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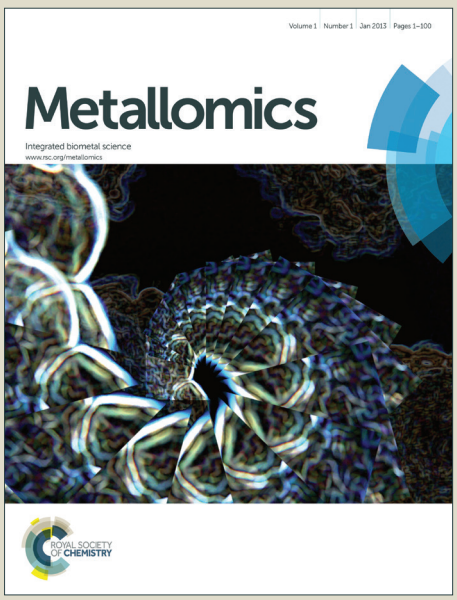

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The biochemical effects of extracellular Zn^{2+} and other metal ions are severely affected by their speciation in cell culture media

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Investigations of physiological and toxicological effects of metal ions are frequently based on *in vitro* cell culture systems, in which cells are incubated with these ions in specialized culture media, instead of their physiological environment. This allows for targeted examination on the cellular or even molecular level. However, it disregards one important aspect, the different metal ion speciation under these conditions. This study explores the role of culture conditions on investigations with zinc ions (Zn^{2+}). Their concentration is buffered by several orders of magnitude by fetal calf serum. Due to the complexity of serum and its many zinc-binding components, zinc speciation in culture media cannot be completey predicted. Still, the primary effect is due to the main Zn^{2+}-binding protein albumin. Buffering reduces the free Zn^{2+} concentration, hereby diminishing its biological effects, such as cytotoxicity and the impact on protein phosphorylation. This is not limited to Zn^{2+}, but is also observed with Ag^{+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, and Ni^{2+}. Usually, the serum content of culture media, and hereby their metal buffering capacity, is only a fraction of that in the physiological cellular environment. This leads to systematic over-estimation of the effects of extracellular metal ions when standard cell culture conditions are used as model systems for assessing potential *in vivo* effects.

Introduction

On average, mammalian cells contain several hundred micromolar of Zn^{2+}, most of it tightly bound to proteins [1]. Frequently, "free" Zn^{2+} is used as an operational term to distinguish an exchangeable pool bound by smaller ligands, such as amino acids, low molecular weight anions, and water, from protein bound Zn^{2+}. Within cells, free Zn^{2+} is thought to serve functions in signal transduction, for example, negatively regulating the activity of protein tyrosine phosphatases (PTP) [2]. Hence, considerable efforts have been made to measure the free intracellular concentrations of Zn^{2+}, which usually lie in the picomolar concentration range [3]. The cellular proteome has a significant buffering capacity for ions such as Zn^{2+} [4]. This is also well documented for the extracellular environment. Metal ions can bind to various extracellular proteins, leading to free concentrations that are orders of magnitude below the total concentration, in particular in the presence of serum or plasma. These contain high concentrations of albumin and several other proteins, which bind transition metal ions [5,6]. For example, next to albumin, which is the major zinc-binding protein in plasma, 12 additional proteins also bind Zn^{2+} [6].

Cell culture-based investigations have been successfully applied for investigating many cellular and molecular mechanisms in heavy metal ion toxicity, including the role of Toll-like receptor 4 in nickel allergy, the immunotoxicity of mercury, and the involvement of Zn^{2+}-toxicity in various pathologies of the central nervous system [7-9]. Still, extracellular metal ion buffering is generally being ignored. In most cases, defined ion concentrations are added to cell cultures, without particular concern regarding the metal ion speciation in the extracellular environment. Nevertheless, it has been shown that protein composition of the culture medium affects the dose-response of Zn^{2+}-induced cytokine secretion in monocytes [10]. In one of the few studies taking into account the speciation of extracellular Zn^{2+}, it was shown that cells die in a narrow concentration range of free Zn^{2+}, which is normally nanomolar [11]. It has been suggested that the free Zn^{2+} concentration is potentially a far more meaningful parameter for describing its biological activity

than the total concentration, and that, analogous to the pH, pZn (the negative decadic logarithm) could be an appropriate measure to describe it [3,11].

This study investigates the impact of metal ion buffering by culture medium on the toxic effects of metal ions, mainly using Zn^{2+} as a model ion. In cell culture media serum levels in general, and albumin in particular, have substantial impact on the toxicity of Zn^{2+}. Under normal culture conditions, the free concentration of this ion is buffered by several orders of magnitude, significantly reducing its toxicity and ability to induce p38 phosphorylation. Accordingly, similar effects on toxicity were observed for several other toxicologically relevant metal ions, namely Cu^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, Ni^{2+}, and Ag^{+}.

Results

Relationship between cytotoxicity and free Zn^{2+}

Culture of Jurkat cells in the presence of different Zn^{2+} concentrations leads to a sharp decline in cellular vitality at concentrations exceeding 100 µM of added Zn^{2+}, with nearly complete toxicity at 250 µM (Fig. 1A). Despite the addition of up to 250 µM Zn^{2+}, free Zn^{2+} in the culture medium remains below 30 nM, being buffered by nearly four orders of magnitude (Fig. 1B). The loss of cellular vitality coincides with a steep increase of free medium Zn^{2+}, indicating the end of a limited buffering capacity of the culture medium, with subsequent uptake into the cells. Accordingly, free intracellular Zn^{2+} is significantly elevated under these conditions (Fig. 1C), even though the free intracellular concentration remains below 4 nM. Notably, Zn^{2+} buffering by culture medium (in the absence of cells) seems to have a component with slow kinetics. There still is a considerable decline of free Zn^{2+} between 4h and 8h, with no further major reduction between 8h and 24h (Fig. 1D).

Fetal calf serum (FCS) as a Zn^{2+}-buffer

Zn^{2+} - buffering in the culture medium is likely mediated by Zn^{2+}-binding serum proteins. Consequently, the lethal concentration (LC_{50}) is reduced from 154.5 µM at 10% FCS (Fig. 1A) to 82.8 µM in the presence of 1% FCS (Fig. 2A). The effect of buffering is not limited to cytotoxicity. Zn^{2+} inhibits PTPs, hereby activating phosphorylation of kinases such as the mitogen-activated protein kinase (MAPK) p38. Western blot analysis shows increased p38 phosphorylation after addition of 100 and 300 µM Zn^{2+} in medium containing 1% FCS, whereas in the presence of 10% FCS, there is only weak phosphorylation at 300 µM (Fig. 2B,C). According to a two-way ANOVA of the densitometric quantification, the concentrations of Zn^{2+} and FCS are both extremely significant factors affecting the phosphorylation of p38 ($p<0.0001$).

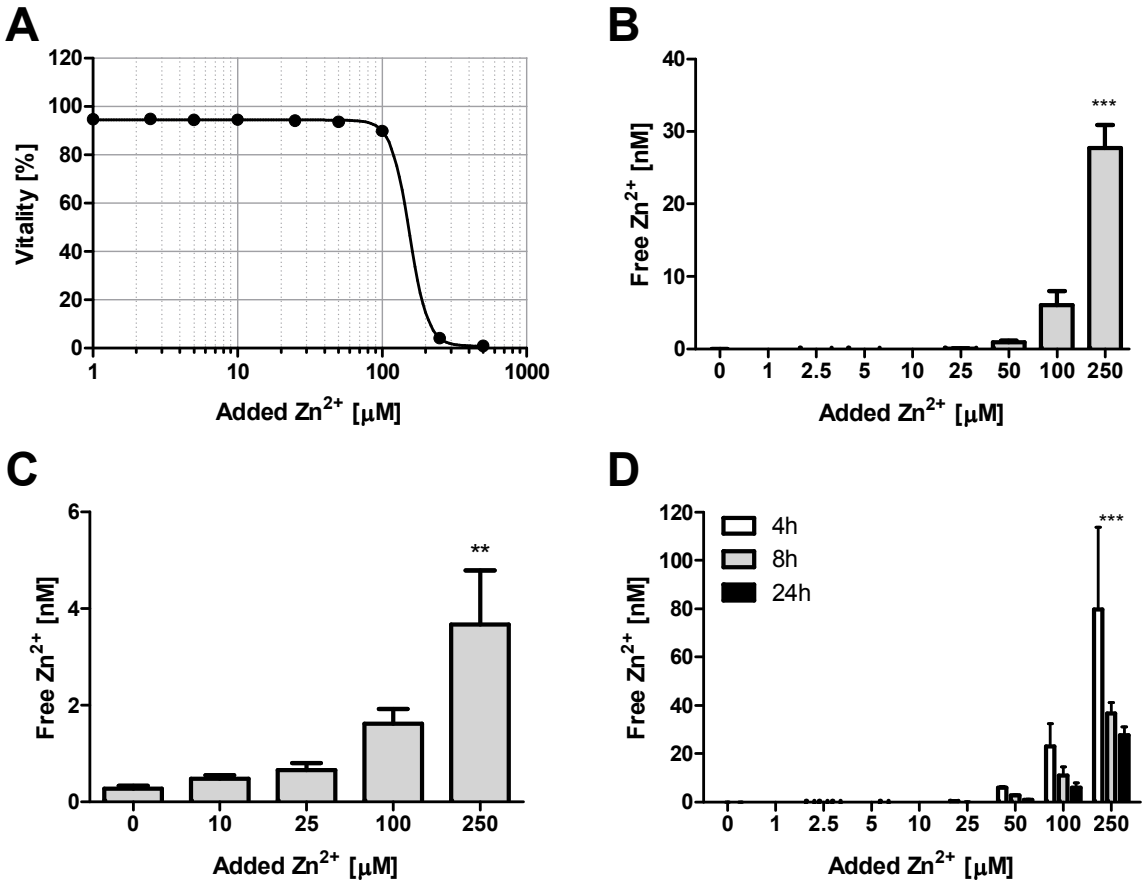

Figure 1: Toxicity and free Zn^{2+} under normal culture conditions. RPMI 1640 cell culture medium containing 10% FCS was supplemented with the indicated concentrations of $ZnSO_4$. (A) After incubation for 24h, the vitality of Jurkat cells was measured by staining with propidium iodide and flow cytometry. A sigmoidal dose-response curve was fitted by non-linear regression. (B) Free Zn^{2+} concentration in cell-free culture medium after 24h, measured with FluoZin-3. (C) Free intracellular Zn^{2+} concentration, measured in Jurkat cells incubated with the indicated concentrations of Zn^{2+} for 2h in normal culture medium and loaded with FluoZin-3. (D) Free Zn^{2+} concentration in media incubated for 4h, 8h, and 24h. All data are shown as means of n=3 independent experiments +SEM. Means significantly different to the untreated controls are indicated (**= $p<0.01$; *** = $p<0.001$; ANOVA with Bonferroni post hoc test).

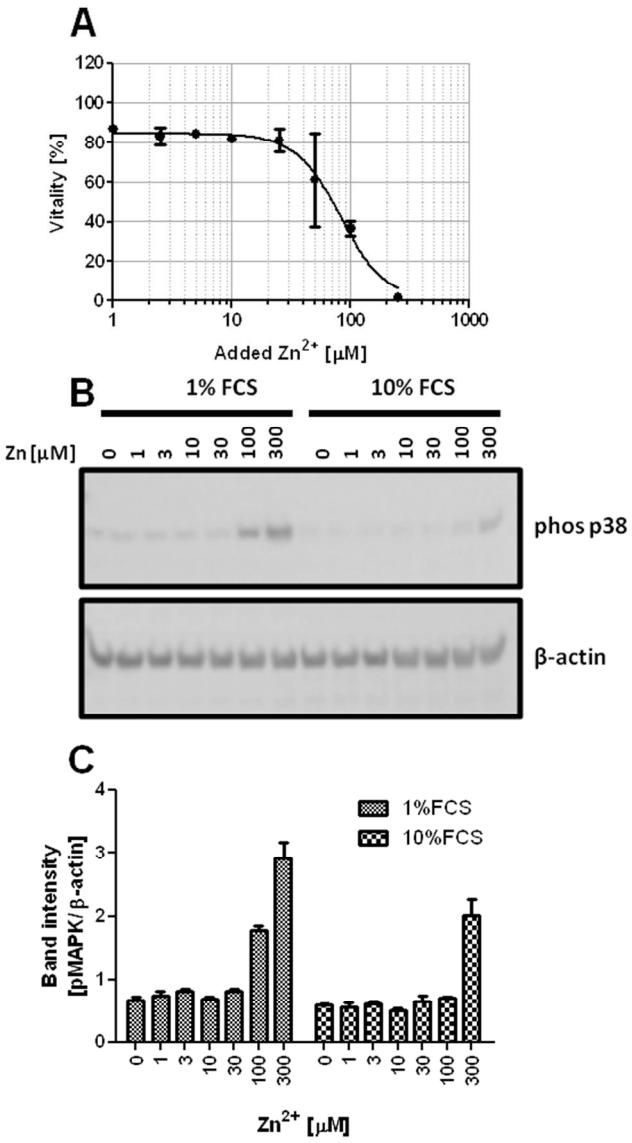

Figure 2: Impact of FCS on Zn^{2+}-mediated p38 activation. Jurkat cells were incubated with the indicated concentrations of $ZnSO_4$. (A) Vitality of cells after 24h incubation in RPMI 1640 cell culture medium containing 1% FCS. A sigmoidal dose-response curve was fitted by non-linear regression. (B,C) Western blot of cells incubated for 30 min either in RPMI 1640 cell culture medium containing 1% or 10% FCS. Data are shown as one representative blot (B) or densitometric quantification (C) of n=3 independent experiments.

Reduced toxicity of Zn^{2+} in the presence of higher FCS concentrations is not limited to Jurkat cells. Three other cell lines, Raw 264.7 (Fig. 3A), BV-2 (Fig. 3B), and L929 (Fig. 3C), showed an equivalent effect when Zn^{2+} toxicity was compared in the presence of 1% and 10% FCS.

To investigate a potential impact of FCS on Zn^{2+} uptake, free intracellular Zn^{2+} was measured in Raw 264.7 cells loaded with FluoZin-3. In the absence of serum, the addition of 1 µM Zn^{2+} causes a transient spike to approximately 75 nM free intracellular Zn^{2+} (Fig. 4A), whereas 1% FCS reduces the effect to 1 nM (Fig. 4B), and it is almost completely abrogated by 10% FCS (Fig. 4C).

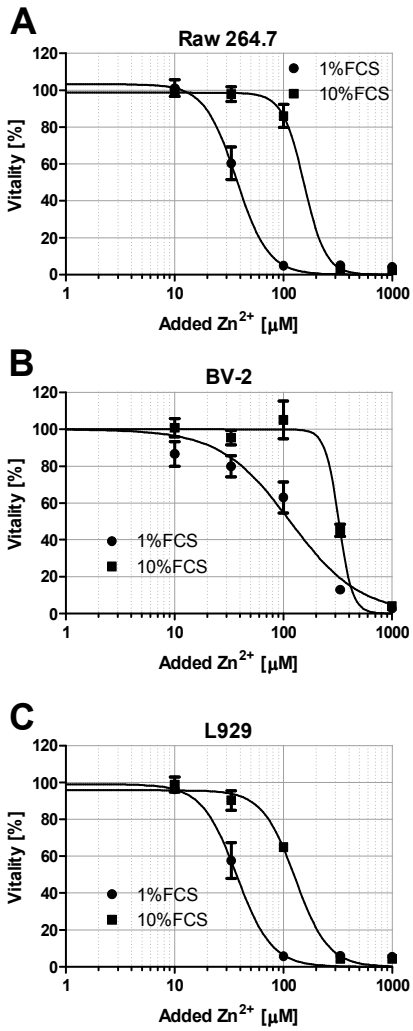

Figure 3: Impact of serum on Zn^{2+} toxicity in different cell lines. Raw 264.7 (A), BV-2 (B), and L929 (C) cells were cultivated for 24h in their respective culture media, supplemented with either 1% or 10% FCS, in the presence of the indicated concentrations of Zn^{2+}. Cellular vitality was determined using the MTT assay and sigmoidal dose-response curves were fitted by non-linear regression. All data are shown as means ± SEM of n=3 independent experiments.

Zn^{2+} buffering by albumin

Serum albumin is the major Zn^{2+}-binding protein in serum [5]. Addition of a physiological concentration of BSA (50 mg/ml, corresponding to approximately 750 µM) to serum-free culture medium abrogates Zn^{2+} toxicity in Raw 264.7 cells (Fig. 5A). In contrast, addition of Na_2HPO_4 (10 mM, triplicating the medium's concentration of phosphate), which can form Zn^{2+}-complexes with low solubility [15], has no effect (Fig. 5A). Cell-free measurements confirm a concentration-dependent buffering of free Zn^{2+} by purified BSA (Fig. 5B). FCS contains growth factors, serving as signals for cellular survival and division [16]. The absence of these factors could render the cells more susceptible to Zn^{2+} toxicity, independently from buffering of the metal ion. However, in the presence of 10% FCS, addition of BSA still reduces Zn^{2+} toxicity (Fig. 5C), indicating that this effect is also observed when the concentration of growth factors remains unchanged.

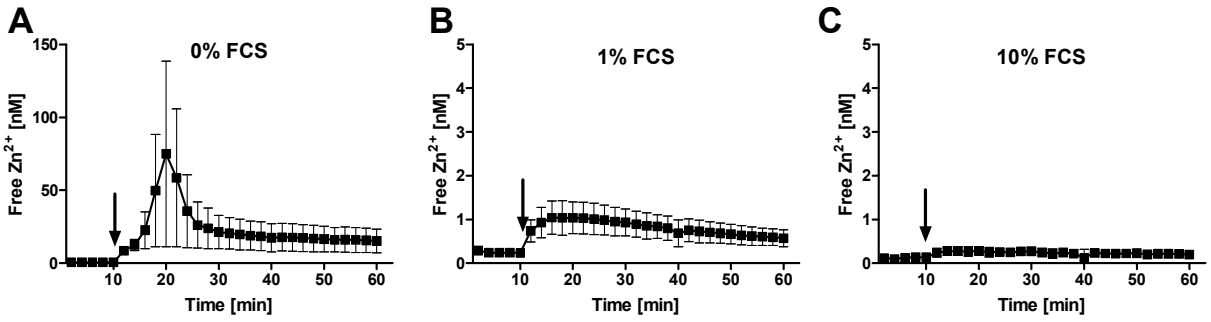

Figure 4: Impact of serum on free Zn^{2+} uptake. Raw 264.7 cells were loaded with FluoZin-3 and free intracellular Zn^{2+} was measured in the presence of 0% (A), 1% (B) or 10% (C) FCS. After 10 minutes recording of the baseline, 1 µM $ZnSO_4$ was added (arrow). Data are shown as means of n=3 independent experiments ± SEM.

PBMC are directly exposed to plasma proteins and Zn^{2+} in the blood. In a buffer system with minimal Zn^{2+}-binding, the nominally physiological concentration of 15 µM Zn^{2+} leads to massive uptake of this ion, which is abrogated by 10 mg/mL (corresponding to approximately 150 µM) BSA (Fig. 5D). This is not due to interference of BSA with the detection of Zn^{2+} by the fluorescent probe, because exposure of PBMC to Zn^{2+} elevated the total cellular Zn^{2+} content,

measured by atomic absorption spectrometry (AAS), an effect which is also significantly reduced by the presence of BSA (Fig. 5E).

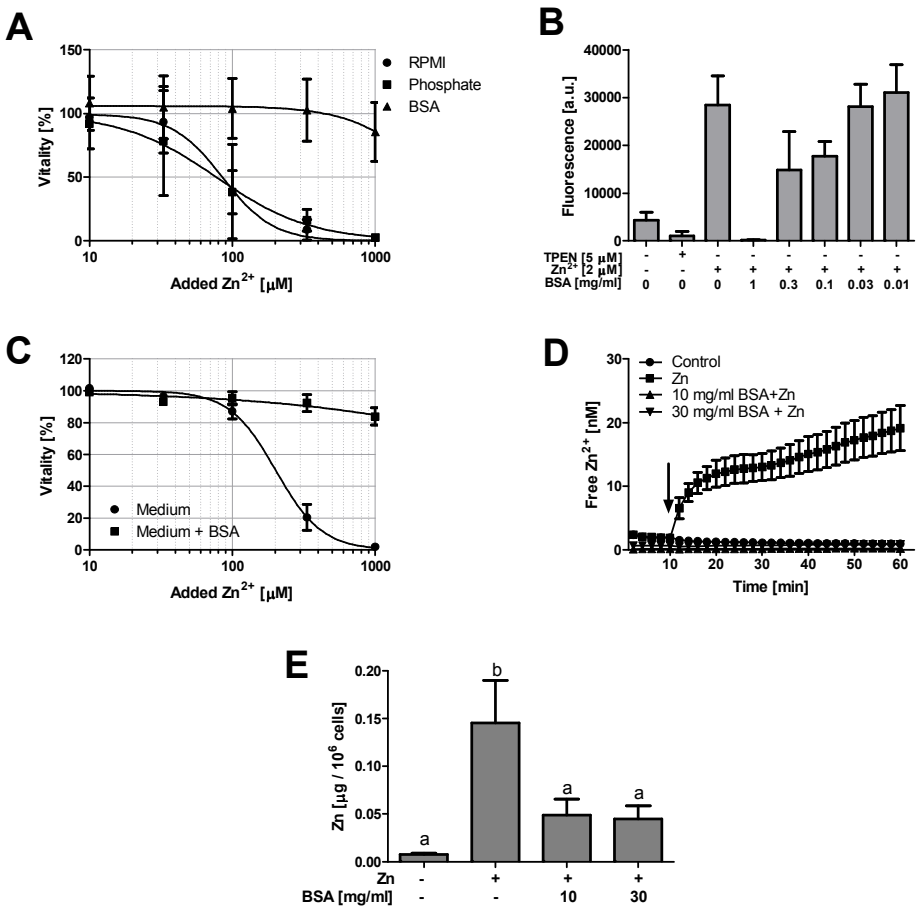

Figure 5: Zn^{2+} buffering by BSA and phosphate. (A) Raw 264.7 cells were cultured for 24h in serum-free RPMI 1640 supplemented with Na_2HPO_4 (10 mM) or BSA (50 mg/ml), followed by a measurement of vitality with the MTT assay. Sigmoidal dose-response curves were fitted by non-linear regression. (B) Cell-free measurement of Zn^{2+} with FluoZin-3 in the presence of the indicated concentrations of BSA. (C) Raw 264.7 cells were cultured for 24 h in normal culture medium (containing 10% FCS) and in medium additionally supplemented with BSA (50 mg/ml). Cellular vitality was determined using the MTT assay and sigmoidal dose-response curves were fitted by non-linear regression. All data are shown as means with SEM from at least n=3 independent experiments. (D) PBMC were isolated from healthy human donors, loaded with FluoZin-3 and, after 10 minutes recording of the baseline, exposed to 15 µM Zn^{2+} (arrow) in the presence of the indicated concentrations of BSA. Data show one representative of n=5 donors (measured in triplicate, ± SEM). (E) Total zinc was measured by AAS after 30 min incubation of PBMC with 15 µM Zn^{2+} in the presence of the indicated concentrations of BSA. Data are shown as means + SEM from n=8 donors. Significantly different means do not share the same letter (One-way ANOVA with Bonferroni post-hoc test).

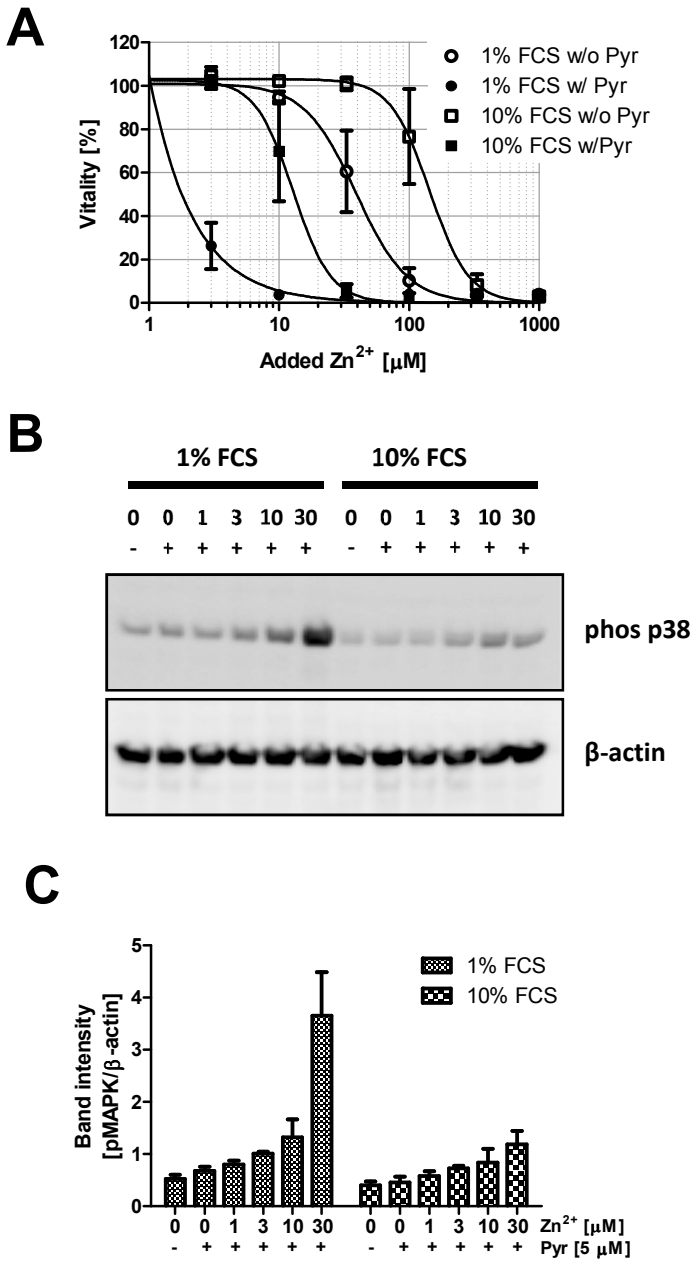

Figure 6: Effect of serum on Zn^{2+} uptake mediated by pyrithione. (A) Raw 264.7 cells were kept in RPMI 1640 cell culture medium containing either 1% (circles) or 10% (squares) FCS. Cellular vitality was determined by the MTT assay after culture for 24h in the absence (empty symbols) or presence (filled symbols) of sodium pyrithione (Pyr, 5 µM) and varying concentrations of $ZnSO_4$ as indicated. Data are shown as means from n=3 independent experiments ± SEM and sigmoidal dose-response curves were fitted by non-linear regression. (B,C) Phosphorylation of p38 MAPK was analyzed by Western blots with Jurkat cells incubated as indicated for 30 min in RPMI 1640 cell culture medium containing 1% or 10% FCS. Data are shown as one representative blot (B) or densitometric quantification (C) of n=3 independent experiments.

Zn^{2+} buffering in the presence of pyrithione

The next experiments are performed to elucidate if the effect of FCS is also observed when pyrithione is present, which is frequently used as an ionophore, facilitating Zn^{2+} uptake into cells. Pyrithione shifts toxicity to lower Zn^{2+} concentrations, but FCS still preserves vitality (Fig. 6A). Additionally, the presence of pyrithione reduces the Zn^{2+} concentration required to induce p38 phosphorylation in Jurkat cells by one order of magnitude (Fig. 2B,C; 6B,C). Nevertheless, the impact of Zn^{2+} is antagonized by FCS; a two-way ANOVA of the densitometric quantification confirms that the concentrations of Zn^{2+} ($p=0.0002$) and FCS ($p=0.0026$) both significantly affect p38 phosphorylation in the presence of pyrithione.

Impact of FCS on the cytotoxicity of other metal ions

Investigation of metal ions in cell culture is clearly not limited to Zn^{2+}, but also deals with many other heavy metal ions, and BSA is known to bind several additional metal ions that have physiological or toxicological relevance [5]. In addition to $ZnSO_4$, the toxicity of $CuSO_4$, $Pb(NO_3)_2$, $CdSO_4$, $HgCl_2$, $NiSO_4$, and $AgNO_3$ is examined in Raw 264.7 cells. For all metal ions investigated, a similar buffering by FCS is observed, and while there is a considerable variation between the different ions due to distinct degrees of their cytotoxicity, the respective LC_{50} values for all ions consistently increase from 1% over 5% to 10% FCS (Fig. 7).

Zn^{2+} toxicity at physiological serum concentrations

To investigate Zn^{2+} toxicity in an experimental setting closer to the *in vivo* situation, the toxicity in culture medium is compared to cells growing in 100% FCS (Fig. 8A). The presence of a physiological serum concentration significantly attenuates the toxic effects of Zn^{2+}. This is confirmed by experiments with PBMC. Culture in serum obtained from the same donor leads to reduced Zn^{2+} toxicity, compared to cell culture medium (Fig. 8B).

	LC_{50} [µM]		
	1% FCS	5% FCS	10% FCS
Zn	37.7	91.4	165.8
Ag	9.1	14.2	24.8
Cu	88.2	182.8	247.6
Pb	52.5	157.4	314.4
Cd	3.1	9.6	24.7
Hg	16.2	17.4	43.7
Ni	319.8	498.2	885.6

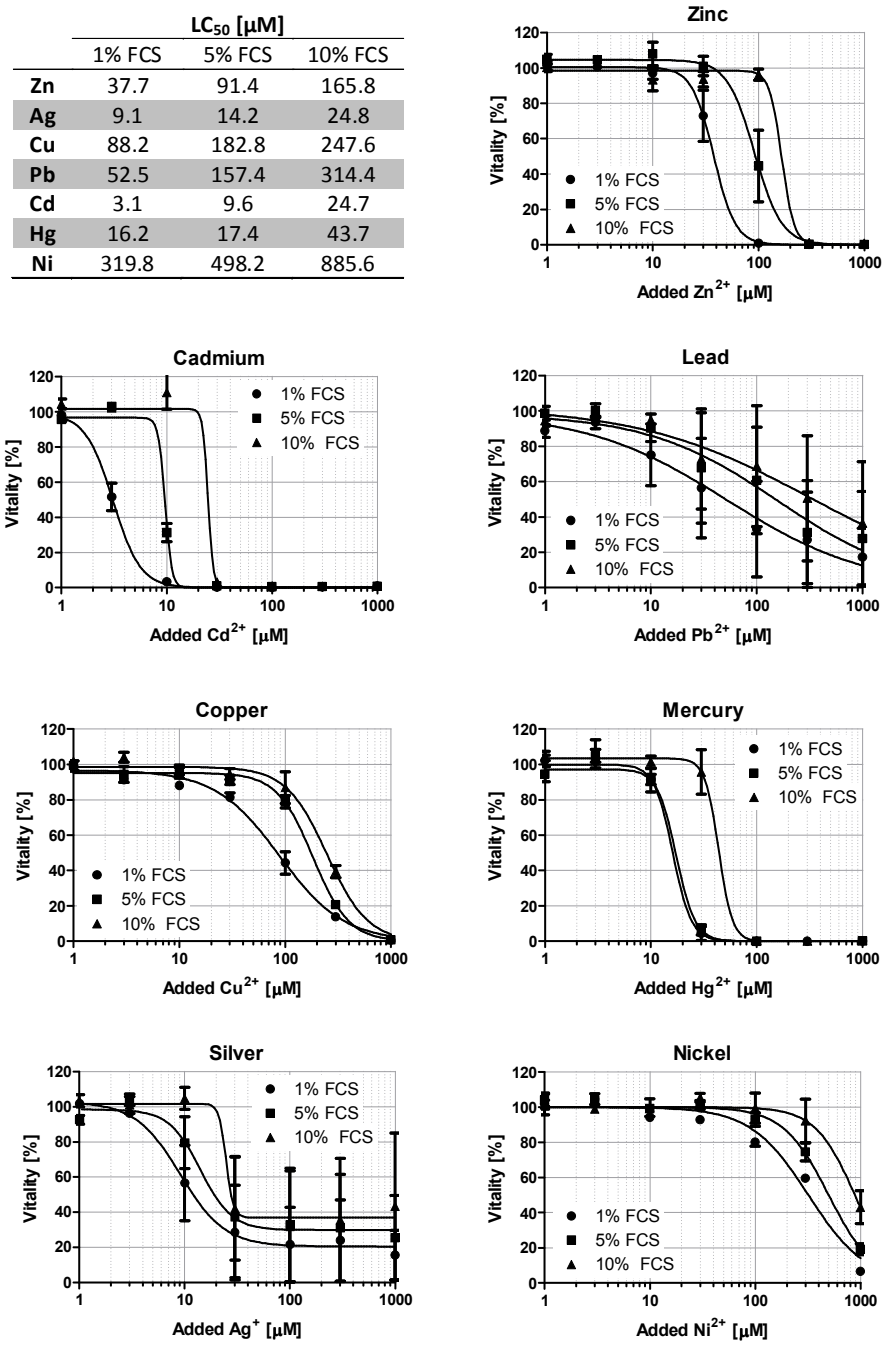

Figure 7: Effect of serum on the toxicity of seven different metal ions. Raw 264.7 cells were grown in RPMI 1640 cell culture medium containing 1% (circles), 5% (squares) or 10% (triangles) FCS. After 24h in

the presence of $ZnSO_4$, $CuSO_4$, $Pb(NO_3)_2$, $CdSO_4$, $HgCl_2$, $NiSO_4$ or $AgNO_3$, the remaining vitality was determined by the MTT assay. All data are shown as means ± SEM from n=3 independent experiments. Sigmoidal dose-response curves were fitted by non-linear regression and the resulting LC_{50} values are indicated in the table.

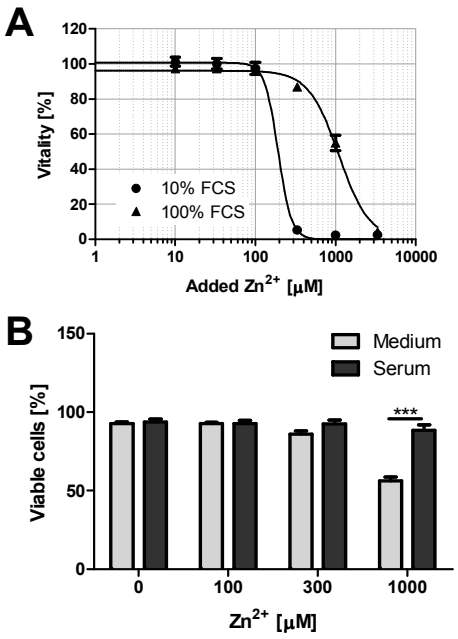

Figure 8: Zn^{2+} toxicity in the presence of 100% serum. (A) Raw 264.7 cells were grown in RPMI 1640 cell culture medium containing 10% FCS (circles), or in 100% FCS (triangles). After 24h in the presence of the indicated concentrations of $ZnSO_4$, the remaining vitality was determined by the MTT assay. All data are shown as means ± SEM from n=3 independent experiments. Sigmoidal dose-response curves were fitted by non-linear regression. (B) PBMC were cultured for 24h in the presence of the indicated concentrations of $ZnSO_4$, either in RPMI 1640 cell culture medium containing 10% FCS or in 100% donor's own serum. The percentage of viable cells was determined by flow cytometry after staining with propidium iodide. Data are shown as means +SEM from n=3 donors (*** = $p<0.001$; ANOVA with Bonferroni post hoc test).

Discussion

The reference range for the total concentration of Zn^{2+} in human plasma is between 76-125 µg/dL (roughly corresponding to 12-20 µM) [14]. In comparison, the free concentration is several orders of magnitude lower [15,16]. This is mainly due to binding by albumin, which is present in a 30 fold molar excess to Zn^{2+} [17], with a reference range in healthy controls between 35-51 mg/ml (corresponding to approximately 500-750 µM) [18]. Albumin has four known metal ion binding sites with different preferences and affinities. Physiologically, Zn^{2+}-binding is mediated by two of these sites, but for one of

them (site B), its affinity for Zn^{2+} and its exact localization in the protein remain unknown [5]. Most reports for the site A, also known as the multi-metal binding site, which is considered the major Zn^{2+} binding site, give an affinity of approximately 100 nM [5]. An exact calculation of the resulting free Zn^{2+} concentration is further complicated by competition between different metal ions present in biological fluids for the binding sites of albumin. Moreover, Zn^{2+} binding is also regulated by additional factors, such as fatty acids [19]. In light of the excess of albumin, the vast majority of Zn^{2+} will be in a protein-bound state even if only site A is considered, resulting in low nanomolar free Zn^{2+} concentrations in biological fluids such as plasma and serum-supplemented cell culture media. We found even lower, sub-nanomolar free Zn^{2+} in our culture media, suggesting a contribution of other binding sites in albumin, but also low molecular weight ligands and other Zn^{2+}-binding proteins, such as α2-macroglobulin and transferrin, contributing to the Zn^{2+} buffering capacity [6,20]. In a comparable manner, cell lysates buffer Zn^{2+} [4], and addition of micromolar concentrations of Zn^{2+} to cell lysates is required to obtain nanomolar concentrations of free Zn^{2+}, for example, for inhibiting PTPs *in vitro* [21].

In the present study extracellular Zn^{2+} was shown to be buffered from micromolar (added) to nanomolar or even picomolar (free) concentrations under normal cell culture conditions. The culture media used in our experiments contain a basal amount of 3 µM Zn^{2+}, originating virtually entirely from the 10% FCS supplement (data not shown). The addition of 250 µM Zn^{2+} killed over 95% of Jurkat cells, while free Zn^{2+} concentrations remained below 30 nM. The effect of buffering seems to be more important than cell type, as similar results were observed in other cell lines. Once the buffering capacity of the culture medium is exceeded, free Zn^{2+} increases to toxic levels, which are still nanomolar. In a similar manner, a different study has previously identified 100 nM as the toxic threshold for free extracellular Zn^{2+} [11].

Albumin has been reported to facilitate the uptake of Zn^{2+} into endothelial cells [22]. If the ion would remain bound after its uptake, it would be invisible to detection by fluorescent probes and also not be contributing to toxicity. Hence, Zn^{2+} may still be taken up in the presence of serum proteins, just not in its free form. This has been investigated by AAS. As shown in figure 5E, the uptake of Zn^{2+} (15 µM added

to extracellular buffer) is significantly lower in the presence of BSA when it is present in tenfold (10 mg/ml, corresponding to 150 μM) or thirtyfold (30 mg/ml, corresponding to 450 μM) molar excess. However, there was no concentration-dependent effect of the addition of BSA. This might reflect that a maximum of albumin-bound Zn^{2+} is already taken up in the presence of 10 mg/ml BSA, with no further effect at 30 mg/ml. Potentially only the uptake of additional, free Zn^{2+} is buffered by the protein. Whereas albumin certainly contributes significantly to buffering, there are also other relevant factors. Zinc phosphate is generally considered to be of very limited solubility [12]. However, calculating zinc speciation in the presence of phosphate with CHEAQS software[23] (for the concentrations used in our experiments, at neutral pH, and in the absence of other metals and ligands) shows that the predominant zinc species (>99%) is the soluble $ZnH(PO_4)_2^{3-}$ complex. Accordingly, the presence of millimolar concentrations of additional phosphate had no effect on Zn^{2+} toxicity, indicating that precipitation of zinc phosphate is negligible under these conditions.

The data in figure 1D indicate that some complexes that reduce free Zn^{2+} levels seem to be forming over time, especially in the first eight hours. However, two aspects should be kept in mind. First, these observations were made after addition of supraphysiological Zn^{2+} concentrations, which might be buffered by ligands that are normally not binding Zn^{2+}. Second, plasma zinc has a high turnover rate, being exchanged approximately 150 times per day [24]. Hence, events with such slow kinetics will have to be taken into account only in cell culture, whereas they should not be of physiological relevance *in vivo*.

High micro- or even millimolar concentrations of Zn^{2+} are frequently applied to cell cultures to elicit a biological response, even though it is widely accepted that such concentrations are not physiologically relevant. Importantly, even the addition of so-called physiological concentrations of Zn^{2+}, for example 10-15 μM, can also not be considered physiological in the absence of buffering proteins. Physiological concentrations of free Zn^{2+} are pico- to low nanomolar [25]. In buffers or serum-free media supplemented with micromolar amounts of Zn^{2+}, the relevant free concentration acting on the cells in the absence of any buffering proteins will exceed the physiological concentrations by several orders of magnitude, even though the nominal total concentration may be identical. Investigations with cell lines or isolated primary

cells are usually performed in culture media, typically containing between 5 and 20% serum, with most experiments in cell culture being performed in the presence of 10% FCS. This constitutes only one tenth of the physiological buffering capacity *in vivo.* The latter leads to a considerable reduction of the effective concentration of metal ions, and a significant over-estimation of the cellular effects that these toxic metal ions may have when toxic effects are deduced from cell culture experiments. For example, 1 mM Zn^{2+} killed virtually all Raw 264.7 cells under normal cell culture conditions (medium with 10% FCS), whereas viability remained well above 50 % when the same amount of Zn^{2+} was added in the presence of 100% FCS. Of note, physiological concentrations of albumin in human plasma are between 35 and 51 mg/ml [18]. Consequently, the data in figure 8 confirm that culture in 100% serum, which is much closer to the physiological conditions that cells encounter *in vivo*, results in strongly elevated tolerance to Zn^{2+} toxicity.

The effects described above are not only relevant for Zn^{2+}, but also for several other metal ions. Albumin has been characterized with regard to binding of Cu^{2+} and Zn^{2+} as a physiological transport mechanism, but has also been shown to bind several toxicologically relevant metal ions such as Ni^{2+}, Co^{2+}, or Cd^{2+} [5]. For Pb^{2+} it has been shown that the presence of FCS affects its solubility, most likely by binding to albumin and other serum proteins [26], and that FCS reduces the uptake of Pb^{2+} into cultured astroglia [27]. As demonstrated in figure 7, buffering by serum is clearly an issue for several metal ions in addition to Zn^{2+}. In conclusion, the complexity of serum and its many zinc-binding components make it impossible to predict the exact zinc speciation in culture media. Especially the interaction of albumin and Zn^{2+} is still subject to investigation. Still, it is clear that free metal ion concentrations are far more relevant for toxicological considerations than total amounts of these metals. The extent to which the metal ions are buffered should be known and taken into consideration when designing an experiment. Notably, FCS is a natural product. Its composition varies between different suppliers, and even batches. Hence, some degree of variation must be expected for metal binding, and the values derived from this study can only serve as an indicator for the extent of Zn^{2+} -binding by FCS. Ideally, free Zn^{2+} is determined under the particular

conditions used by each lab individually. As recently postulated, metal ion buffers are an appropriate and recommendable tool for applying controlled extracellular concentrations of free metal ions [11]. Otherwise, experiments in cell culture might lead to a systematic over-estimation of the potential effects of extracellular metal ions *in vivo*.

Experimental

Materials. FluoZin-3 acetoxymethyl ester and FluoZin-3 tetrapotassium salt were both from Invitrogen (Karlsruhe, Germany). $ZnSO_4$ x 7 H_2O was obtained from Merck (Darmstadt, Germany). Bovine Serum Albumin (BSA) was from PAA (Cölbe, Germany). N,N,N',N',-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), and Na-pyrithione were purchased from Sigma-Aldrich (Germany). HNO_3, H_2O_2 and H_2O for atomic absorption were of appropriate quality for trace element analysis (TraceSelect, Fluka, Germany). All other reagents were of analytical purity and obtained from standard sources.

Cell culture. All cells were cultured at 37°C and saturated humidity in a mixture of 95% air and 5% CO_2. The human acute T-cell leukemia cell line Jurkat was grown in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, 1 mM Na-pyruvate, and non-essential amino acid supplement (all from Lonza, Verviers, Belgium). Raw 264.7 murine macrophages and L929 murine fibroblasts were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, and the murine microglial cell line BV-2 in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 2 mM L-glutamine, 100U/ml penicillin, and 100µg/ml streptomycin. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral venous blood from healthy consenting donors by density gradient centrifugation with Ficoll as described [28], and cultured in the same medium as Raw 264.7 cells. For standard cell

culture, all media were supplemented with 10% low endotoxin fetal calf serum (FCS, obtained from PAA Germany) that had been heat-inactivated at 56°C for 30 min prior to use. Alternative serum concentrations used for the experiments are indicated in the respective figure legends.

Cytotoxicity tests. All cells were seeded onto 96 well plates and incubated for 24 h under the conditions described in the respective figure legends. Subsequently, cells in suspension culture (Jurkat, PBMC) were collected by centrifugation, taken up in Phosphate Buffered Saline (PBS), incubated with propidium iodide (1 µg/ml) for 10 min at room temperature and dye uptake (indicating membrane damage as a result of cell death) was analyzed by flow cytometry on a Becton Dickinson FACSCalibur (BD Biosciences, Heidelberg, Germany). A control with Na_2SO_4 confirmed that the toxicity was not caused by the anion. Adherent cells (Raw 264.7, BV-2, L929) were incubated for three hours with 0.01 % (w/v) Methylthiazolyldiphenyl-tetrazolium bromide (MTT) in normal culture medium. Cells were then lysed in isopropanol and the absorption determined at 570 nm, using a reference wavelength of 700 nm, on a Tecan Sunrise well plate reader (Crailsheim, Germany). Data were analyzed with GraphPad Prism software version 5.01, applying a non-linear regression, fitting a sigmoidal dose-response curve with variable slope as a function of the logarithm of concentration.

Free Zn^{2+} measurements in cell culture medium. Complete culture medium was preincubated (w/o cells) for 24 h at 37°C, 5% CO_2, at saturated humidity in 96 well plates (100 µl/well). Zinc sulfate was added and the medium was incubated under the same conditions for additional 24h. FluoZin-3 (tetrapotassium salt) was added to a final concentration of 1 µM. Samples were equilibrated for 30 min, wells were sealed with adhesive sealing foil (Nunc, Roskilde, Denmark) to preserve the atmosphere during the measurements, ensuring conditions identical to the ones during cell culture. The resulting fluorescence was recorded on a Tecan Ultra 384 fluorescence well plate reader (Tecan, Crailsheim, Germany) at 37°C, using excitation and emission wavelengths of 485 nm and 535 nm, respectively. Free zinc concentrations were calculated using the formula by Grynkiewicz et al. [29]. For the determination of F_{min} and F_{max}, medium was incubated with TPEN (10 µM) or $ZnSO_4$ (10 mM), respectively.

Intracellular free Zn^{2+} measurements. Free intracellular Zn^{2+} was measured as previously described [30]. Briefly, cells were loaded with 1 µM FluoZin-3 acetoxymethyl ester for 30 min at 37°C and their fluorescence was measured on a FACSCalibur (BD Biosciences, Heidelberg, Germany) flow cytometer (Jurkat) or on a Tecan Ultra 384 fluorescence well plate reader (Raw 264.7, PBMC), using the same wavelengths as above. Free zinc concentrations were calculated using a dissociation constant for the Zn^{2+}/FluoZin-3 complex of 8.9 nM [4], determining the minimal and maximal fluorescence after addition of TPEN (50 µM) or a combination of Zn^{2+} (100 µM) and the ionophore pyrithione (50 µM) for at least 15 min.

Atomic absorption spectrometry (AAS). PBMC (2×10^6) were incubated as indicated in the respective figure legend for 30 min in a buffer containing 25 mM HEPES, pH 7.35, 120 mM NaCl, 5.4 mM KCl, 5 mM glucose, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM NaH_2PO_4. Cells were dissolved in a mixture (1:1) of ultrapure HNO_3 (67%) and H_2O_2 (30%) and dried at 85°C over night. The residue was dissolved in ultrapure water containing 0.2% (v/v) HNO_3. Samples were analyzed by flame AAS on a Perkin Elmer AAnalyst 800 instrument.

Western blot. Western blot analysis was performed as described [12]. Antibodies against phosphorylated p38 (phos p38, Thr180/Tyr182) and total β-Actin were obtained from New England Biolabs, Germany. Densitometry was performed with ImageJ (http://rsb.info.nih.gov/ij/index.html).

Statistical analysis. Statistical significance was calculated by one- or two-way Analysis of Variance (ANOVA), depending on the number of variables, followed by Bonferroni post hoc test, using GraphPad Prism Version 5.01 (GraphPad Software, USA). A p value ≤ 0.05 was considered statistically significant. All experiments have been performed at least three times independently with comparable results.

Notes and references

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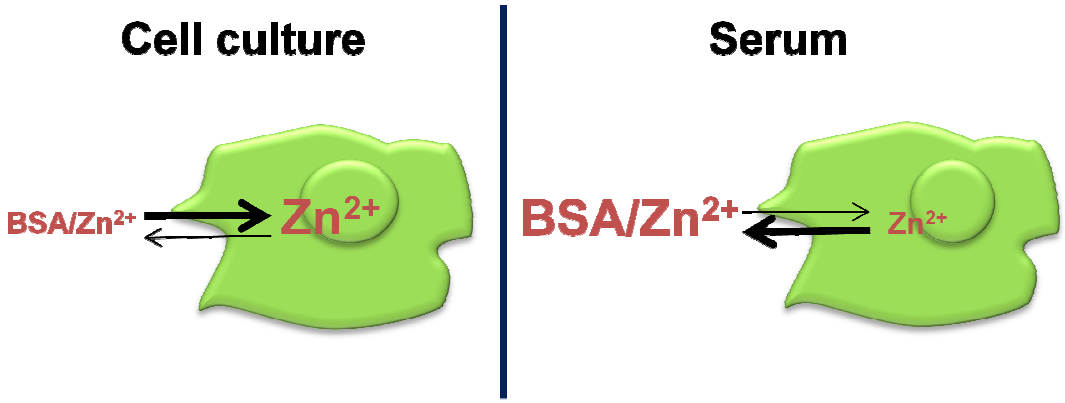
Table of contents entry



Differential speciation and lower zinc buffering by less bovine serum albumin (BSA) in cell culture medium leads to altered zinc homeostasis compared to the cellular environment *in vivo*.