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Effect of Zinc Oxide and Zinc Chloride on Human and Swine Intestinal Epithelial Cell Lines.

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ABSTRACT.

Zinc (Zn) salts are often used as nutritional additives in order to promote gut health. The aim of the present study was to assess the effect of two widely used additives in feedstuff, on the intestinal epithelium. In particular, the effect of zinc oxide (ZnO) and zinc chloride ($ZnCl_2$) was investigated in human (INT-407) and porcine (IPI-2I) cell line models. The effect of Zn sources on IPI-21 and INT-407 cell lines was evaluated by a colorimetric viability test using an incubation period of 3 and 24 hours under serum-free conditions. INT407 and IPI-2I showed to be a suitable model of the intestine and a simple tool to investigate the role of Zn supplements. INT407 showed to be the most sensible model to Zn supplements considered, whereas IPI-2I were more resistant. The results of this study contribute to determine the role of zinc in human and swine intestinal epithelium. However, further in vivo experiments may be done to clarify the contribution of Zn supplements in gut health and to improve Zn supplementation in animal feed and in human formulations.

1 Introduction

Zinc (Zn) is an abundant trace element in the body recognized as essential micronutrient. At intestinal level, Zn is involved in the modulation of immune system, affecting both non-specific and acquired immunity (Fraker et al., 2000; Frieke, 2000), in mucosal resistance to infection, in the restoration of mucosal barrier integrity and the promotion of antibody production against intestinal pathogens (Sargeant et al., 2011). The absorption of Zn occurs primarily in the small intestine and is influenced by different dietary factors such as the presence of Zn antagonists (e.g. calcium, phytate, fibers). Low molecular weight binding ligands such as citrate, picolinate and aminoacids enhance its absorption (Lönnerdal, 2000). Zn is supplemented in human multivitamin formulations and in animal feedstuff in either inorganic or organic forms. In pig livestock, Zn has been included as nutritional additive at concentration lower than 150ppm as indicated in the Reg. CE 1831/2003. Whereas, pharmacological concentrations of Zn >1000ppm are commonly used as alternative to in-feed antibiotics (Poulsen, 1995). Generally, inorganic Zn salts (e.g. zinc oxide) are the most commonly forms supplemented in animal diets. It has been previously demonstrated that in swine livestock the supplementation of 150ppm of Zinc Oxide (ZnO) improves gut health (Rosselli et al 2005). Also, ZnO at pharmacological concentrations reduces the incidence of diarrohea in the weaned piglets (Owusu-Asiedu et al., 2003). New approaches are necessary to optimize zinc supplementation in feedstuffs in order to improve animal health and to control zinc release in the environment. Moreover, further investigations into the mechanism by which zinc supplementation improves intestinal swine condition may shed light on the role of zinc in human gut level. Studies conducted *in vitro* reported that ZnO inhibits bacterial growth, plays a pivotal role in maintaining epithelial barrier integrity and function, may improve mucosal repair and paracellular permeability (Roselli et al, 2003). Nevertheless, despite the wide use of Zn oxide in in vivo trials, few studies were focused on its effect at cellular level (Sargeant et al., 2010; Sargeant et al., 2011). The importance of intestinal cell models of human and pig origins in *in vitro* platforms for preclinical research is well recognised (Langerholc et al., 2011). In this context, *in vitro* studies can provide evidences regarding the mechanism of action of specific dietary compounds at cellular level, as well as discover any cytotoxic effects of these molecules. Cell culture models have been often used to evaluate the cellular mechanisms of several compounds (Baldi et al, 2004; Rebucci et al, 2007). In addition, *in vitro* animal and human cell models represent a suitable alternative to in vivo animal experiments (Cencič & Langerholc, 2010) for the determination of Zn supplements effects. In light of that, the aim of the present study was to assess the effect of two widely used additives in feedstuff, on the intestinal epithelium. In particular, the effect of ZnO and $ZnCl_2$ was investigated in human (INT-407) and porcine (IPI-2I) cell line models.

2 Materials and Methods

2.1 Cell line and cell culture conditions

The Human embryonic intestinal cell line (INT-407), a non-transformed epithelial cell line, originally derived from ileum of a 2-month-old human embryo, was obtained from the

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American Type Culture Collection. INT-407 were routinely cultivated into 75 cm^2 tissue culture flasks in RPMI-1640 medium supplemented with 200mM glutamine, 1% non-essential aminoacids (NEAA) and 10% Fetal Bovine Serum (FBS). Cells used in this work were between passages 36-40.

The IPI-2I cell line, derived from the ileum of an adult boar and immortalized by transfection with an SV40 plasmid (pSV3) (Kaeffer et al., 1993), was obtained from American Type Culture Collection. IPI-2I were routinely cultivated into 75cm2 tissue culture in DMEM-F12 supplemented with 4mM glutamine, 0.024UI/ml insulin and 10% Fetal Bovine Serum (FBS). Cells used in this work were between passages 5-12.

Cells were cultured in an atmosphere of 5% CO2 at 37 °C until sub-confluence. Cell monolayers were washed with phosphate buffered saline (PBS) and trypsinized with 0.25% trypsin-EDTA. After 48 hours, cells were detached and re-suspended in culture medium to a concentration of 2.5 x 105 cells/ml. Portions (200 µl) of cell suspension were dispensed into sixty wells of a ninety-six-well tissue culture plates.

2.2 Zinc oxide and zinc chloride solutions

A stock solution of 400mM ZnO (Zn content 80.34%) was prepared in 5% of acetic acid. Starting from this stock, ZnO treatment solutions (50, 200, 1000 and 4000 µM) were dissolved in serum-free medium.

A stock solution of $ZnCl_2$ (Zn content 47.97%) was prepared as described in Merck Index instructions (1989). In detail, 1 g of $ZnCl_2$ was dissolved in 0.25 ml of 2% HCl. Starting from this stock, ZnCl2 treatment solutions (50, 200, 1000 and 4000 µM) were dissolved in serum-free medium.

IPI-2I and INT-407 were exposed to treatment solutions in concentration mentioned above (200µl) of ZnO and $ZnCl_2$ for the following 3 and 24h. In particular, for each cell lines we tested four concentrations of Zn sources in triplicate (3 wells per treatment) at two incubation times (3 and 24h). Moreover, at least two independent experiments were performed. The concentrations of ZnO and $ZnCl_2$ were selected on the basis of preliminary assays (data not shown) whereas the times of exposure were selected on the basis of the intestinal transit time in according to Roselli et al. (2003).

2.3 Evaluation of the effect of different zinc sources on cell viability: MTT test

The effect of zinc sources on IPI-21 and INT-407 cell lines was evaluated by MTT test using an incubation period of 3 and 24 hours under serum-free conditions. In particular, after removing the treatment solutions, 150µl MTT stock solution (5mg/ml) in PBS was added to each well and the plates were incubated for 1.5 h in a humidified chamber. The reaction was accomplished by removing the incubation solution and adding 150µl dimethyl sulfoxide to dissolve the formazan. The optical density of dimethyl sulfoxide (540 nm) was determined on a Biorad 680 microplate reader. Cells incubated with culture medium alone representing 100% viability, were included as negative controls in all experiments. This assay measured the production of the chromophore formazan from (4,5 – dimethylthiazol – 2 - yl) - 2,5 -

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diphenyltetrazoliumbromide (MTT) (Sigma-Aldrich). Formazan was produced in viable cells by the mitochondrial enzyme succinate dehydrogenase. The percentage of cell viability induced by treatments was calculated as follows:

$$\%\ \text{cell viability} = \frac{\text{mean optical density of treated cells}}{\text{mean optical density of control cells}} * 100$$

3 Statistical analysis

At least three replicates (3 wells per treatments) at each incubation time were performed and two independent experiment were conducted. The data are presented as means and standard error (SE) and analyzed by one-way ANOVA (SAS, 2010). Duncan's post-hoc multiple range test was used, with $P < 0.05$ considered statistically significant. Different concentrations of the two zinc sources were tested using a model including the systematic effects of the source of zinc (n = 2), concentrations of zinc (four levels). The effect of the assay (n = 2) was included as a blocking factor.

4 Results

INT-407 cells have been exposed to a wide range of ZnO and ZnCl2 concentrations in order to establish the cell viability in response to zinc treatments (figure 1). In particular, INT-407 cell line treated for 3h with 50µM of ZnO increased cell viability up to 120% of the control. The same tendency in term of enhancement of cell viability was observed after 3 and 24h of exposure to 50 µM of $ZnCl_2$. Higher concentrations of ZnO and $ZnCl_2$ have affected cell viability in a dose-dependent way. A significative ($P < 0.05$) reduction of cell viability was observed.

As shown in Figure 2, IPI-2I have shown a different susceptibility to ZnO and ZnCl2 treatment. In particular after 3h of incubation, ZnO did not significantly inhibited IPI-2I cell viability, which remained about 80% at 50, 200 and 1000µM. Whereas, a significative ($P < 0.05$) reduction in IPI-21 cell viability was observed at the highest zinc oxide concentration tested (4000 µM). A significative reduction ($P < 0.05$) was observed at all concentrations of ZnO tested after 24h of incubation. A similar dose and time dependent effect was also observed when IPI-21 cells were treated with increasing concentrations of $ZnCl_2$ for 3 and 24h.

Overall, a dose-response effect was observed after ZnO and $ZnCl_2$ treatments in both the intestinal cell lines considered suggesting that *in vitro* cell models are suitable for studying the effect of zinc additives on cell viability. In particular, it was observed that after 3 hours of incubation, which correspond to the transit time for several nutrients, cell viability was maintained by the lowest ZnO and $ZnCl_2$ presence in the medium.

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Figure 1. Effect of increasing concentrations of ZnO and $ZnCl_2$ on cell viability of INT-407 after 3h and 24h of treatment measured by MTT test. The graph shows the percentages of cell viability over control. Values significantly different from proliferation obtained with 0 µM Zn sources (control medium 100% viability) are indicated: a, $P < 0.05$

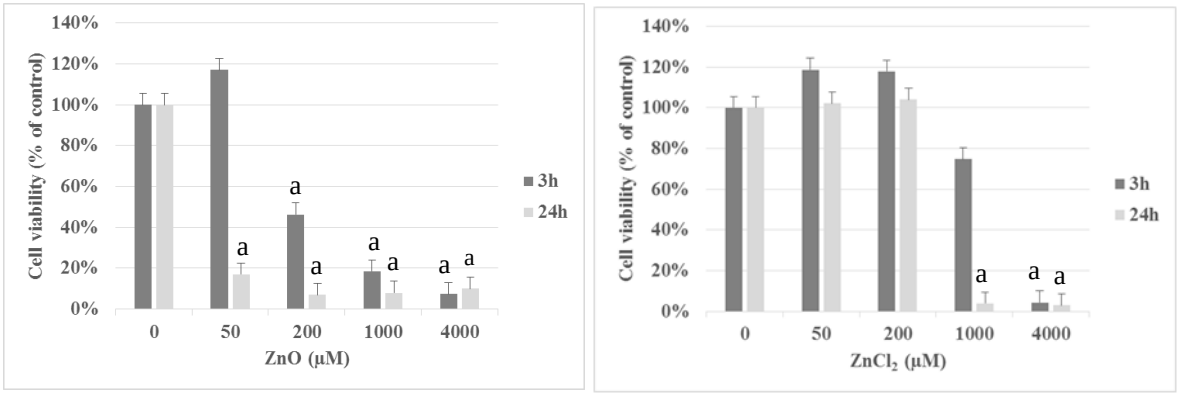
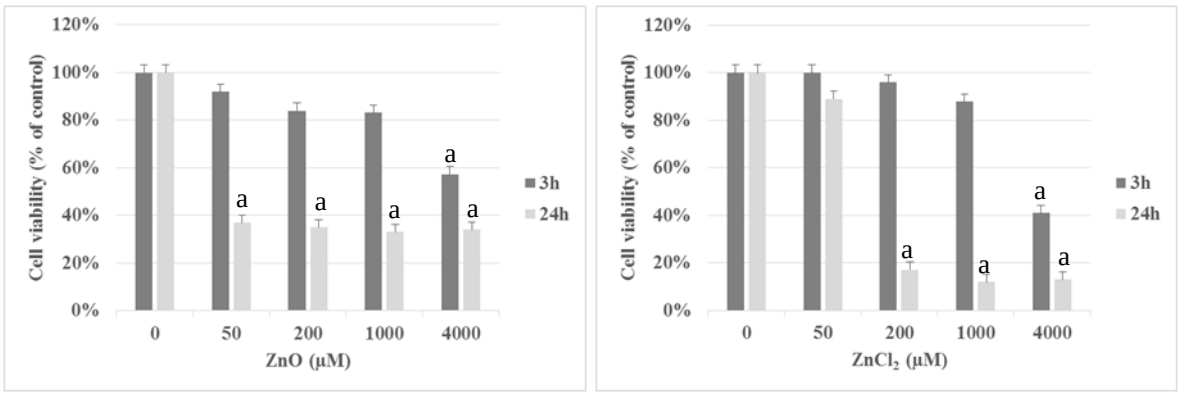


Figure 2. Effect of increasing concentrations of ZnO and ZnCl2 on cell viability of IPI-2I after 3h and 24h of treatment measured by MTT test. Values significantly different from proliferation obtained with 0µM Zn sources (control medium 100% viability) are indicated: a, $P < 0.05$



5 Discussion and Conclusion

Cell models are reaching growing interest among biological studies because of their wide use among the scientific community. They are becoming more realistic and representative of the in vivo environment providing an economic and ethical alternative to in vivo animal testing (Langherolc, 2010). Further, cell culture models meet the EU requirements for the reduction of the number of animal experimental models whenever possible, following the three R paradigm (Reduce, Refine, Replace). Intestinal *in vitro* model are of great importance in food and nutritional research, they represent an essential method to characterize the mechanism of action of several dietary compound in an easy and repeatable way. Since intestinal cell model

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simplify biological systems, they are widely used in toxicological and bioavailability tests of a wide range of dietary compounds, which are inevitable for bringing products to the market (Cencic & Langherolc, 2011).

MTT assay is a viability assay that measures the metabolic activity the cells. MTT assay used in this study represent a simple and useful method to preliminary qualify the effect of a large number of compounds (Zn, tocopherol, probiotics) at cellular level (Baldi et al., 2004; Rebucci et al., 2007). Faller et al. (2002) reported that this assay showed a better correlation with in vivo assay than other bioassays. Cell-based model represents simplify biological system and may be used in the evaluation of nutritional additives effects. In this study, two intestinal epithelial cell lines from different species have been considered. This is essential in order to better characterize the response of dietary compounds on a specific epithelium. Despite the wide use of Zn as animal feed supplement, little studies have focused on its effect *in vitro*. The intestinal cell lines INT-407 and IPI-2I showed to be suitable models of the intestine and represent a simple tool to investigate the role of Zn supplements.

INT-407 showed to be the most sensible model to Zn supplements considered in this study, whereas IPI-2I showed to be the most resistant. Viability of IPI-2I cells is reduced at ZnO and ZnCl2 concentrations >1000uM, whereas Sargeant et al. (2010) found a reduction of IPEC-J2 cell viability at concentrations >100µM of ZnO. However, the lowest concentrations of both ZnO and $ZnCl_2$ considered in this study have maintained the viability of INT-407 and IPI-2I underlining the beneficial role of Zn on human and swine intestine. In particular, it was observed that after 3 hours of incubation, which correspond to the transit time for several nutrients, cell viability was enhanced by the lowest ZnO and $ZnCl_2$ concentrations tested, indicating that these compounds may have a beneficial role for human and swine intestinal epithelium. Overall, these results contribute to determine the role of zinc in human and swine intestinal epithelium. In general, this study confirm that the use of *in vitro* cell-based models for screening the biological activity of single compounds at specific concentrations and in a strictly controlled environment could offer novel insight in the field of human and animal nutrition, making *in vitro* models an essential tool in biological studies. However, cell-based models may not reflect the *in vivo* condition of the intestinal cells in their natural state in the intact organism, where bioavailability, metabolism, binding and transport proteins may influence the biological effect of zinc sources. Therefore, further in vivo experiments are necessary in order to extend the results obtained *in vitro*, to clarify the contribution of Zn supplements in gut health, and to improve zinc supplementation in animal feed and in human formulations.

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