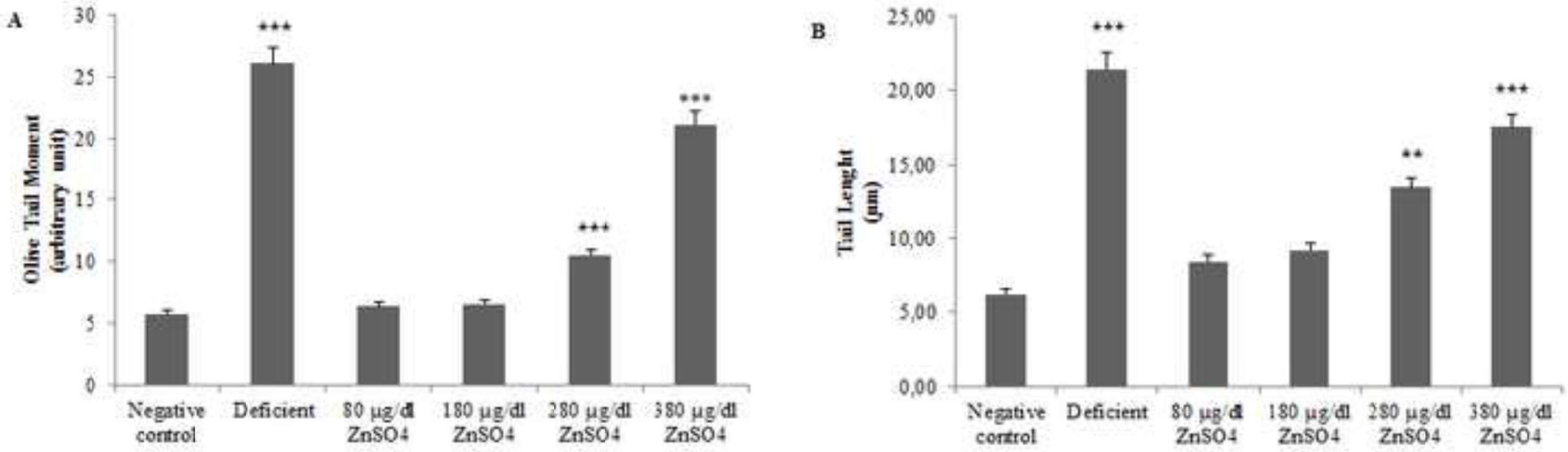
Figure 1. Olive tail moment (A) and tail length (B) in peripheral lymphocytes. Data are means ± SE. Kruskal-Wallis Contrast test through the Statgraphics® 5.1 software was used. Significance for the comparison between the negative control and all the experimental points was marked with asterisks (**$p < 0.01$; *** $p < 0.001$).

Table 2. Nuclear division index, apoptotic cells, necrotic cells, micronuclei, nucleoplasmic bridges and nuclear buds in peripheral lymphocytes.

Data are means ± SE. Groups not sharing the same letter are significantly different from each other (*P* values refer to ANOVA analysis: $p < 0.05$). NDI= nuclear division index; AC= apoptotic cells; NC= necrotic cells; MN= micronuclei; NPB= nucleoplasmic bridges; NBuds= nuclear bud.

Figure

Click here to download Figure Figure 1.tif



Table

	ZnSO4 Level (µg/dl)	
Culture	Before	After
Negative control	60.08 ± 0.00017	60.08 ± 0.00017
Deficient	12.18 ± 0.0004	12.18 ± 0.0004
Lower level	12.18 ± 0.0004	80.05 ± 0.0001
Average level	12.18 ± 0.0004	180. 03 ± 0.00009
Upper limit	12.18 ± 0.0004	280.06 ± 0.0001
Excess	12.18 ± 0.0004	380.01 ± 0.00003

Table

	CBMNcyt assay					
Culture	NDI	AC %	NC %	MNi ‰	NPBs ‰	NBuds ‰
Negative control	1.76 ± 0.13	3.5 ± 0.57a	0.5 ± 0.55a	1.00 ± 0.30a	0.00 ± 0.00	0.00 ± 0.00
Deficient	1.68 ± 0.13	10.5 ± 0.57c	5.5 ± 0.58b	2.10 ± 0.41b	2.30 ± 0.15	0.00 ± 0.00
80 µg/dl ZnSO4	1.78 ± 0.13	2.5 ± 0.56a	1.5 ± 0.57a	1.30 ± 0.34a	6.50 ± 0.25	1.00 ± 0.10
180 µg/dl ZnSO4	1.82 ± 0.13	2.5 ± 0.58a	1.5 ± 1.73a	0.90 ± 0.29a	1.00 ± 0.10	1.00 ± 0.10
280 µg/dl ZnSO4	1.64 ± 0.13	7.5 ± 4.04b	3 ± 2.31a,b	2.10 ± 0.41b	10.0 ± 0.30	2.00 ± 0.14
380 µg/dl ZnSO4	1.52 ± 0.12	11 ± 2.31c	9 ± 3.46c	3.20 ± 0.47c	1.00 ± 0.10	0.00 ± 0.00