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RESEARCH PAPER

Zinc deficiency or excess within the physiological range increases genome instability and cytotoxicity, respectively, in human oral keratinocyte cells

Razinah Sharif · Philip Thomas · Peter Zalewski · Michael Fenech

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Abstract Zinc (Zn) is an essential component of Zn-finger proteins and acts as a cofactor for enzymes required for cellular metabolism and in the maintenance of DNA integrity. The study investigated the genotoxic and cytotoxic effects of Zn deficiency or excess in a primary human oral keratinocyte cell line and determined the optimal concentration of two Zn compounds (Zn Sulphate ($ZnSO_4$) and Zn Carnosine (ZnC)) to minimise DNA damage. Zn-deficient medium (0 μM) was produced using Chelex treatment, and the two Zn compounds $ZnSO_4$ and ZnC were tested at concentrations of 0.0, 0.4, 4.0, 16.0, 32.0 and 100.0 μM. Cell viability was decreased in Zn-depleted cells (0 μM) as well as at 32 μM and 100 μM for both Zn compounds ($P < 0.0001$) as measured via the MTT assay. DNA strand breaks, as measured by the comet assay, were found to be increased in Zn-depleted cells compared with the other treatment groups ($P < 0.05$). The Cytokinesis Block Micronucleus Cytome assay showed a significant increase in the frequency of both apoptotic and necrotic cells under Zn-deficient conditions ($P < 0.05$). Furthermore, elevated frequencies of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBuds) were observed at 0 and 0.4 μM Zn, whereas these biomarkers were minimised for both Zn compounds at 4 and 16 μM Zn ($P < 0.05$), suggesting these concentrations are optimal to maintain genome stability. Expression of PARP, p53 and OGG1 measured by western blotting was increased in Zn-depleted cells indicating that DNA repair mechanisms are activated. These results suggest that maintaining Zn concentrations within the range of 4–16 μM is essential for DNA damage prevention in cultured human oral keratinocytes.

Keywords Zinc · Cytotoxicity · DNA damage · Genomic stability · Human oral keratinocytes · Micronuclei

Abbreviations

HOK	Human oral keratinocytes
CBMN-Cyt	Cytokinesis block micronucleus cytome assay
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MNi	Micronuclei
NPBs	Nucleoplasmic bridges
NBuds	Nuclear buds
Zn	Zinc

R. Sharif · P. Thomas · M. Fenech (✉)
CSIRO Food and Nutritional Sciences,
Adelaide, SA, Australia
e-mail: michael.fenech@csiro.au

R. Sharif · P. Zalewski
School of Medicine, Faculty of Health Sciences,
University of Adelaide, Adelaide, SA, Australia

R. Sharif
Program of Nutrition, School of Healthcare Sciences,
Faculty of Health Sciences, Universiti Kebangsaan Malaysia,
Kuala Lumpur, Malaysia

Introduction

Extensive research has been undertaken to investigate the effect of zinc deficiency on DNA damage events in both in vitro and in vivo systems (Ho and Ames 2002; Ho et al. 2003; Song et al. 2009a; Song et al. 2009b; Song et al. 2009c; Yan et al. 2008). However, little is known on the effect of zinc concentration on epithelial cells within the buccal mucosa. Buccal cells can be easily obtained in a minimally invasive manner and have been used extensively

in human in vivo studies investigating the genotoxic effects of environmental and lifestyle factors (Nersesyan and Chobanyan 2010; Nersesyan et al. 2010; Nersesyan 2006; Nersesyan and Adamyan 2004; Li et al. 1998; Munoz et al. 1987; Piyathilake et al. 1995; Prasad et al. 1995; Ramirez and Saldanha 2002; Thomas et al. 2010). Determining the nutritional requirements for optimal genome integrity in human tissue is a high priority for disease prevention as risk for developmental and degenerative diseases increases with elevated rates of genetic damage (Fenech 2010). However, the mineral micronutrient requirement for DNA damage prevention in ectodermal tissue such as buccal mucosa is virtually unexplored.

In order to better understand the effect of zinc on genome integrity in buccal cells, Human Oral Keratinocyte (HOK) primary cells were used as a model (Mitchell et al. 2010). Oral keratinocytes play a major role in cell protection by providing a major barrier to physical, microbial and chemical agents that may potentially cause local cell injury. Their involvement in proinflammatory processes involve the production of cytokines either constitutively or after a variety of stimuli (Lundqvist et al. 1994), implying that they may potentially participate in controlling oral infections through an inflammatory process involving different interleukins, such as IL-1β and IL-18 (Rouabhia et al. 2002). Oral keratinocytes express a variety of differentiation markers, which are influenced by calcium-induced changes in the transcription of target genes (Presland and Dale 2000). These cells share major structural and functional features as well as same gene expression patterns with the well-characterised dermal keratinocytes and provide a suitable model for cells of the buccal mucosa (Mitchell et al. 2010).

To date, there are no studies on the effect of Zn on genomic stability in primary HOK cells even though Zn is recognised as one of the key minerals that are essential in maintaining DNA integrity (Ho 2004; Dreosti 2001). The Cytokinesis Block Micronucleus Cytome (CBMN-Cyt) assay has been identified as currently the best validated biomarker of DNA damage and cytotoxicity that is sensitive to nutritional status as well as being associated with and predictive of degenerative diseases (Fenech 2010). Thus, this assay was chosen as the primary outcome measure to investigate the potential genomic stability effects of Zn, depending on its concentration and its source compound. To provide some insight into potential molecular mechanisms, the expression of important DNA damage response proteins was measured such as γ-H2AX, Caspase 3, p53, PARP and OGG1 (Ho 2004) and metallothionein was investigated as a biomarker for Zn status (Hambidge 2003).

In this present study, it was hypothesised that both deficiency and excess of Zn could cause DNA damage and cytotoxicity, and the effect of Zn on these DNA damage events may be different depending on the different chemical forms. In addition, we aimed to define the optimal concentrations of Zn for genome stability of cultured human oral keratinocytes and to identify those biomarkers in the CBMN-Cyt assay that are most sensitive to alterations in Zn concentration.

In order to test these hypotheses, two Zn compounds were compared; Zinc Sulphate ($ZnSO_4$) as the most commonly used form of Zn in research studies and Zinc Carnosine (ZnC) as a novel form of Zn that is increasingly being used as a dietary supplement possessing health-promoting effects for gastrointestinal function (Mahmood et al. 2007).

Materials and methods

HOK cell culture and study design

HOK cells are human oral keratinocytes isolated from the normal human oral mucosa and were obtained from the ScienCell Research Laboratories (Cat No 2610; ScienCell, Ca, USA) (Fig. 1). Cells were cultured in Oral Keratinocyte Medium (OKM) which is a complete medium for optimal growth of normal human oral keratinocytes in vitro. OKM consists of 500 ml of basal medium, 5 ml of oral keratinocyte growth supplement (OKGS, Cat. No. 2652 ScienCell Research Laboratories, Ca, USA) and 5 ml of penicillin/streptomycin solution (P/S, Cat. No. 0503, ScienCell Research Laboratories, Ca, USA). The medium is serum-free, HEPES and bicarbonate buffered and has a pH of 7.4. Cells were cultured in T75 cm^2 flasks coated with poly-L-lysine (Sigma, USA) solution in an incubator at 37°C in a 5% CO_2 humidified atmosphere. HOK cells were cultured in 500 μl volumes in 24-well plates (Thermo Fisher Scientific, NY, USA) at an initial density of 2×10^3 cells/ml (based on growth curve—data not shown) for 9 days and the medium was replaced every 3 days (Fig. 2b). Viable cell counts were performed via a trypan blue exclusion assay as described previously (Phillips 1973). For all experiments, cells were cultured in Zn-depleted medium (0 μM) to which $ZnSO_4$ (Sigma Aldrich St. Louis, MO, USA) or ZnC (Hamari Chemicals Osaka, Japan) was added to obtain Zn concentrations of 0.4, 4.0, 16.0, 32.0 and 100.0 μM. Zn-depleted medium was prepared as follows: HOK medium was mixed with 10% Chelex-100 (Sigma, St. Louis, MO, USA) for 2 h and the cycle of depletion was repeated again for another 4 h. The chelex-treated medium was filter-sterilised prior to use in cultures.

The experiments were performed by doing 6 replicate assays or cultures per dose, and the experiments were repeated 6 times to allow an accurate estimate of intra- and inter-experimental variation.

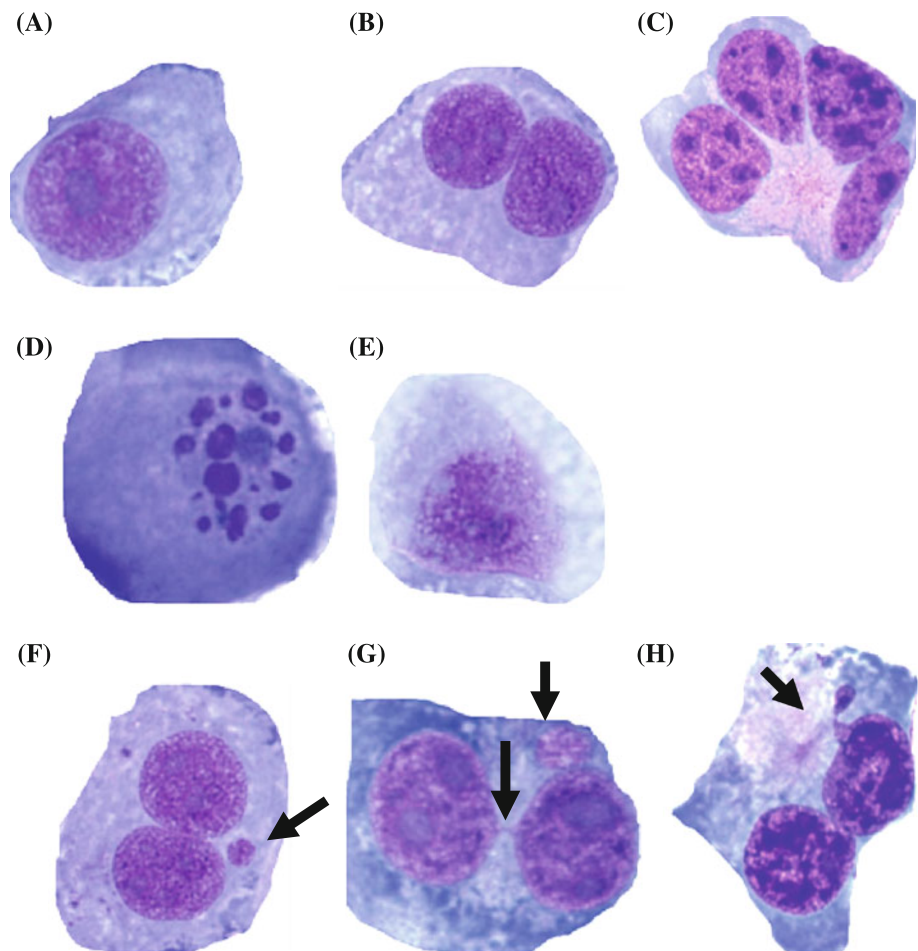

Fig. 1 Photomicrographs of human oral keratinocyte cells scored in the CBMN-Cyt assay (×1,000); **a** mononucleated cell; **b** binucleated cell; **c** multinucleated cell; **d** apoptotic cell; **e** necrotic cell; **f** binucleated cell containing a micronucleus (indicated by *arrow*); **g** binucleated cell containing a nucleoplasmic bridge and a micronucleus (indicated by *arrow*); and **h** binucleated cell containing a nuclear bud (indicated by *arrow*)

Inductively coupled plasma optical emission spectrometry (ICPOES)

ICPOES was used to determine Zn levels both in media and within cells after culturing for 9 days. Analysis was conducted at the Waite Analytical Services (W.A.S.—School of Agriculture and Wine, University of Adelaide). Briefly, either 2 ml of medium or cell pellets (4×10^6 cells) were incubated with 4% nitric acid and hydrogen peroxide, diluted and analysed by ICPOES as described previously (Verbanac et al. 1997). The intra- and inter-assay coefficient of variation (CV) for the Zn measurements was 9.18 and 10.59%, respectively.

MTT cell viability assay

The level of viable cells was measured using the MTT assay as described previously (Mosmann 1983). Briefly, 100 µl of HOK cells (1×10^3 cells/ml) were cultured at different Zn concentrations for 9 days in 96-well plates (Thermo Fisher Scientific, NY, USA) coated with poly-L-Lysine (Sigma, St. Louis, MO, USA). Medium was changed on day 3 and day 6. Ten microlitres of MTT salt solution (5 mg/ml—Sigma, St. Louis, MO, USA) was added on day 9 to each well and incubated for 4 h. Solubilising solution [10% Sodium Dodecyl Sulphate, SDS (Sigma, St. Louis, MO, USA)] in 0.01 M HCl (BDH, Analar, England) was added to the plate and further incubated overnight at 37°C. Absorbance was read with an ELISA microplate reader (SpectraMax 250, Molecular Devices, CA, USA) and the difference in optical density at 650 and 570 nm measured. The intra- and inter-assay coefficient of variation (CV) for the MTT assay was 18.16 and 25.29%, respectively.

Comet assay

The comet assay was used in this study to measure DNA strand breaks and alkaline-labile sites in cells cultured for 9 days. The assay was conducted under alkaline conditions as described previously (Singh et al. 1988; Tice et al. 2000) with slight modification for use with a high throughput CometSlide HT (Trevigen Inc. Cat 4252-02 K-01). Hundred cells were randomly selected from each spot and

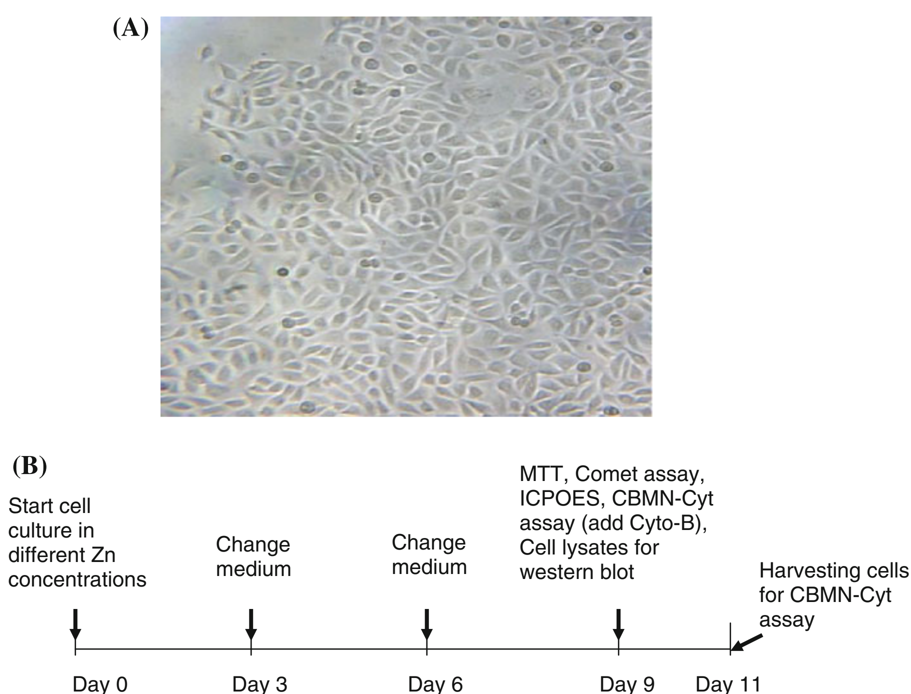

Fig. 2 **a** Photomicrograph of human oral keratinocyte cell line (×40). **b** Schematic diagram for 9-day tissue culture protocol for HOK cells testing for cytotoxic and genotoxic effects of Zn. *HOK* human oral keratinocytes, *MTT* [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; *ICPOES* inductively coupled plasma optical emission spectrometry, *CBMN-Cyt* cytokinesis block micronucleus cytome assay, *Cyto-B* cytochalasin B. The initial concentration of cells was 2×10^3 cells/ml on day 0 and cells were cultured in 500 µl volumes

scored with online software (Tritek—http://autocomet.com/main_home.php) for tail moment and tail intensity. Tail moment (tail length × DNA density) and tail intensity (% DNA in tail) were used as indicators of DNA damage. The intra- and inter-assay CV for the tail moment measured was 21.49 and 32.22%, and for tail intensity was 12.63 and 17.94%, respectively.

CBMN-Cyt assay

In the CBMN-Cyt assay, DNA damage biomarkers are scored in cytokinesis-blocked binucleated cells. The DNA damage biomarkers scored are micronuclei (MNi, a biomarker for whole chromosome loss or chromosome breakage), nucleoplasmic bridges (NPBs, a biomarker of DNA misrepair and/or telomere to telomere end fusions) and nuclear buds (NBuds, a biomarker of gene amplification) (Fenech 2007). Cytochalasin B (Sigma, St. Louis, MO, USA—4.5 µg/ml) was added and cells further incubated for another 48 h (37°C, 5% CO_2). Cells were then harvested onto microscope slides on day 11 using a cytocentrifuge as per the manufacturer's instructions (Shandon Products, UK). Slides were air-dried for 10 min, fixed in Diff-Quik fixative for 10 min and stained using Diff-Quik stains (Lab Aids, Australia).

A total of 3,600 cells were scored per dose (treatment) (100 × 6 slides × 6 experiments) and classified to determine the ratios of mononucleate, binucleate (BN), multinucleate, apoptotic and necrotic cells. These ratios were used to determine the nuclear division index (NDI) which is a biomarker of cytostasis where cytostatic effects are readily estimated from the ratio of mono-, bi- and multinucleated cells. The NDI provides a measure of the proliferative status of the viable cell fraction. It is therefore an indicator of cytostatic effects, and in the case of lymphocytes, it is also a measure of mitogenic response, which is useful as a biomarker of immune function (Fenech 2007). NDI is calculated according to the method of Eastmond and Tucker (Eastmond and Tucker 1989). Five hundred viable cells are scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei and calculate the NDI using the formula $NDI = (M1 + 2M2 + 3M3 + 4M4)/N$, where M1–M4 represent the number of cells with 1–4 nuclei and N is the total number of viable cells scored (excluding necrotic and apoptotic cells). The NDI is a useful parameter for comparing the mitogenic response of lymphocytes and cytostatic effects of agents examined in the assay.

Cytotoxicity events were assessed by the frequency of necrotic and apoptotic cells. A total of 18 000 BN cells per dose (treatment) (500 × 6 slides × 6 experiments) were scored for genome damage indices (MNi, NPBs and NBuds). The scoring criteria for these cells are based on those originally described by (Fenech 2007). Photomicrographs of the different cell types and nuclear anomalies scored in the CBMN-Cyt assay are shown in Fig. 1. The intra-assay and inter-assay CV for CBMN-Cyt assay biomarkers was as follows: Apoptotic cells (23.82, 48.73%); Necrotic cells (31.63, 45.56%); NDI (2.19, 5.23%); MNi

(73.21, 102.58%); NPB (62.61, 86.37%); NBuds (57.13, 91.58%), respectively.

Western blotting

Equal amount of protein lysate (20 mg/lane) was separated using SDS–PAGE on a 4–12% bis–Tris gel (Invitrogen, CA, USA) and transferred to nitrocellulose membrane (Amersham Hybond™ ECL™, GE Healthcare, UK). The membranes were probed with antibodies to specific proteins as follows: mouse anti-poly-ADP-ribose polymerase (PARP; BD Pharmingen, CA, USA), rabbit anti-8-oxoguanine glycosylase (OGG1; Novus Biologicals, USA), mouse anti-p53 (Calbiochem, USA), mouse anti-metallothionein (MT; Dako, Denmark), mouse anti γ-H2AX (Millipore, USA) and mouse anti β-actin (Sigma, St. Louis, MO, USA). Bound antibodies were detected using either goat anti-mouse IgG horseradish peroxidase or goat anti-rabbit IgG horseradish peroxidase (Dako, Denmark) and developed with Amersham™ ECL™ western blotting detection reagent (GE Healthcare, UK). Bands of interest were acquired using luminescent image analyser LAS-4000 (FujiFilm, Tokyo, Japan), and quantification of bands on Western blot membrane was performed using the Multi Gauge version 3.0 program (FujiFilm, Tokyo, Japan). The data are presented as a ratio of protein expression relative to β-actin. The intra-assay and inter-assay CV for protein expression was as follows: Caspase 3 (10.86, 17.76%); Metallothionein (13.34, 18.60%); PARP (14.14, 16.69%); OGG1 (27.39, 35.74%); p53 (7.36, 10.19%); γ-H2AX (17.68, 26.11%), respectively.

Statistical analysis

For each treatment, six replicate measurements were performed within each of six separate experiments performed on separate days ($n = 6$). All end points measured were tested for Gaussian distribution by using the Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* tests for data with Gaussian distribution was performed to compare the effects of different Zn concentrations. The non-parametric Friedman test followed by Dunn's multiple comparison test was used for data that did not exhibit Gaussian distribution. Two-way ANOVA was used in this study to measure the difference in effects between $ZnSO_4$ and ZnC and the % variance for biomarker results that could be explained by Zn concentration and Zn compound used. Data are expressed as mean ± standard error with $P < 0.05$ considered statistically significant. Statistical analyses were performed using Prism 5.0 (GraphPad Inc., San Diego, CA).

Results

Cellular zinc concentrations

Zn-depleted cells showed a significant reduction in cellular Zn levels ($P < 0.0001$) and there was no effect on other divalent metals (Copper and Iron) (Table 1). Cells supplemented with either $ZnSO_4$ or ZnC showed a significant dose-related increase in cellular Zn ($P < 0.0001$) with increased concentration of Zn in medium (Fig. 3a). The increment, however, appeared to be slightly greater for $ZnSO_4$ relative to ZnC based on the observed trends and the associated % variance estimates (effect of type of Zn compound: 1.47%, $P = 0.0004$, effect of concentration: 92.92%, $P < 0.0001$).

Effect of Zn concentrations on cell viability measured via MTT assay

A significant decrease in viable cells in Zn-depleted cultures and with excess Zn ($P < 0.0001$) is shown in Fig. 3b. Cell viability appeared to be optimal between the range 4–6 µM for both ZnC and $ZnSO_4$. At 32 µM, both Zn compounds showed a significant decrease in viable cell number, and at 100 µM, both Zn compounds exhibited severe cytotoxic effects ($P < 0.0001$). Two-way ANOVA analysis showed better viability for cells treated with $ZnSO_4$ compared with ZnC, at 4, 16 and 32 µM ($P < 0.05$), but the greatest % variance was attributable to Zn concentration (effect of type of Zn compound: 9.59%, $P < 0.0001$, effect of concentration: 68.63%, $P < 0.0001$).

Effect of Zn concentrations on DNA strand breaks measured via comet assay

Comet assay was used to determine DNA strand breaks and alkali-labile sites. DNA damage is often associated with cell death; therefore, it is critical that the highest dose tested should not induce excessive cytotoxicity. Hence, treatment with $ZnSO_4$ and ZnC at 100 µM was excluded as cell viability was less than 5%. Two endpoints were measured in this assay: tail moment (TM) and tail intensity (TI). TI represents percentage of DNA in the tail and TM represents a measure of tail length multiplied by the measure of DNA in the tail as a metric for DNA migration (Olive et al. 1990). Figure 4a–d shows a significant increase in both TM and TI for Zn-depleted cells ($P < 0.05$). A reduction in both TM and TI was observed with increasing zinc concentrations. However, at 32 µM, values for TM and TI started to increase, suggesting a U-shaped dose–response curve. There were no significant differences in values for both TM and TI for cells treated

Table 1 Levels of iron and copper for various zinc concentrations

Zinc concentrations	0 μM	ZS 0.4 μM	ZS 4 μM	ZS 16 μM	ZS 32 μM	ZC 0.4 μM	ZC 4 μM	ZC 16 μM	ZC 32 μM
Iron (μg/million cells)	0.08 ± 0.00022	0.078 ± 0.00015	0.08 ± 0.00020	0.081 ± 0.00022	0.08 ± 0.00017	0.08 ± 0.00019	0.082 ± 0.00021	0.08 ± 0.00023	0.081 ± 0.00018
Copper (μg/million cells)	0.19 ± 0.00021	0.20 ± 0.00022	0.21 ± 0.00018	0.22 ± 0.00021	0.20 ± 0.00017	0.20 ± 0.00021	0.21 ± 0.00022	0.21 ± 0.00023	0.20 ± 0.00024

ZS zinc sulphate, *ZC* zinc carnosine

with $ZnSO_4$ or ZnC (Tail moment—effect of type of Zn compound: 0.45%, $P = 0.3950$, effect of concentration: 66.70%, $P < 0.0001$; Tail Intensity—effect of type of Zn compound: 0.01%, $P = 0.9121$, effect of concentration: 55.87%, $P < 0.0001$).

Effect of Zn concentration on baseline levels of cytotoxicity and chromosome damage measured by the CBMN-Cyt assay

Zn-depleted cells showed the highest percentages of necrotic and apoptotic cells ($P < 0.05$) (Fig. 5a–d). Increasing concentrations of Zn showed a reduction in the percentage of both necrotic and apoptotic cells ($P < 0.05$). Figure 5e–f shows a reduction in NDI for Zn-depleted cells ($P < 0.05$), while NDI increased with increasing Zn concentration indicating a cystostatic effect of Zn deficiency. There was no difference in apoptosis, necrosis and cytostasis (NDI) between $ZnSO_4$ and ZnC at any of the concentrations tested (Apoptosis—effect of type of Zn compound: 0.00%, $P = 0.9798$, effect of concentration: 55.73%, $P < 0.0001$; Necrosis—effect of type of Zn compound: 0.06%, $P = 0.8107$, effect of concentration: 49.13%, $P < 0.0001$; NDI—effect of type of Zn compound: 5.27%, $P = 0.0432$, effect of concentration: 31.83%, $P < 0.05$).

In order to measure chromosome damage, MNi, NPBs and NBuds were scored in binucleated cells as shown in Fig. 1. Figure 6a–f shows a significant increase in frequency of MNi and NPBs for Zn-depleted cells ($P < 0.05$). A non-significant increase in NBuds frequency was observed in Zn-depleted cells. Zn-supplemented cells showed a dose-related reduction in DNA damage events compared with Zn-depleted cells ($P < 0.05$). Lowest DNA damage was observed at 4 μM and 16 μM and this trend is similar for both $ZnSO_4$ and ZnC. There were no significant differences in the frequencies of MNi, NPBs, NBuds for cells treated with $ZnSO_4$ and ZnC (MNi—effect of type of Zn compound: 0.66%, $P = 0.4884$; effect of concentration: 30.41%, $P < 0.05$; NPBs—effect of type of Zn compound: 0.08%, $P = 0.8155$, effect of concentration:.26.90%, $P < 0.05$; NBuds—effect of type of Zn compound: 0.14%, $P = 0.7556$, effect of concentration: 26.03%, $P < 0.05$).

Western blot analysis

Western blot was used to measure expression of key proteins involved in the molecular mechanisms by which cells respond to Zn status or genotoxic and cytotoxic events induced by Zn depletion or excess. Higher levels of PARP, OGG1 and p53 were observed in Zn-depleted cells ($P = 0.065$, <0.0001, <0.05, respectively) (Fig. 7a–c);

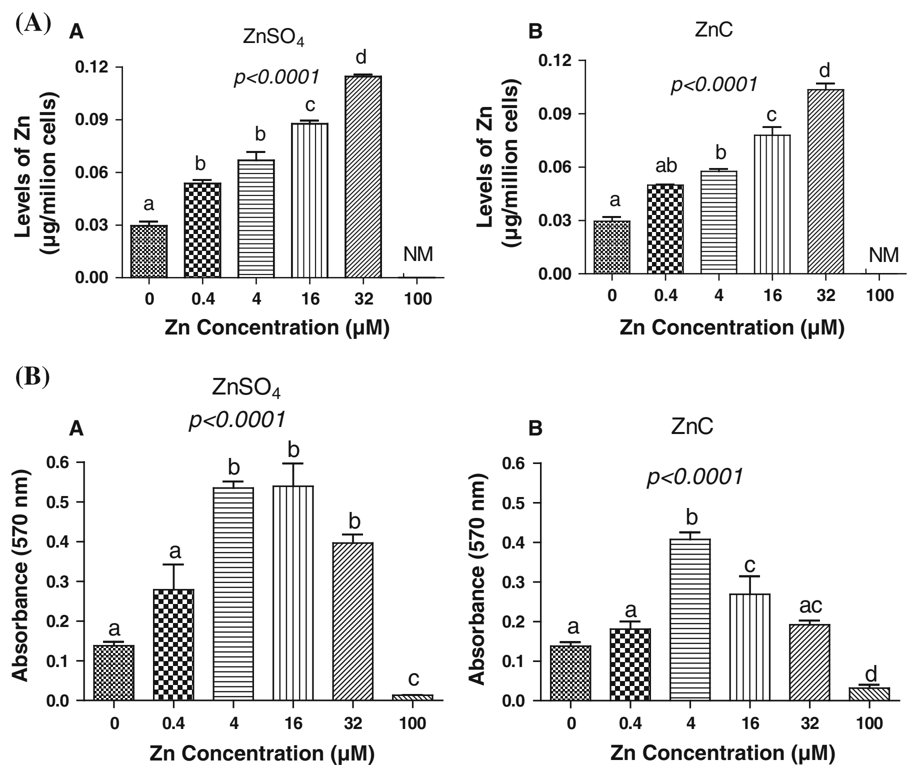

Fig. 3 **a** Concentration of intracellular zinc in HOK cells treated with *a* $ZnSO_4$ and *b* ZnC, at increasing concentrations of Zn. 0 μM represents cells grown in Zn-depleted medium. Groups not sharing the same letter are significantly different to each other (*P* values refer to One-way ANOVA analysis: $P < 0.05$). Results shown are mean ± standard error ($n = 6$). *NM* not measurable. **b** Level of viable cells in cultures treated with *a* $ZnSO_4$ and *b* ZnC at increasing Zn concentration measured via MTT assay. Groups not sharing the same letter are significantly different to each other (*P* values refer to One-way ANOVA analysis: $P < 0.05$). Results shown are mean ± standard error ($n = 6$)

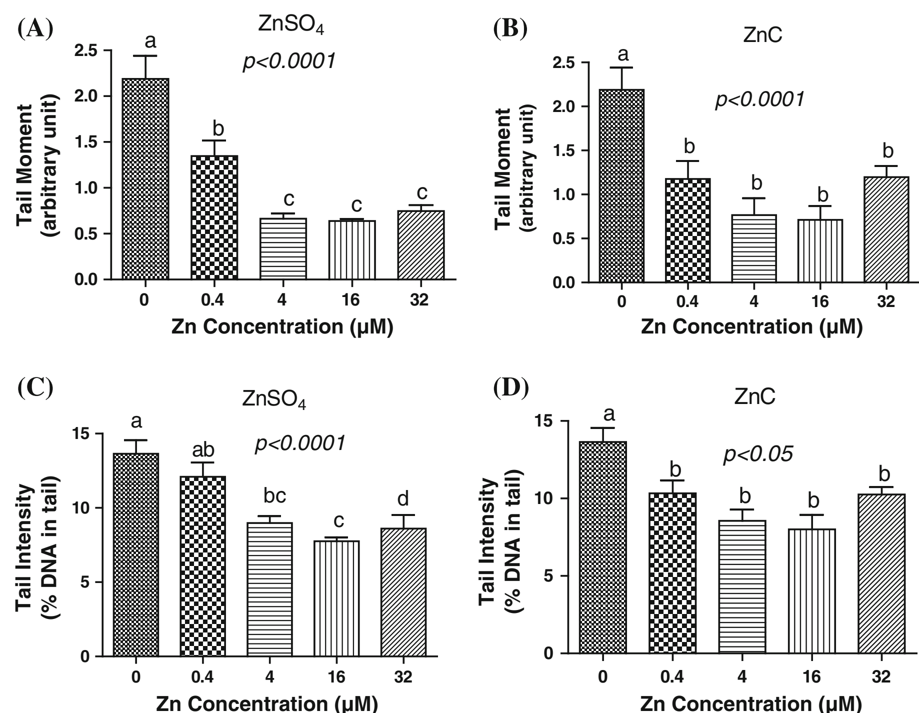

Fig. 4 Tail moment values for cells treated with **a** $ZnSO_4$ and **b** ZnC; Tail intensity values for cells treated with **c** $ZnSO_4$ and **d** ZnC at increasing concentrations of Zn. Groups not sharing the same superscript letter are significantly different to each other (*P* values refer to One-way ANOVA analysis: $P < 0.05$). Results are shown as mean ± standard error ($n = 6$)

however, Zn-supplemented cells did not show any significantly different levels of these proteins at Zn concentrations ranging from 0.4 to 32 μM. Increased levels of γ-H2AX levels were observed in Zn-depleted cells suggesting increased DNA breaks, and γ-H2AX expression was minimised at 4 μM Zn in $ZnSO_4$ cultures ($P < 0.05$) (Fig. 7d). Zn-depleted cells also showed higher expression of Caspase 3 as compared to the other groups ($P < 0.05$)

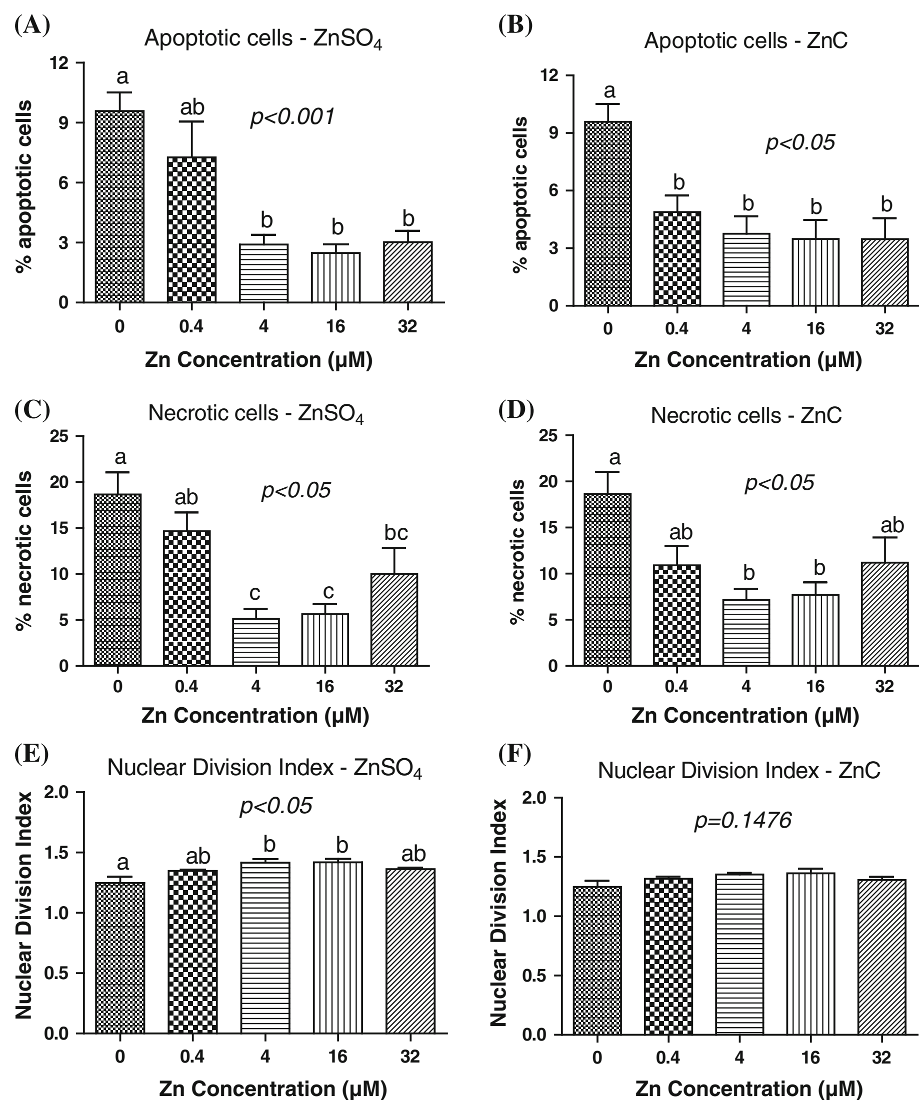

Fig. 5 Cytotoxicity and cytostatic end points with increasing Zn concentration scored using CBMN-Cyt assay in HOK cells on day 11: **a** percentage of apoptotic cells from cultures treated with $ZnSO_4$, **b** percentage of apoptotic cells from cultures treated with ZnC, **c** percentage of necrotic cells from cultures treated with $ZnSO_4$, **d** percentage of necrotic cells from cultures treated with ZnC, **e** NDI of cultures treated with $ZnSO_4$, and **f** NDI of cultures treated with ZnC. Groups not sharing the same letter are significantly different to each other. Results are shown are mean ± standard error ($n = 6$). (P values refer to One-way ANOVA analysis: $P < 0.05$)

(Fig. 7e) indicative of apoptosis activity induced via Caspase 3. Levels of metallothionein (MT) increased in a dose-dependent manner and were significantly higher in cells grown at 16 and 32 μM compared with all other groups confirming the relevance of this biomarker as an indicator of Zn status (Fig. 7f). There was no difference in any of the protein expression biomarkers between $ZnSO_4$ and ZnC at any of the concentrations tested (PARP: effect of type of Zn compound: 1.56%, $P = 0.1794$, effect of concentration: 49.12%, $P < 0.0001$; OGG1: effect of type of Zn compound: 1.92%, $P = 0.0291$, effect of concentration: 70.55%, $P < 0.0001$; p53: effect of type of Zn compound: 0.55%, $P = 0.0004$, effect of concentration: 95.67%, $P < 0.0001$; γ-H2AX—effect of type of Zn compound: 0.15%, $P = 0.6699$, effect of concentration: 59.43%, $P < 0.0001$; Caspase 3: effect of type of Zn compound: 0.10%, $P = 0.5738$, effect of concentration: 81.59%, $P < 0.0001$; MT—effect of type of Zn compound: 0.21%, $P = 0.2059$, effect of concentration: 91.79%, $P < 0.0001$).

Cytotoxicity and genotoxicity effect of HOK cells in optimal medium

Data on HOK cells grown in optimal medium [Oral Keratinocyte Medium (OKM) which is a complete medium for optimal growth of normal human oral keratinocytes in vitro] are presented in Table 2. OKM consists of 500 ml of basal medium, 5 ml of oral keratinocyte growth supplement (OKGS, Cat. No. 2652 ScienCell Research Laboratories, Ca, USA) and 5 ml of penicillin/streptomycin solution (P/S, Cat. No. 0503, ScienCell Research Laboratories, Ca, USA). The optimal culture medium used which consists of basal medium with additional oral keratinocytes growth supplement and penicillin/streptomycin solution contains 4 μM of Zn. Cells grown in optimal medium did not show any cytotoxicity and genotoxicity effects as compared to Zn-depleted cells. The relationship between DNA damage and cells grown in optimal medium was very similar to cells grown in additional 4 μM of Zn.

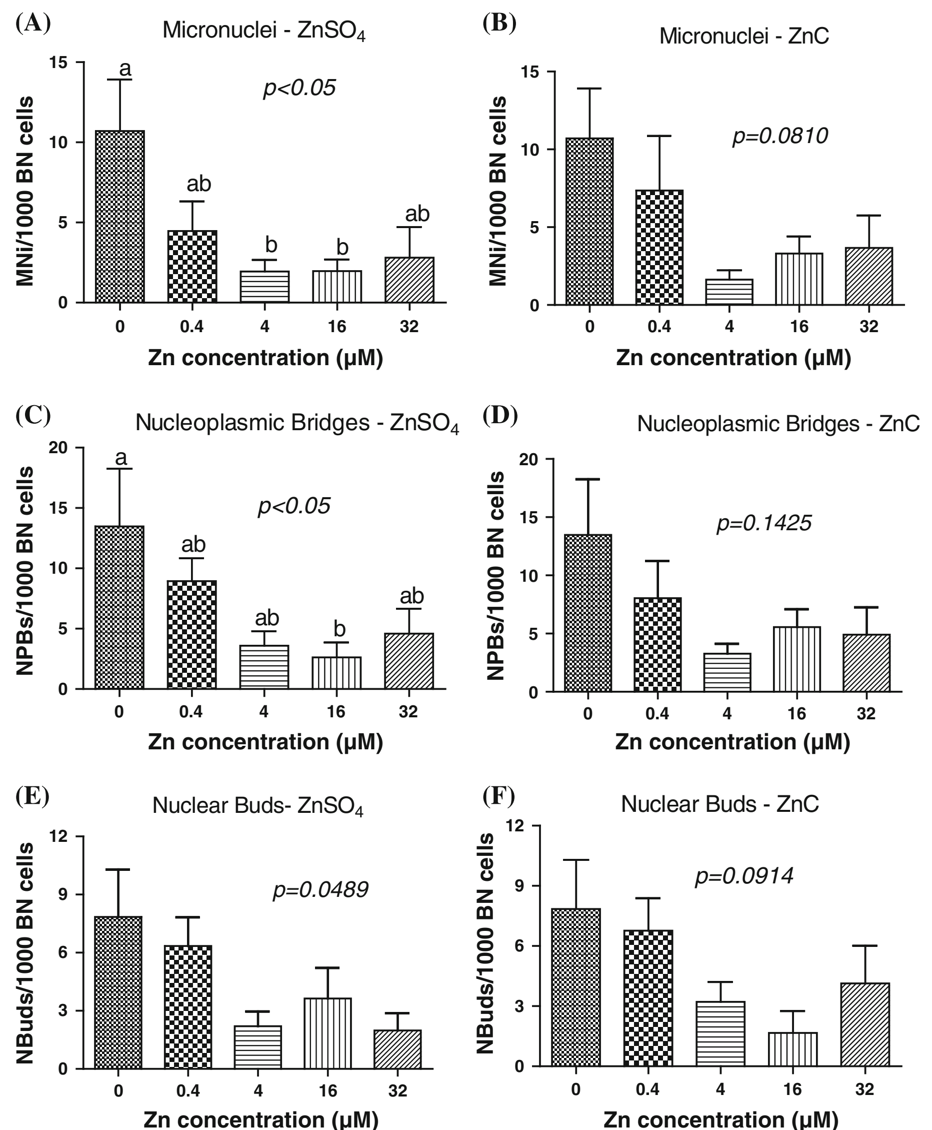

Fig. 6 DNA damage biomarker end points scored using CBMN-Cyt assay in HOK cells on day 11 in relation to increasing Zn concentration: **a** frequency of MNi in 1000 BN cells from cultures treated with $ZnSO_4$, **b** frequency of MNi in 1000 BN cells from cultures treated with ZnC, **c** frequency of NPBs in 1000 BN cells from cultures treated with $ZnSO_4$, **d** frequency of NPBs in 1000 BN cells from cultures treated with ZnC, **e** frequency of NBuds in 1000 BN cells from cultures treated with $ZnSO_4$, and **f** frequency of NBuds in 1000 BN cells from cultures treated with ZnC. Groups not sharing the same letter are significantly different to each other. (*P* values refer to One-way ANOVA analysis: $P < 0.05$). Results shown are mean ± standard error ($n = 6$)

Discussion

The results of this study show that both Zn deficiency and Zn excess increase cytotoxicity and DNA damage events in HOK cells and that Zn deficiency induces the expression of DNA damage response and/or repair proteins. This study highlights the critical role of Zn in genome maintenance and identifies the optimal concentration of Zn required for maintaining genomic stability in human oral keratinocytes.

Bioavailability of Zn is controlled by Zn homoeostatic mechanisms that involve Zn uptake, efflux and Zn distribution in cells (Murgia et al. 2006). When comparing dose–response effects of zinc concentrations in medium and intracellular zinc (Fig. 3) and metallothionein expression (Fig. 7), it is evident that intracellular zinc measurements are more reliable and more specific biomarker of Zn status. In this present study, cellular Zn in cells treated with $ZnSO_4$ was slightly higher compared with cells treated with ZnC. This may be due to the chelating property of carnosine in ZnC that may result in reduced bioavailability compared with $ZnSO_4$ (Sharif et al. 2011). Carnosine from ZnC may have contributed to the protective effects of this compound and could explain why ZnC was as efficacious in preventing DNA damage and cytotoxicity as $ZnSO_4$ despite a lower intracellular Zn concentration. Carnosine is an antioxidant, derived from the amino acids β-histidine and L-alanine, and has been shown to protect against X-ray irradiation and promote wound healing (Klebanov et al. 1998; Hipkiss 1998; Hipkiss and Chana 1998; Hipkiss et al. 1997; Hipkiss et al. 1998). Several potential antioxidant mechanisms which maybe involved include: (a) its chelating action against metal ions; (b) superoxide-dismutase (SOD)-like activity; and (c) ROS and free radical scavenging (Babizhayev et al. 1994; Chan and Decker 1994; Chan et al. 1994). Similarly, it is possible that some observed effects of $ZnSO_4$ may have been influenced not

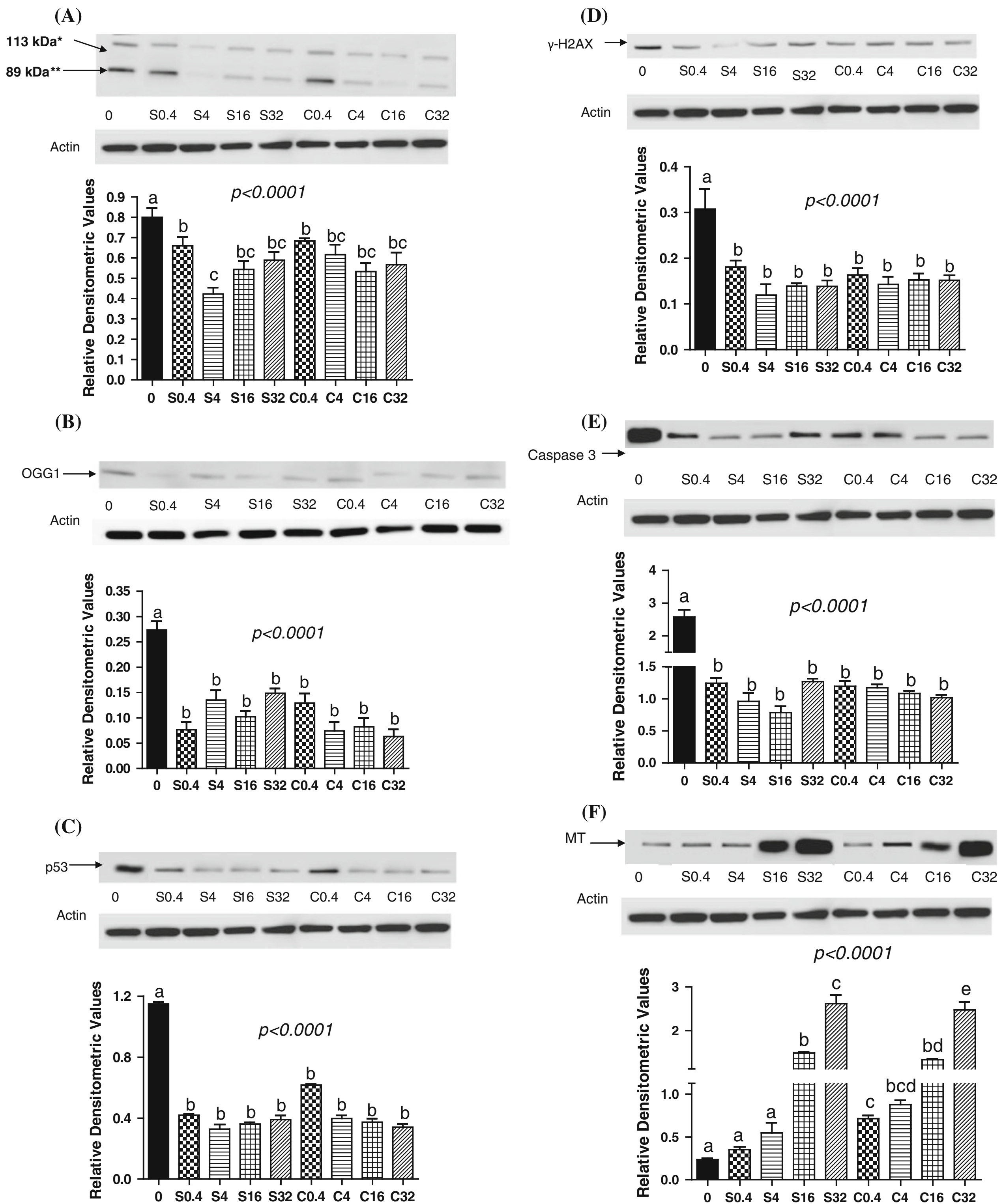

Fig. 7 The effects of zinc concentration in HOK cells on levels of **a** Poly (ADP-ribose) polymerase (PARP); **b** 8-oxoguanine glycosylase (OGG1); **c** p53; **d** γ-H2AX; (**e**) Caspase 3; **f** Metallothionein (MT) expressed in HOK cells following 9 days of culture, as determined by western blot analysis. *S* zinc sulphate, *C* zinc carnosine, 0: Zinc depleted cells, 0.4: 0.4 μM ZnS and C, 4: 4 μM ZnS and C, 16: 16 μM ZnS and C, 32: 32 μM ZnS and C, *intact PARP segments; **cleaved PARP segments. Representative western blot are shown in the inserts. Relative densitometric values are values for each sample divided by *β*-actin. Groups not sharing the same letter are significantly different to each other. (*P* values refer to One-way ANOVA analysis: $P < 0.05$). Results shown are mean ± standard error (n = 6)

Table 2 Levels of zinc, cytotoxicity and genome stability measurements for HOK cells cultured in standard normal medium

Levels of zinc (µg/million cells)	Absorbance (570 nm)	Tail moment (arbitrary unit)	Tail intensity (% DNA in tail)	Apoptotic cells (% apoptotic cells)	Necrotic cells (% necrotic cells)	NDI (NDI indices)	Micronuclei (MNi/1000 BN)	Nucleoplasmic bridges (NPB/1000 BN)	Nuclear buds (NBuds/1000 BN)
0.066 ± 0.004	0.585 ± 0.021	0.629 ± 0.043	8.809 ± 0.407	2.735 ± 0.447	4.780 ± 1.011	1.417 ± 0.029	1.275 ± 0.655	2.920 ± 1.340	1.650 ± 0.793

only by Zn but also by the SO_4^{2-} ion. However, evidence for this possibility is lacking in the literature. The observation that metallothionein expression was not different between ZnC and $ZnSO_4$ suggests that differences in Zn status are unlikely to explain any differences in genotoxicity and cytotoxicity that may be observed with these two Zn compounds. High zinc concentrations may also compete for uptake of other divalent cationic metals causing toxic effects not directly dependent on zinc. However, no significant difference was found in the levels of copper and iron in the different zinc concentrations.

Supplementation with Zn at 4 and 16 µM proved to be most beneficial in reducing genomic instability in vitro. Similar findings were found in a previous study conducted using the WIL2-NS lymphoblastoid cell line using the same protocol and approach (Sharif et al. 2011). Both deficient (Zn-depleted medium, 0 µM) and the higher concentrations of Zn (32 and 100 µM) were found, in both this study on HOK cells and the one on WIL2-NS cells, to induce elevated levels of cell death and DNA strand breaks suggesting a U-shaped dose response in epithelial and lymphoblastoid cells. The physiological range for Zn is reported to be between 2 and 15 µM (Chang et al. 2006). Our data showed that physiological concentrations of Zn had beneficial effect on the genomic stability of HOK cells, whereas at lower concentration of Zn and maximal pharmacologic concentration of 100 µM, Zn appeared to cause severe cellular toxicity. However, this finding is in contrast to that reported by Sliwinski et al. (Sliwinski et al. 2009) who showed that $ZnSO_4$ at 100 µM did not affect the viability of primary human lymphocytes suggesting that sensitivity to Zn toxicity may be dependent on cell type or cell culture conditions. The mechanism of how Zn excess can cause toxicity is still unknown and is likely to be different to that caused by Zn deficiency.

Reduced cell viability was shown in Zn-depleted cells as measured via MTT assay and this is supported by an increase in both apoptotic and necrotic cells, observed via the CBMN-Cyt assay. Low cellular Zn concentration was found to induce programmed cell death in various cell types including fibroblasts, hepatocytes, T-cell precursors, glioma and testicular cells (Ho and Ames 2002; Ho et al. 2003; Yan et al. 2008; Bao and Knoell 2006; Yamaguchi et al. 2009). In addition, Zn depletion has also been associated with caspase activation and increases in apoptosis in airway epithelial cells, lung and hepatocytes (Truong-Tran et al. 2001; Hennig et al. 1999). Under low Zn conditions, Caspase 3 was reported to be involved in apoptosis induction via the intrinsic cell death pathway (Clegg et al. 2005). Our data confirmed that Zn-depleted cells caused an increase in the activation of Caspase 3, in parallel with a higher percentage of apoptotic cells as measured via the CBMN-Cyt assay. Previous literature has shown that ZnC

has a strong cytoprotective effect that might be due to the synergistic effect of Zn and L-carnosine (Nishida et al. 2010; Omatsu et al. 2010). However, there was no difference for the effect of the 2 different Zn forms when 2-way ANOVA analysis was performed.

In this study, we used both the comet assay and CBMN-Cyt assay to obtain more comprehensive data on how zinc affects genome stability. The alkaline comet assay provides a measure of single and double strand breaks in DNA and alkaline-labile abasic sites that are induced but remain unrepaired. In contrast, micronuclei and nucleoplasmic bridges may occur due to mis-repair of DNA strand breaks which leads to the formation of acentric chromosome fragments and dicentric chromosomes (Fenech 2007). An increase in DNA strand breaks was found in Zn-depleted cells, which is consistent with previous studies (Ho and Ames 2002; Ho et al. 2003; Yan et al. 2008; Song et al. 2009a; Song et al. 2009b; Song et al. 2009c; Sharif et al. 2011). Higher concentrations of Zn further reduced DNA strand breaks (4 and 16 μM), but at the highest concentration tested (32 μM), an increase in DNA strand breaks was observed suggesting a potential genotoxic effect for both Zn compounds when present in excess; however, this increase may have also been affected by a contribution of damaged DNA in necrotic cells which tended to increase at 32 μM Zn. However, the mechanism by which Zn excess can cause toxicity remains unclear. Zn-depleted cells also showed higher expression of γ-H2AX which is a sensitive molecular marker for DNA strand breaks (Mah et al. 2010). These results further confirmed the hypothesis that low Zn can cause genome instability via an increase in DNA strand breaks.

In the CBMN-Cyt assay, Zn-depleted cells expressed a higher frequency of MNi, NPBs and NBuds, while Zn-supplemented cells showed significant reductions in DNA damage events ($P < 0.05$). To date, this is the first cytogenetic study to investigate the effects of various concentrations of Zn on MNi, NPBs and NBuds expression simultaneously in HOK cells. The first study using the micronucleus assay, reported in 2001, showed that Zn dimethyl and Zn diisonyldithiocarbamate at 1.53, 15.3 and 153.3 μM did not induce any MNi in human peripheral blood lymphocyte culture (Zenzen et al. 2001). In contrast, Santra et al. (2002) showed that induction of MNi in Zn chloride-treated human lymphocytes at 0.15 and 0.3 mM is significant compared with negative controls, but this did not occur in a dose-dependent manner (Santra et al. 2002). In our recent study, it was found that concentrations of Zn at 4 and 16 μM reduce DNA damage events in WIL2-NS lymphoblastoid cell line relative to lower or higher concentrations (Sharif et al. 2011). This suggests that a narrow physiological range of Zn between 4 and 16 μM is required for optimising chromosomal stability. Another previous investigation conducted in vivo found excess Zn acetate can induce significant increases in both MNi and sister chromatid exchange in bone marrow cells in an Algerian mouse model (Tapisso et al. 2009).

Several mechanisms that could lead to MN formation include simultaneous excision repair of damaged (e.g. 8-oxo-deoxyguanosine) or inappropriate bases (e.g. uracil) incorporated into DNA that are in close proximity and on opposite complementary DNA strands (Fenech et al. 2010). Such simultaneous repair events will lead to DNA double strand breaks and MN formation (Fenech and Crott 2002; Bull and Fenech 2008). In addition, hypomethylation of cytosine in centromeric and pericentromeric repeat sequences can also lead to MN formation (Fenech et al. 2010). There are very few studies that have investigated the relationship between Zn and hypomethylation. However, in one of the previous studies, Zn deficiency was found to reduce the utilisation of methyl groups from SAM in rat liver, resulting in genomic DNA hypomethylation and histone hypomethylation (Duerre and Wallwork 1986; Wallwork and Duerre 1985). This could lead to an increase in MN formation in Zn-depleted cells, as observed in the present study due to whole chromosome loss and malsegregation caused by altered structure of pericentromeric DNA and failure of kinetechore assembly at centromeres (Fenech et al. 2011).

In the current study, it was also found that Zn depletion caused an increase in NPBs which may be due to DNA break mis-repair or telomere end fusion, possibly caused by accelerated telomere shortening or deletion and/or base damage in the telomere sequence (Murnane 2006). Telomere stability has been extensively correlated with the risk of cancer (Callen and Surralles 2004; Engelhardt et al. 1997; Griffith et al. 1999; Meeker 2006; Plentz et al. 2003; Sieglova et al. 2004), and few studies have investigated the relationship of Zn with telomere stability (Nemoto et al. 2000; Bae et al. 2007; Liu et al. 2004). Accumulation of cells with short telomeres, which may lead to telomere end fusions, was found to be associated with reduced Zn status in hypertensive patients (Cipriano et al. 2009). Human Tankyrase 1 (TANK1) that plays a key role in maintaining telomere integrity has a Zn-binding domain in its catalytic site and is therefore dependent on Zn for its function (Lehtio et al. 2008). TANK1 is a member of the growing family of (poly ADP-ribose) polymerases (PARPs) that interacts with ADP-ribosylases the telomere-binding protein TRF1 (Smith et al. 1998). The role of TANK1 involves displacing TRF1 from telomeric DNA and suggests that TANK1 may be a positive regulator of telomere length in telomerase-expressing cells (Smith and de Lange 2000; van Steensel and de Lange 1997). It is plausible that optimal Zn concentrations may reduce genomic instability events and the risk of cancer possibly by enhancing telomere stability

and limiting the incidence of telomere end fusions (Bull and Fenech 2008; Callen and Surralles 2004). This may lead to reduced formation of dicentric chromosomes that are expressed as NPBs (Fenech 2006).

An increase in DNA damage events in Zn-depleted cells may be due to compromised DNA repair functions. In this study, it was found that p53 expression is increased under low Zn conditions, which is consistent with other previous studies (Ho and Ames 2002; Ho et al. 2003; Song et al. 2009c; Yan et al. 2008). p53 is a Zn-finger transcription factor that regulates both G1 and G2 checkpoints and plays a crucial role in regulating DNA repair (Fanzo et al. 2002; Fanzo et al. 2001). It is plausible that low Zn may up-regulate p53 levels via oxidative DNA damage and the induction of ATM kinase, which can be activated by cellular stress and can phosphorylate and stabilise p53 protein (Siliciano et al. 1997). Thus, Zn status can indirectly affect p53 protein levels, yet Zn is also an integral component of p53 at its Zn-finger domain. However, although p53 is activated, other previous studies have found that DNA-binding activity of p53 is impaired by zinc deficiency (Yan et al. 2008; Ho and Ames 2002; Song et al. 2009c). Therefore, p53 which plays the role as a transcription factor may also be dysfunctional when Zn is deficient and may cause activation for gene transcription of some DNA damage response genes to be compromised. Besides p53, we also assessed two other DNA damage response and repair proteins, PARP and OGG1.

Zn is an essential component for PARP-1, which binds via its Zn domain to DNA strand breaks thereby assisting in the recruitment of DNA repair complexes (Mazen et al. 1989). PARP was found to be increased in Zn-depleted cells and this is consistent with another in vivo study that investigated the effect of Zn deficiency in rats (Song et al. 2009c). The induction of PARP under Zn depletion conditions as observed in our study suggests the accumulation of DNA damage at the molecular level (Song et al. 2009c) which coincided with a higher frequency of MNi, NPB and NBuds and higher DNA strand breaks. A positive correlation between cellular poly(ADP-ribosyl)ation and Zn status in human peripheral blood mononuclear cells (Kunzmann et al. 2008) was reported, further indicating that Zn is required for PARP activity.

Zn is also part of OGG1, another Zn-finger protein which is a DNA repair enzyme that is crucial in base excision repair by removing 8-hydroxy-2′-deoxyguanosine, one of the more prevalent oxidative DNA damage events (Boiteux and Radicella 2000). This current study showed that OGG1 expression is significantly increased in Zn-depleted cells suggesting that oxidative stress is one of the mechanisms by which Zn deficiency may cause DNA damage. Song et al. (2009a, b, c) hypothesised that there may be a potential hierarchy of induction of Zn-deficient response proteins when Zn concentration is sub-optimal (Song et al. 2009c), and our previous finding using WIL2-NS cells is consistent with this hypothesis because OGG1 was highly expressed in low Zn but the expression of PARP was only slightly affected in the same conditions (Sharif et al. 2011). However, in this study, the increment in OGG1 and PARP expression in HOK cells in Zn-deficient medium relative to 4 μM Zn was not markedly different.

Besides the involvement of Zn in certain DNA damage responses and DNA repair protein function, an increase in oxidative stress may also contribute to DNA damage events. In this study, the levels of metallothionein (MT) expression were measured to test whether this is one of the possible induced mechanisms that may exert a genome-protective effect (Ho 2004). Zn can modulate the expression of metallothionein that is involved in cellular defence against free radicals and oxidative stress (Dreosti 2001). Our results showed that Zn-depleted cells have a lower MT expression indicating that MT is not involved in protecting against Zn deficiency-induced oxidative stress. Cells with low levels of MT are more susceptible to DNA damage and apoptotic death following exposure to stress stimuli, including oxidative stress, whereas prior induction of MT appears to offer cellular protection against oxidants (Chimienti et al. 2001; Jourdan et al. 2002; Dineley et al. 2003; Maret et al. 2002). These results suggest that DNA damaging effects of Zn deficiency may be exacerbated by lower MT concentration.

In conclusion, both $ZnSO_4$ and ZnC were shown to provide optimal cell viability and minimal DNA damage at 4 and 16 μM Zn concentration. Higher concentrations of Zn (32 and 100 μM) or Zn deficiency ($\leq$0.4 μM) may cause adverse cellular cytotoxic and genotoxic effects, respectively. Although mechanisms of how Zn deficiency can affect genome integrity is becoming clearer with the involvement of Zn in p53, PARP and OGG1, the mechanism by which Zn excess can cause toxicity is still unknown. It is evident that more attention should be given to Zn levels in culture medium which should be carefully maintained at an optimal concentration to maintain genome stability. Future studies should be aimed at determining whether observations for this cell type also apply to other human epithelial cells depending on their genotype for Zn transport, Zn metabolism, Zn-finger protein and enzymes that require Zn as a cofactor.

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Conflict of interest None declared.

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