

# Molecular response of 4T1-induced mouse mammary tumours and healthy tissues to zinc treatment

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**Abstract.** Breast cancer patients negative for the nuclear oestrogen receptor  $\alpha$  have a particularly poor prognosis. Therefore, the 4T1 cell line (considered as a triple-negative model) was chosen to induce malignancy in mice. The aim of the present study was to assess if zinc ions, provided in excess, may significantly modify the process of mammary oncogenesis. Zn(II) ions were chosen because of their documented antitumour effects. Zn(II) is also known to induce the expression of metallothioneins (MT) and glutathion (GSH). A total dose of zinc sulphate per one gram of mouse weight used in the experiment was 0.15 mg. We studied the expression of *MT1*, *MT2*, *TP53* and *MTF-1* genes and also examined the effect of the tumour on antioxidant capacity. Tumour-free mice had significantly higher expression levels of the studied genes ( $P < 0.003$ ). Significant differences were also revealed in the gene expression between the tissues ( $P < 0.001$ ). The highest expression levels were observed in the liver. As compared to brain, lung and liver, significantly lower concentrations of MT protein were found in the primary tumour; an inverse trend was observed in the concentration of Zinc(II). In non-tumour mice, the amount of hepatic hydrosulphuryl groups significantly increased by the exposure to Zn(II), but the animals with tumour induction showed no similar trend. The primary tumour size of zinc-treated animals was 20% smaller ( $P = 0.002$ ); however, no significant effect on metastasis progression due to the zinc treatment was discovered. In conclusion, Zn(II) itself may mute the growth of primary breast tumours especially at their early stages.

## Introduction

Breast cancer is one of the most frequent cancers in females worldwide. Patients negative for the nuclear oestrogen receptor ER- $\alpha$  (oestrogen receptor  $\alpha$ ) have a particularly poor prognosis (1). Therefore, we focused on neoplastic processes induced in mice by ER- $\alpha$ -negative tumours. 4T1 cell line was chosen to induce malignancy. This cell line is considered as a triple-negative model. Apart from ER- $\alpha$ , this cell line lacks the protein expression of p53 (2), Wnt-5a and PgR (progesterone receptor) (3).

The 4T1 mammary carcinoma cell line was isolated by Miller *et al* (4) growing as an adherent epithelial type *in vitro*, and are characterized as murine mammary carcinoma cells. When introduced orthotopically into BALB/c mice, 4T1 cells rapidly divide and spontaneously metastasize from the mammary fat pad to several organs including liver, lungs, lymph node and brain, as well as into bones (5). Since these tumours closely imitate advanced human breast cancer, the 4T1 cell line serves as a model for stage IV of breast cancer (6).

Many epidemiological studies have dealt with a close link between higher breast tissue zinc levels and the development of breast cancer (7,8). Compared with the adjacent tissues, the zinc accumulation in the tumour tissue correlated with the higher expression of cellular zinc importing proteins, prompting that tumour cells are profiting from the increase of zinc uptake (9). Furthermore, a low level of zinc efflux transporter ZnT1 was found in breast tumour cells, which caused higher levels of zinc, confirming a deregulation of zinc transport in proliferating tumour cells (10,11). The expression of ZIP6, 7 and 10 (zinc-influx transporters) has been also associated with the breast cancer growth and metastasis (8,12-14). Breast cancer seems to be rare in its acquisition of zinc, indicating a potential zinc demand for the development of breast malignancy (8).

Proper supervision of cytosolic zinc is highly needed for maintaining the redox status of the cells, because both excessive and reduced zinc levels can induce oxidative stress (15).

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As the excessive zinc is toxic, detoxification of this ion has a crucial role in sustaining cell homeostasis.

Zinc and other heavy metals are known to induce the expression of metallothioneins (MT) (16-19). MT expression was shown to be increased during oxidative stress (20,21) to protect cells against cytotoxicity (22,23) and DNA damage (24,25). Metallothionein (MT) was also reported as a possible negative regulator of apoptosis (26). The main transcription factor involved in the metal regulation of MT expression is MRE-binding transcription factor-1 (MTF-1) (27). The free zinc is capable to bind MTF-1 and translocate it to the nucleus (28). In the nucleus, MTF-1 binds to metal responsive elements (MRE) in the MT promoter and triggers the MT expression to recoup for metal toxicity. Nevertheless, some studies have demonstrated that only breast cancer epithelial cells with the intact p53 can induce metallothionein (MT) synthesis after metal exposure (29,30). Accordingly, it was also shown, that a disruption of the p53 function sensitizes breast cancer MCF-7 cells to cisplatin (31).

Consequently, the aim of the present study was to evaluate the effect of excessive zinc supplementation on metallothionein 1 and 2, metal-regulatory transcription factor 1 and TP53 expression in a mouse model with the 4T1-induced advanced breast cancer. The studied mammary tumour arose from 4T1 cells with non-functional p53 and zinc transporters set to zinc accumulation (13,32); thus, the hypothesis of the toxicity of excessive zinc for the tumour tissue was tested. Furthermore, the effect of tumour on the antioxidant capacity of tissues was examined.

## Materials and methods

**Chemical and biochemical reagents.** RPMI-1640 medium, fetal bovine serum (FBS) (mycoplasma-free), penicillin/streptomycin and trypsin were purchased from PAA Laboratories GmbH (Pasching, Austria). Phosphate-buffered saline (PBS) was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Ethylenediaminetetraacetic acid (EDTA), Zinc(II) sulphate (BioReagent grade, suitable for cell cultures), RIPA buffer and all other chemicals of ACS purity were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless noted otherwise.

**Cell culture and culture conditions.** For *in vivo* and *in vitro* studies, the 4T1 cell line was used (cell line was kindly provided by Dr Lucia Knopfova, Masaryk University, Brno, Czech republic). The 4T1 cells grow as adherent epithelial type *in vitro*, and are characterized as murine mammary carcinoma cells [American Type Culture Collection (ATCC) catalogue no. CRL-2539, 2004]. The cells were cultured in RPMI-1640 medium (PAA Laboratories) supplemented with 10% fetal bovine serum (PAA Laboratories), 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, and 4.5 g/l glucose and kept in a 37°C humidified incubator with 5% CO<sub>2</sub> in air.

**Cell number quantification.** Total cell content was analysed using the Casy model TT system (Roche Applied Science, Penzberg, Germany) and the following protocol: first, calibration was performed from viable and necrotic cells. For necrotic cells, 100 µl cell suspension and 800 µl Casy Blue

solution was mixed and left for 5 min at room temperature. Subsequently, 9 ml Casy Tone was added. To prepare a viable cell standard, 100 µl of cell suspension was mixed with 10 ml of Casy Tone. All subsequent measurements were made in 100x diluted 100 µl cell suspension. Prior to each measurement, background was subtracted. All samples were measured in duplicates.

**Zinc(II) treatment of 4T1 cells.** Once the cells grew up to 50-60% confluence in the culture, the growth media were replaced by fresh medium for 24 h to synchronize the cell growth. The 4T1 cells were then treated with zinc sulphate (0-100 µM) in fresh medium for 48 h.

**MTT viability assay.** The MTT assay was used to determine cell viability. The suspension of cells in the growth medium was diluted to a density of 5,000 cells/1 ml and 200 µl were transferred to wells 2-11 of standard microtiter plates. The medium (200 µl) was added to the first and to the last column (1 and 12). The plates were incubated for 2 days at 37°C to ensure the cell growth. The medium was removed from columns 2 through to 11. Columns 3-10 were filled with 200 µl of the medium containing different concentrations of zinc (0, 25, 50, 75 and 100 µM). As a control, columns 2 and 11 were fed with the medium only. The plates were incubated for 24 h; then, the medium was removed and exchanged with a fresh medium, daily three times. After that, columns 1-11 were fed with 200 µl of the medium with 50 µl of MTT (5 mg/ml in PBS) and incubated for 4 h in a humidified atmosphere at 37°C, wrapped in the aluminium foil. After that, the medium-MTT was exchanged with 200 µl of 99.9% DMSO to dissolve MTT-formazan crystals. Then, 25 µl of glycine buffer was added to all wells with DMSO and the absorbance was recorded at 570 nm (VersaMax microplate reader; Molecular Devices, Sunnyvale, CA, USA) (33).

**Real-time impedance based cell growth and proliferation assay.** The impedance-based real-time cell analysis (RTCA) xCELLigence system was used according to the instructions of the supplier (Roche Applied Science and ACEA Biosciences, San Diego, CA, USA). The xCELLigence system consists of four main components: RTCA DP station, RTCA computer with integrated software and disposable E-Plate 16. Firstly, the optimal seeding concentration for proliferation and cytotoxic assay was determined. Optimal response was found in the well containing 10,000 cells. After seeding a total number of cells in 200 µl of medium to each well in E-Plate 16, the attachment and proliferation of the cells were monitored every 15 min. Duration of all experiments was 200 h. Results are expressed as relative impedance using manufacturer's software (Roche Applied Science and ACEA Biosciences) (33).

**Tumour development and zinc supplementation.** Eight-week-old 40 Balb/c female mice (weight 21-24 g) were equally distributed to 2x2 groups according to the tumour presence (tumour/control) and zinc supplementation (supplemented/not supplemented). The 4T1 metastatic breast cancer cell line was used to induce the neoplastic process. The 4T1 cells used in the tumour development study were freshly harvested at 70% confluence. The 4T1 cells (1x10<sup>5</sup>) in 20 µl of PBS and Matrigel

(1:1 ratio) were administered into the fourth mammary fat pad under general anesthesia (1% Narkamon + 2% Rometar 0.5 ml/100 g of weight).

The intraperitoneally administered dose of zinc was a half-LD<sub>50</sub> dose as determined by Domingo *et al* (34) (total dose 0.15 mg/g of zinc sulphate). A total amount of 0.15 mg/g of zinc sulphate divided into 4 doses (0.025 + 0.05 + 0.05 + 0.025 mg/g) was applied to the mice intraperitoneally. The mice were fed *ad libitum*, and observed daily. The cages were kept in a climate-controlled room and cleaned weekly. Tumour volume (length x width<sup>2</sup> x 0.5) was measured twice per week following the equation utilized in similar tumour development studies (35,36). After 1 month, the animals were sacrificed, the tumour weight and volume were measured and the following organs were isolated: primary tumour, brain, liver, lung, spleen and kidney. RNA was isolated by using TriPure reagent (Roche, Boston, MA USA) and qRT-PCR was performed with  $\beta$ -actin as housekeeping gene.

The use of the animals followed the European Community Guidelines as accepted principles for the use of experimental animals. The experiments were performed with the approval of the Ethics Commission at the Faculty of Medicine, Masaryk University, Brno, Czech Republic.

**Tissue sample preparation.** Approximately 0.01 g of the tissue in 500 ml of PBS was mechanically disintegrated using Ultra-Turrax T8 homogenizer (IKA-Werke, Staufen, Germany) placed in an ice bath for 3 min at 25,000 rpm.

The homogenates were kept at 99°C in a thermomixer (Eppendorf 5430; Eppendorf, Hamburg, Germany) for 15 min with shaking. The denatured homogenates were centrifuged at 4°C, 15,000 x g for 30 min (Eppendorf 5402; Eppendorf). Heat treatment effectively denatures and removes thermolabile and high molecular mass proteins from the samples. The prepared samples were used for metallothionein and sulfhydryl group analyses.

**Electrochemical detection of Zinc(II) ions.** Electrochemical analyser Model Metrohm AG (Herisau, Switzerland) was used for the determination of Zn(II). The analyser (757 VA Computrace) employs a conventional three-electrode configuration with the hanging mercury drop electrode (HMDE) as a working electrode: 0.4 mm<sup>2</sup>, Ag/AgCl/3M KCl as a reference electrode and a platinum auxiliary electrode. Differential pulse voltammetric measurements were carried out under the following conditions: deoxygenating with argon for 60 sec; deposition potential: -1.3 V; time of deposition: 240 sec; start potential: -1.3 V; end potential: 0.15 V; pulse amplitude: 0.025 V; pulse time: 0.04 sec; step potential: 5.035 mV; time of step potential: 0.3 sec. For more details see Masarik *et al* (33).

**RNA isolation and reverse transcription.** High pure total-RNA isolation kit (Roche) was used for isolation. The medium was removed and the samples were twice washed with 5 ml of ice-cold PBS. The cells were scraped off, transferred to clean tubes and centrifuged at 20,800 x g for 5 min at 4°C. After this step, a lysis buffer was added and RNA isolation was carried out according to manufacturer's instructions. The isolated RNA was used for cDNA synthesis. RNA (600 ng) was transcribed

using Transcriptor first strand cDNA synthesis kit (Roche), which was applied according to manufacturer's instructions. The cDNA (20  $\mu$ l) prepared from the total-RNA was diluted with RNase-free water to 100  $\mu$ l and the amount of 5  $\mu$ l was directly analysed by using the 7500 RT-PCR system (Applied Biosystems).

**Quantitative real-time polymerase chain reaction.** q-PCR was performed in triplicate using the TaqMan gene expression assay system with the 7500 RT-PCR system (Applied Biosystems) and the amplified DNA was analysed by the comparative Ct method using  $\beta$ -actin as an endogenous control for metallothionein *MT2*, *MT1*, *MTF-1* and *TP53* gene expression quantification. The primer and probe sets for  $\beta$ -actin (assay ID: Mm00607939\_s1), *MT2* (Mm04207591\_g1), *MT1* (Mm00496660\_g1), *TP53* (Mm01731290\_g1) and *MTF-1* (Mm00485274\_m1) were selected from TaqMan gene expression assays (Life Technologies, Waltham, MA, USA). q-PCR was performed under the following amplification conditions: total volume of 20  $\mu$ l, initial incubation at 50°C/2 min followed by denaturation at 95°C/10 min, then 45 cycles at 95°C/15 sec and at 60°C/1 min.

**Electrochemical detection of metallothionein.** Electrochemical detection was used to quantify metallothionein in the thermolysate samples. The detection was carried out using Autolab Analyser (Eco Chemie, Utrecht, The Netherlands) with the classic three-electrode arrangement using differential pulse voltammetry Brdicka reaction. The analysed sample was accumulated on the surface of a working electrode, which was represented by the hanging mercury drop electrode. After the accumulation, the detection proceeded in a supporting electrolyte containing cobaltic (cobalt<sup>3+</sup>) salt in ammonia buffer of pH 9.6 (37).

**Analysis of the total content of sulfhydryl groups.** The concentration of sulfhydryl groups (SH groups) in the tissues was determined using 0.2 mmol/l 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reported by Ellmann (38) (the intra-assay CV was 3.5% and the inter-assay CV was 5.5%). Total sulfhydryl group content values were expressed in  $\mu$ mol/g of proteins.

**Statistical analysis.** The data were tested for normality and the log-normal distributed data were recalculated. Factorial ANOVA and the Bonferroni post-hoc test were used for the statistical analysis. Pearson correlations were performed to reveal dependence between the variables. Software Statistica 12 (StatSoft, Inc., Tulsa, OK, USA) was employed to perform the statistical analysis. P-level of <0.05 was considered statistically significant.

## Results

### *In vitro* effects on the 4T1 cell line

**Effect of zinc(II) on 4T1 viability.** In the first step, an analysis of 4T1 cell line viability after Zinc(II) treatment was performed to adjust the optimal dose administered to animals in the next step. For the proliferation and viability assay, zinc sulphate treatment concentrations were optimized as follows: 0 (control), 25, 50, 75 and 100  $\mu$ M. Consequently, IC<sub>50</sub> of zinc sulphate

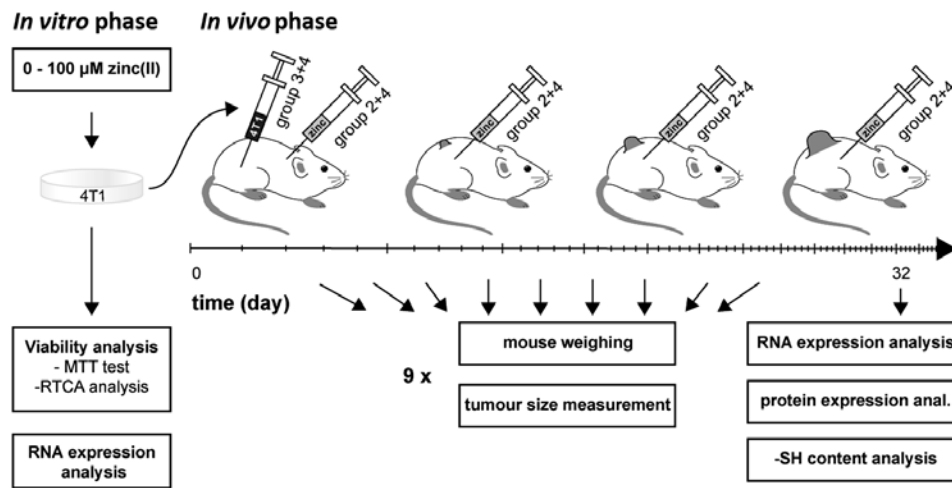


Figure 1. Experimental flow chart. Animals were divided into 4 groups: no zinc treatment, no tumour induction (group 1); zinc-treated, no tumour induction (group 2); no zinc treatment, tumour induction (group 3); zinc treatment and tumour induction (group 4); (group 4 is shown). Zinc was administered in 4 doses; the animals were weighed and the tumour size was measured 9 times during the experiment. After the experiment, selected tissues were analysed.

for the 4T1 cell line was determined. The  $IC_{50}$  value obtained from the MTT cytotoxicity assay was  $83.7 \mu\text{M}$  (Fig. 2A). The  $IC_{50}$  value obtained by using the impedance-based real-time cell analysis (RTCA) system was also determined for the same period of time as the MTT assay (48 h after the beginning of the experiment). The  $IC_{50}$  value was  $52.8 \mu\text{M}$  (Fig. 2B).

**Effect of zinc(II) on 4T1 gene expression.** In the next step, the expression of *MT1* and *MT2* in the 4T1 cell line after the zinc sulphate treatment was detected. The following Zinc(II) concentrations were used to analyse the effect on expression: 0, 25, 50, 75 and  $100 \mu\text{M}$ . Using one-way ANOVA, no significant effect of the 'zinc concentration' factor was determined for *MT1* and *MT2* expression. No correlation between *MT1* or *MT2* expression and zinc sulphate concentration was found (Fig. 2C).

#### Mouse models of breast tumours

**Effect of zinc(II) on animal weight.** Animals were divided into the following groups: i) control, ii) control administered with Zinc(II), iii) 4T1-induced tumour mice, iv) 4T1-induced tumour mice administered with zinc. The experimental scheme is illustrated in Fig. 1. To verify whether the administered Zinc(II) doses cause symptoms of intoxication, the mice were weighed during the experiment. There was no significant trend during the treatment. Additionally, one-way ANOVA did not reveal any significant weight difference between the groups (Fig. 3A). Based on this finding it can be concluded, that Zinc(II) does not have any serious whole-body effects.

**Effect of Zinc(II) on tumour size.** Tumours arose in all animals with the 4T1 breast cancer cell application, and no tumour was found in animals without the 4T1 tumour induction. The autopsy of organs revealed a clear progression of metastases to liver, lungs, bones and spleen with occasional metastases to brain and intestines in both groups (zinc sulphate untreated or treated).

Concurrently, the tumour size was measured during the experiment to verify whether the Zinc(II) administration

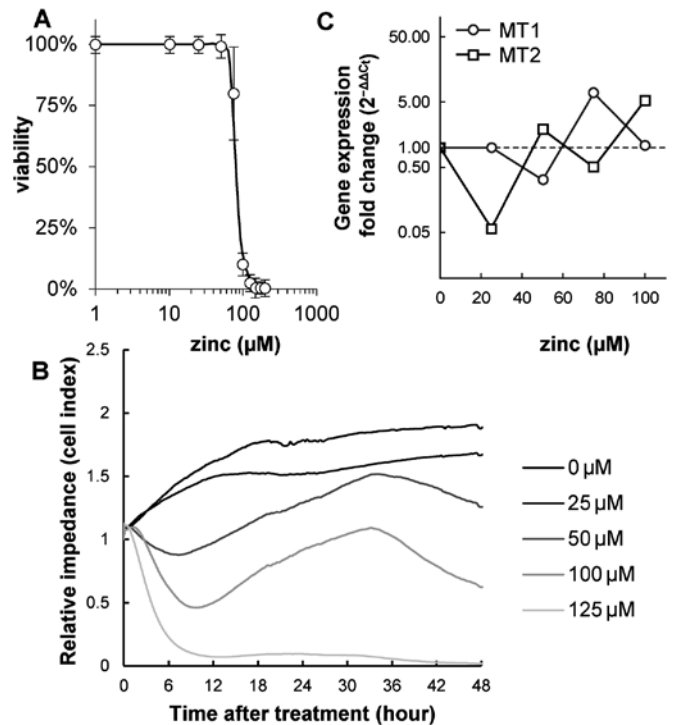


Figure 2. 4T1 cell line analysis. Analysis of viability after Zinc(II) treatment using MTT (A) and real-time impedance-based cell analysis system (B). For  $IC_{50}$  refer to the Results. (C) Analysis of *MT1* and *MT2* gene expression after the zinc treatment. No significant trend was observed.

affects the tumour tissue. Using one-way ANOVA after the adjustment to the treatment time, there was a significant effect of the treatment group,  $F(1, 137)=7.2804$ ,  $P=0.008$ . Tumour reduction up to 28% was observed in the zinc-treated subjects (Fig. 3B).

**Gene expression.** The effect of Zinc(II) supplementation was analysed for association with the gene expression profile of metallothionein 1 and 2 (*MT-1* and *MT-2*), metallothionein transcription factor *MTF-1* and *TP53*. The effect of tumour

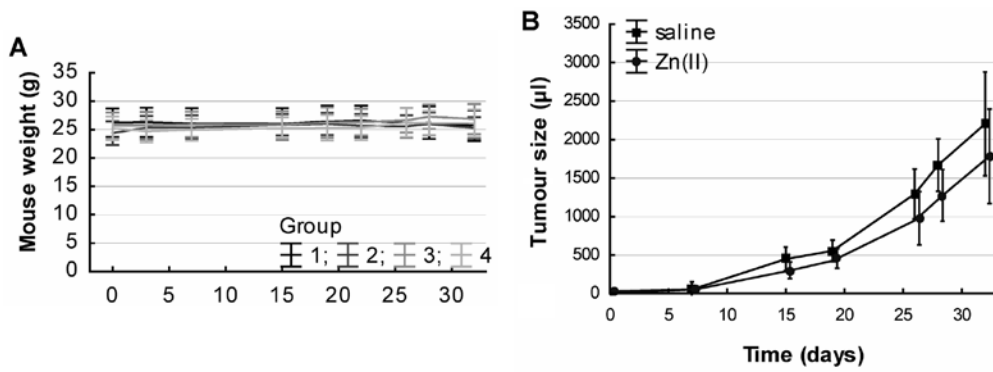


Figure 3. (A) Mouse weight and tumour size (B) measurements. Data are displayed as a mean  $\pm$  SD. Significant difference in the tumour size was observed between groups 3 (tumour induced, no zinc treatment) and 4 (tumour induced, zinc-treated) after the adjustment for time.

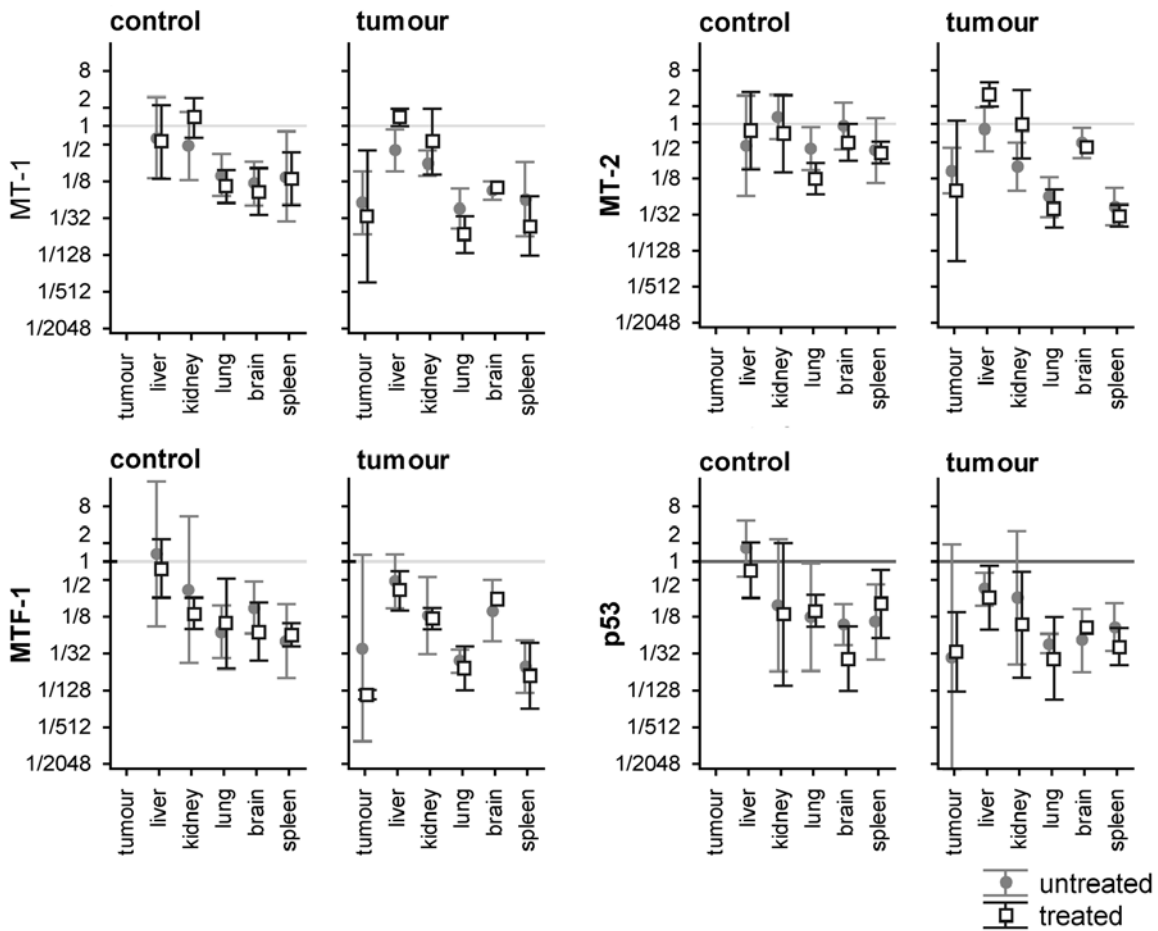


Figure 4. Relative expression levels of genes in selected tissues. Expression related to untreated liver tissue in mice without induced tumours. For details of the statistical significance refer to the Results. Data are the mean and 95% confidence intervals.

induction, Zinc(II) treatment, and tissue type on gene expression was analysed.

Using multivariate ANOVA, we observed a significant effect of the tissue type on the gene expression level,  $F(20, 481.86)=12.96$ ,  $P<0.001$  after the adjustment of all other variables. As expected, the highest expression levels of all the above-mentioned genes were observed in hepatic and kidney tissues. On the other hand, the expression of all genes was significantly lower in the lung tissue, spleen and in the primary tumour as assessed by Bonferroni post-hoc testing.

Using multivariate ANOVA, there was a significant effect of tumour induction on the gene expression level,  $F(4, 145)=11.36$ ,  $P<0.001$  after the adjustment of all other variables; the expression of all of the above-mentioned genes was lower in the tumorous tissues. Consequently, the effect of treatment was analysed using the same test after the adjustment of all other variables. However, the expression of the selected genes did not differ significantly in relation to this factor,  $F(4, 145)=1.40$ ,  $P=0.24$ . Therefore, a combined effect of the Zinc(II) treatment, tumour induction and tissue type was

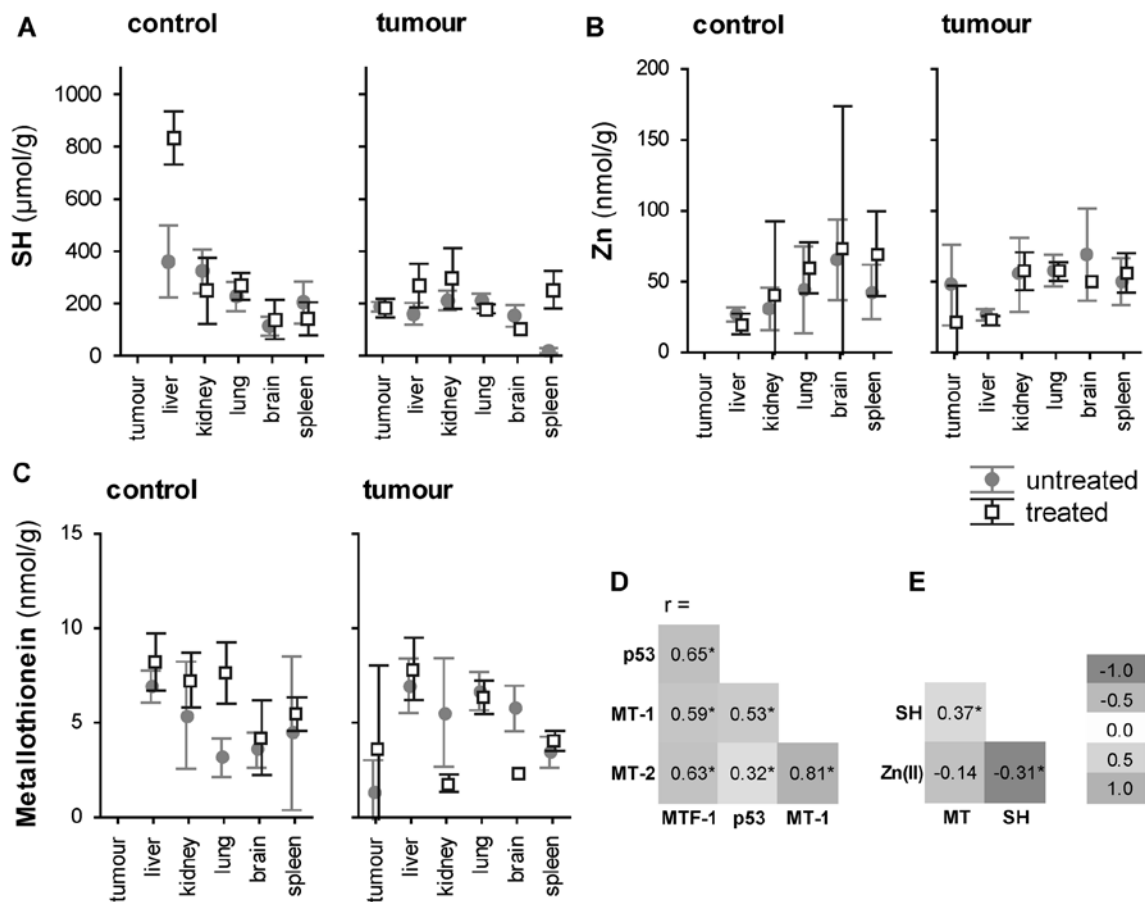


Figure 5. (A) Analysis of sulfhydryl group content, (B) zinc level and (C) metallothionein protein expression in selected tissues. Correlation between parameters at RNA level (D) and (E) correlation between zinc content, metallothionein protein expression and sulfhydryl group content. Data are the means and 95% confidence intervals. Correlations displayed as Pearson  $r$ . Asterisk indicates a significance level at  $P < 0.05$ .

analysed in relation to gene expression to reveal whether the effect of treatment is recorded only in the individual types of tissues or only in the tumour-induced cases.

The ANOVA test revealed, that the expression of genes is significantly affected by the combined effect of Zinc(II) treatment\*tumour induction\*tissue,  $F(20, 481,86)=1.64$ ,  $P=0.04$  (Fig. 4). To reveal the trends of individual genes, the Bonferroni post-hoc test was performed using the 'tissue-by-tissue' manner. There was a strong co-expression pattern of the genes with significant positive correlations; all correlations were significant at  $P < 0.01$  with the highest correlation observed between *MT1* and *MT2* ( $r=0.82$ ) and the lowest (but still significant) correlation observed between *TP53* and *MT2* ( $r=0.32$ ) (Fig. 5D). With regard to the metallothionein expression, significant differences were recorded in the lung tissue; animals with induced tumours had significantly lower *MT1* and *MT2* levels than the controls. While no other 'intra-tissue' trends were observed in the *MT1* isoform, we recorded significant trends of *MT2* levels in the spleen and liver tissue. Similarly as in the lungs, the *MT2* expression was significantly lower in the tumorous tissue than in the control animals. By contrast, an inverse trend was apparent in the liver tissue; the expression of *MT2* in untreated controls was significantly lower than in treated mice with the induced tumours.

In summary, the expression levels of *MT*, *TP53* and *MTF-1* are affected mostly by the tissue type. Tumour induction and Zinc(II) treatment affect the expression of individual genes only, namely *MT1* and *MT2*.

*Analysis of sulfhydryl group compounds, MT protein expression and zinc(II) ions.* Experiments analysing the RNA levels pointed to a significant variation of MT in relation to tumour induction. MTs have a high content of cysteine residues that bind various heavy metals, therefore an analysis of sulfhydryl group content was carried out. The total-SH content does not reflect only MT, but also other redox-active compounds, reduced glutathione (GSH) in particular. Thus, MT was determined electrochemically. In addition, Zinc(II) levels were detected.

A correlation between these variables was determined. While only a weak positive correlation was found between the content of -SH groups and MT ( $r=0.37$  at  $P < 0.001$ ), Zinc(II) correlated with the -SH group content negatively,  $r=-0.31$  at  $P < 0.001$  (Fig. 5E). Thus, -SH and metallothionein were interpreted separately in the following statistical tests.

Similarly to the analysis of gene expression, the effect of tissue on the concentration of sulfhydryl groups (-SH), metallothionein protein (MT) and Zinc(II) was assessed using ANOVA. Similar to the mRNA level, the concentrations of

these substances differed between the tissues. While there were significantly lower concentrations of MT in the primary tumour as compared to brain, lung and liver, an inverse trend was observed in the concentration of Zinc(II): the concentration of Zinc(II) was significantly higher in the tumorous tissue compared to that recorded in the liver.

Consequently, the effect of tumour induction on the level of MT, -SH and zinc was assessed. A significantly 1-5-fold lower -SH content was identified in animals with the induced tumours using post-hoc testing. MT and zinc did not differ significantly.

There was a significant effect of the treatment on the metallothionein protein level, -SH groups and zinc level,  $F(3, 113)=4.90$ ,  $P=0.003$  (Fig. 5A-C). Post-hoc testing revealed a significantly 1.4-fold higher content of -SH groups in treated animals at  $P<0.05$ . This trend was highlighted namely when the combined effect of treatment\*tissue type\*tumour induction on the -SH concentration was analysed (Fig. 5A). On the other hand, the metallothionein protein and zinc levels were also elevated in the treated animals, but below the level of statistical significance.

## Discussion

The aim of the present study was to assess if zinc ions provided in excess may significantly modify the process of mammary oncogenesis in mice. Zn(II) ions were chosen because of their documented antitumour effect and inhibition of copper ions, which are known as pro-oxidant and angiogenic factors (32,39-41). Previously, it was also demonstrated in breast cancer cells that zinc ions can inhibit NF- $\kappa$ B, which is exceedingly activated in breast cancer with a hormone independent phenotype (42,43). Moreover, zinc may have a direct impact on cell death in prostate cancer cells (44). Even physiological concentrations of zinc cause quick discharge of cytochrome *c* from the inner membrane of mitochondria. Thereafter, cytochrome *c* triggers the caspase pathway and activates apoptosis (45). Zinc is also involved in epigenetic mechanisms (46,47). From the epigenetic perspective, zinc deficiency leads to decreased DNA and histone methylation (48), which may be due to the fact that histone deacetylases and histone lysine methyltransferases are zinc-dependent enzymes (49-52). Inhibitors of histone deacetylase were also shown to reactivate the oestrogen receptor in oestrogen receptor-negative breast cancer cells (53). Other effects of zinc ions were expected through the oxidative stress and metallothionein-mediated pathways.

In the present study, the intraperitoneally administered dose of zinc was a half-LD<sub>50</sub> dose as determined by Domingo *et al* (34) (total dose 0.15 mg/g of zinc sulphate). Using different dietary zinc doses at different time periods, Hou *et al* (40) found that doses <1 mg/g do not result in anorexia and body weight loss in mice. Accordingly, zinc doses administered in our study caused no symptoms of intoxication, and there was no body weight loss in the treated mice as compared with the control group. The studied mammary tumour arose from 4T1 cells with non-functional p53 and zinc transporters set to zinc accumulation (13), thus, the hypothesis about the toxicity of excessive zinc for the tumour tissue was tested. Fan and Cherian (29) and Ostrakhovitch *et al* (30) demonstrated

that only breast cancer epithelial cells with the intact p53 can induce metallothionein (MT) synthesis after the metal exposure. In agreement with these findings, no significant changes in *MT1* or *MT2* mRNA expression after the zinc sulphate treatment of the 4T1 cell culture were found. No correlation between *MT1* or *MT2* expression and zinc sulphate concentration was established. Neither was a significant change in *MT1*, *MT2*, *TP53* and *MTF1* gene expression found in the tumour tissue after Zinc(II) exposure. The tumour-free mice had significantly higher expression levels of the studied genes ( $P<0.003$ ).

Significant differences were also revealed among the tissues ( $P<0.001$ ). The highest expression levels were observed in liver, which indicates the important role of this organ in the oxidative stress processes and in the homeostasis of metal ions. In the tumour-free mice, the amount of hepatic sulfhydryl groups was significantly increased by the exposure to zinc sulphate, but animals with tumour induction did not show a similar trend. In contrast, no such trend was observed in metallothionein (compare Fig. 5A and C). The difference as well as the relatively weak correlation between MT and -SH can be elucidated as follows: GSH is an important non-protein -SH compound, together with MT, which includes an unusually high cysteine content, these two -SH substances represent important defence against stress (54). This is why the total -SH content reflects mostly those compounds. Thus, the zinc-induced -SH elevation reflects an increase of GSH content in liver and emphasizes the importance of liver tissue in the regulation of zinc(II).

The above confirms the significant effect of breast tumour on the antioxidant capacity of the whole organism and shows the inability of the exhausted body to cope with oxidative stress conditions. According to Feng *et al* (55) in patients with malignant breast cancer the overall antioxidant/oxidant balance is significantly disrupted as compared with healthy controls. Kasapovic *et al* (56) also showed that breast carcinoma is associated with elevated plasma lipid peroxidation and with a concomitant decrease in the antioxidant capacity of blood cells.

In conformity with our hypothesis, the primary tumour size of zinc-treated animals was 23% smaller  $F(1, 114)=9.71$ ,  $P=0.02$  using ANOVA after adjustment for time; however, no significant effect on metastasis progression due to the zinc treatment was discovered. As a further confirmation of excessive zinc toxicity for breast cancer cells, Al-Qubaisi *et al* (57) found that the IC<sub>50</sub> value of NiZn ferrite nanoparticles in normal breast MCF-10 cells was almost 15 times higher than that in breast cancer MCF-7 cells lines. Surprisingly, no significant changes in the final zinc accumulation between tissues or due to the zinc treatment or tumour bearing were found. Nevertheless, Woo and Wu (32) reported that MNU-induced mammary tumourigenesis resulted in a zinc concentration in the mammary tumour higher than in the mammary gland, whereas the body zinc-distribution profile was not affected. Skrajnowska *et al* (58) demonstrated that rat diet supplemented with zinc or copper increased the Cu content in the cancerous tissue as compared to normal tissue but did not lead to changes in the final Zn levels. We hypothesize that the tumour cells with the highest zinc accumulation succumbed to cell death, which is why the final zinc levels remained unchanged.

In contrast to our study, some other studies have shown that the growth of mammary carcinomas is suppressed rather by zinc depletion (59-61). McQuity *et al* (62) discovered that mammary tumour growth was markedly inhibited in the zinc-deficient group and only slightly inhibited in the zinc-adequate and zinc-excess pair-fed groups of rats. This difference might be due to the different cell lines used in the studies. Pories *et al* (60), Dewys *et al* (61) and McQuity *et al* (62) used Walker 256 cells. Mills *et al* (59) used R323OAC mammary adenocarcinoma cells. Walker 256 cells arose spontaneously in the mammary gland of a pregnant albino rat and were shown to be of hematopoietic (i.e., monocytoid) origin (63). The R323OAC rat mammary adenocarcinoma is a transplantable tumour model, which can be grown *in vitro*, but unlike the 4T1 cells, it does not metastasize spontaneously (64). It seems there are no significant differences between the normal rat mammary tissue and the oestrogen receptor-deficient R323OAC tumour with regard to the number of prolactin binding sites, specificity of binding, or the affinity of the receptor for prolactin (65). In contrast, 4T1 cells represent a typical triple-negative breast cancer cell line (ER, PR, HER2) (66), they are highly metastatic, display predominantly epithelial characteristics, though they also express the mesenchymal marker VIM (67). Similar to human breast cancer, 4T1 metastatic disease spreads from the primary tumour. Also, the progressive dissemination of 4T1 metastases to lymph nodes and other organs is analogous to human breast cancer (68). This evidence indicates that 4T1 should be a more favourable breast cancer model for the assessment of zinc treatment. According to the present study and to the results of other authors (8,69,70), zinc itself may mute the growth of primary breast tumours especially at the early stages.

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