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Effects of Cobalt on Gene Expression of Zinc Transporters in TM4 Sertoli Cells

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Abstract

The *in vivo* administration of cobalt in male mice causes reproductive toxicity resulting in decreased testicular weight followed by reduced sperm concentration and fertility. However, the co-administration of zinc with cobalt *in vivo* mitigates reproductive toxicity and histological damage to the germinal epithelium. Zinc is a key signaling molecule which is tightly controlled intracellularly by two families of zinc transporters, ZNT 1-10 and ZIP 1-14. They regulate intracellular zinc levels and distribution of zinc into organelles. Since Sertoli cells regulate spermatogenesis and are one of the last surviving cells observed histologically in the cobalt-treated testis, we investigated the mechanism of cobalt reproductive toxicity using a TM4 Sertoli cell line. Cobalt treatment of TM4 cells altered over 5500 genes as measured by microarray analysis. Validation of specific changes by quantitative PCR showed significant changes in the gene expression of *slc39a9* and *a13* (ZIP 9 and13) and *slc30a4*, *a5*, *a7* (ZNT 4, 5 and 7) zinc transporters. After 24 hours of treatment with Co 33 μM, maximum reduction of gene expression of *slc30a4*, *a5* and *a7* efflux transporters to 4.08, 2.86 and 2.67% of controls was observed, respectively. Expression of *slc39a9* and *a13* influx transporter genes was maximally decreased to 3.22 and 2.79% of controls, respectively. These data support the hypothesis that cobalt reproductive toxicity may be mediated, in part, by alteration of zinc transport and homeostasis in TM4 Sertoli cells.

Keywords: Metal toxicity; Zinc signaling; Zinc transporter; Cobalt

Abbreviations

Co: Cobalt; Zn: Zinc; PCR: Polymerase Chain Reaction; MT-1 or -2: Metallothioneins 1 or 2; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

Introduction

The reproductive toxicity of metals, including Cobalt (Co) [1,2] and Cadmium (Cd) [3,4] in male rodents is well established *in vivo*. The toxic effects observed with chronic administration of Co include decreased testicular weight, degeneration of the germinal epithelium, and reduced sperm production resulting in decreased fertility. These reproductive effects of Co are slowly reversible over 20 weeks after discontinuation of administration [2,5]. Histopathology of the testis after chronic Co treatment shows ubiquitous damage to the germinal epithelium with replacement of spermatocytes, spermatids and spermatozoa by Giant cells and fatty depositions. However, the Sertoli cells remain intact even with severe damage to the germinal epithelium [5]. Measurement of Co levels in the whole testis shows an elevation of Co in the testis with chronic treatment in the virtual absence of germinal epithelium, suggesting that Co is associated with the remaining somatic cells, e.g. Sertoli cells [6].

Interestingly, mice treated concomitantly with Zn and Co *in vivo* exhibit partial mitigation of cobalt's toxic reproductive effects in the testis [7]. Zinc (Zn) is a cofactor for over 300 enzymes critical for biochemical processes, and is required for function of more than 2000 transcription factors [8], including the metal responsive element-binding transcription factor MTF-1 [9]. Zn binds to about 10% of proteins in humans. Therefore, Zn must be tightly regulated at the cellular level because disruption of Zn homeostasis can cause pathological conditions, including male infertility. Zn is necessary for normal spermatogenesis and reproductive function in the male [10]. Both Zn deficiency and excess cause impairment of spermatogenesis in mice [11] and humans [12].

There are two major families of Zn transporters in mammalian species that control cytoplasmic Zn levels and subcellular distribution of Zn within the cell, ZNT (*slc30*) and ZIP (*slc39*) transporters, which function to move Zn in opposite directions. ZNT family of transporters (1-10) moves Zn out of the cytoplasm across the cellular membrane into the extracellular space, or sequesters Zn into intracellular compartments [13]. ZIP transporter family (ZIP1-14) primarily moves Zn from the extracellular space or from intracellular stores into the cytoplasm [8].

Zn transporters are ubiquitously expressed in mammalian tissues including the testis, and specifically Sertoli cells. Metal ions including Zn, Cd and Co have been shown to induce or repress gene and protein expression of both families of Zn transporters [8,11,13-16], as well as metal binding proteins metallothioneins 1 and 2 (MT1 and MT 2) [17]. Mutations of the Zn transporters are associated with disease states including a subtype of Ehlers-Danlos syndrome in mice and humans (ZIP13, *slc39a13*) [18] and lethal milk disorder in mice (ZNT4, *slc30a4*) [19].

Since the Sertoli cell regulates the process of spermatogenesis [20] and remains intact during Co reproductive toxicity, the role of the

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Table 1: Effect of Cobalt Treatment on Microarray Gene expression. TM4 Sertoli cells were treated for 24 hours with 0 or 33μM cobalt *in vitro*. Total RNA was quantitated using Affymetrix Mouse 2.0 MT gene array. Data is shown as fold change from control, using a 2-fold change as threshold. All values are significantly different from controls (p≤0.021).

Solute Carrier gene (slc)	Expression Level: (Fold Change)	Transport Function:
11a2	0.37	Metal transporters,including Fe, Mn, Co, Cd, Ni, Pb.
25a12	0.17	Ca^{++}-dependent exchange cytoplasmic glutamate with mitochondrial aspartate
25a24	0.48	Ca^{++}-dependent mitochondrial transporter, protects against oxidative stress-induced cell death
30a4	0.21	Zn transporter out of the cytoplasm, sequestration into an intracellular compartment.
30a5	0.44	Zn transporter- regulates cellular Zn homeostasis. Required for the activation of Zn-requiring enzymes, e.g., alkaline phosphatases.
30a7	0.31	Zn transport facilitator from cytoplasm into Golgi. Regulates cellular Zn homeostasis.
39a9	0.48	Zinc-influx transporter.
39a13	0.50	Zinc-influx transporter.
41a1	0.42	Magnesium transporter

Table 2: Primers designed for qPCR measurement of gene expression of *slc30* (ZNT) and *slc39* (ZIP) transporters, Metallothioneins 1 and 2, and GAPDH.

Gene	Product size (bp)	Forward Primer 3' to 5'	Reverse primer 3' to 5'
Zip 1	300	CAGTAGGGCTGCTGACACTCAT	GTTCCTTGTAAGCCAGCGTG
Zip 9	242	AGCGTGCGAAGAAGACACTT	CGAAGCACGCCACTCTCTTA
Zip 13	165	GCCCTGGTGAATGTGTTACC	GAGTCTCATTGCTCGCTGTG
Znt 4	255	TCACTCTGCTTGCTTTGTGG	GACCGGACTGGTTCAACAGA
Znt 5	251	ATGTTTGAAGGCTGTGGGAC	GCAGCAAGGTCCTTAGTGGT
Znt 7	217	TGCTATCAGAGGGTGCAACA	CCTTTGACTACATTGCCGCA
MT 1	190	GTCCTCTAAGCGTCACCACG	AGGAGCAGCAGCTCTTCTTG
MT 2	205	TCTCGTCGATCTTCAACCGC	GCACTTGTCGGAAGCCTCTT
GAPDH	452	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Sertoli cell in Co toxicity was investigated *in vitro* using TM4 Sertoli cells, a murine derived epithelial cell line [21]. Because Co levels are elevated *in vivo* in the testis with chronic toxicity, we explored the effect of Co on gene expression in TM4 Sertoli cells. We also measured the cellular levels of Co and Zn after Co treatment *in vitro*. Since previous studies showed concurrent Zn treatment *in vivo* reduced Co reproductive toxicity *in vivo*, the impact of Co on Zn transporters is of particular interest. We conducted these studies to determine if Co toxicity is mediated through Sertoli cell mechanisms, and whether disruption of Zn homeostasis is involved.

Materials and Methods

TM4 Sertoli cell line obtained from ATCC was grown to confluence in Ham's F-12/DMEM with 5% (v/v) FBS in a Forma Scientific 5% CO_2 incubator in T75 cm^2 flasks. Early passages cells (6.5 X 10^5) were plated into 6-well (SA-9.6 cm^2) culture ware (Falcon B-D) for individual treatments. Non-confluent cultures were treated with control (fresh media), or Co media containing cobaltous chloride hexahydrate (Sigma Aldrich) at a concentration of 10, 33, or 100 μM for 24 hours. Cells were harvested by detaching cells with a sterile scraper from well surface, and collected in cell lysis solution. Cells were then disrupted by passing the lysis suspension with cells rapidly through a 23G needle 3 times.

RNA Isolation

Total RNA isolation was performed using RNeasy RNA kit (Qiagen). DNA was then digested using an RNase-free DNase (Invitrogen). Total RNA was stored at -20°C until reverse transcribed and assayed for expression of specific messages by microarray analysis or quantitative PCR.

Reverse transcription

Isolated total RNA from each sample (1.5 μg) was used as the template for reverse transcription to generate cDNA using random hexamer primers with 1 μL reverse transcriptase (iScript, BioRad) under standard synthesis conditions.

Microarray analysis

In collaboration with Dr. Jamie Barth of the Proteogenomics Core Facility at the Medical University of South Carolina (MUSC) the research resources at the core facilities were utilized to carry out this study. The facility is equipped with a complete Affymetrix system, comprising a GeneChip® Scanner 3000 7G with AutoLoader, hybridization oven and fluidics workstation. This is complemented by a BioRadiCycler real time PCR machine and an Agilent 2100 Bioanalyzer. Bioinformatic analysis of significant changes was evaluated in collaboration with the Microarray facility to identify important pathways involved in the Co effects on gene expression. Affymetrix Murine microarray 2.0 ST was used to evaluate gene expression changes in response to cobalt treatment in TM4 cells. Single channel microarrays with 30,000 transcripts were used for screening control and cobalt-treated TM4 Sertoli cells. Three samples from the treatment groups 0, 10 and 33 μM Co were analyzed on triplicate microarrays.

Real-time PCR

Quantitative PCR was performed using Eva Green SsofastSupermix (BioRad) under cycling conditions optimized for each specific primer target. Specific forward and reverse primers were designed for each target using Primer3 software and evaluated for specificity for target using PrimeBlast (NCBI) (Table 2). Primers synthesized by Integrated DNA Technologies (IDT) were used to amplify cDNA from genes affected by cobalt treatment as identified by microarray analysis. PCR assays were done with a BioRad C1000 Thermal cycler with CFX96 Real-time PCR system, with data management software CFX Manager used for analysis. PCR products were analyzed using a high resolution Melt Curve Analysis program (BioRad). Changes in gene expression were normalized to GAPDH and expressed as per cent control or folds change over control. Gene expression was statistically evaluated by ANOVA, using a significance level of $p < 0.05$.

Mass spectrometry

TM4 cells were grown as described above in T-75 flasks (SA-75 cm^2) to a concentration of ~ 10^7 cells. Co media was added to 2 flasks of cells for each time point at concentrations of Co (0- 100 µM). Cells were incubated with Co for 0, 4, 8, or 24 hours at each concentration of Co. Cells were harvested after removal of media and a brief rinse with PBS. Cells were scraped from surface and collected into 0.5 mL microfuge tubes. After a brief spin for 10 sec, all fluid was removed and cells were weighed. Cells were frozen and stored at -80°C, then later transferred to the Mass Spectrometry Facility in the Dept. of Chemistry & Biochemistry at the University of South Carolina, Columbia, SC, for analysis of total cellular Co and Zn by mass spectrometry.

Samples were digested using 0.3 mL of nitric acid (optima grade, Fisher) and 0.9 mL of hydrochloric acid (optima grade, Fisher) in Teflon digestion vessels on a hot block at 120°C for 5 hours. The digestates were brought to 10 mL with 2% nitric acid. The final solution was analyzed without further dilution. Finnigan ELEMENT XR double focusing magnetic sector field inductively coupled plasma-mass spectrometer (SF-ICP-MS) was used for the analysis of Co and Zn. For sample introduction, a 0.2 ml/min Micromist U-series nebulizer (GE, Australia), quartz torch and injector (Thermo Fisher Scientific, USA) were used. Sample gas flow was at 1.08 mL/min. The forwarding power was 1250 W. A 5-point calibration curve was used for Co and Zn (High-Purity Standards). The calibration range was from 1 to 100 ppb. The R squared values for the initial calibration curves were > 0.999. Data was analyzed using 2-way ANOVA with a statistical significance value of $p \leq 0.05$.

Results

Microarray data showed over 5500 significant alterations in gene expression in the TM4 Sertoli cell line in response to Co 33 µM exposure *in vitro*. Gene expression of nearly a dozen solute transporters in the *slc* family was affected. Table 1 shows the significant changes seen in transporters of metal ions and metal binding proteins in response to cobalt treatment. The gene expression of members of the two major families of Zn transporters, *slc30* (ZNT) and *slc39* (ZIP), was significantly affected by Co treatment at a concentration of 33 µM for 24 hours. In Figure 1A and 1B, the validation of microarray changes in gene expression is shown as measured by quantitative PCR. For ZNT4, 5 and 7 Zn efflux transporters, maximum reduction of *slc30a4*, *a5* and *a7* gene expression was observed from treatment with 33 µM Co for 24 hours (Figure 1A). Expression of ZIP 9 and 13 Zn influx transporter genes *slc39a9* and *a13* was maximally decreased by treatment with Co 33 µM for 24 hours (Figure 1B). However, gene expression of *slc39a1*, ZIP 1, a major efflux Zn transporter, was not significantly affected by Co exposure over the range of 10-100 µM over a 24-hour time course (Figure 2). A non-significant trend was observed at Co 10µM for *slc30 a4*, *a5* and *a7*, as well as *slc39a9* and *a13*. In contrast to the Zn transporter genes, expression of metal

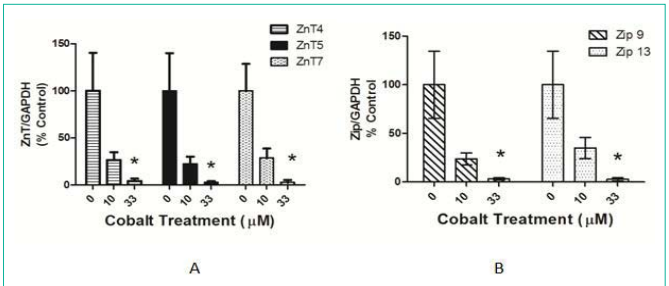

Figure 1: Effect of cobalt treatment on zinc transporter gene (*slc30* and *slc39*) expression. TM4 Sertoli cells were treated for 24 hours *in vitro* with 0, 10 or 33µM Co and gene expression was measured by qPCR. Data is presented as (*slc30*) ZNT or (*slc39*) ZIP, normalized to GAPDH gene expression and shown as % Control ±SEM. A. ZnT 4, 5 and 7 zinc transporter gene expression. B. ZIP 9 and 13 zinc transporter gene expression. *Expression significantly different from control, p≤0.05.

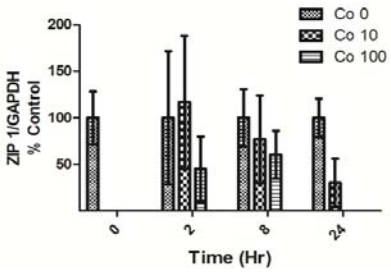

Figure 2: Effect of cobalt treatment on zinc transporter gene (slc39a1) expression. TM4 Sertoli cells were treated *in vitro* with cobalt 0, 10 or 100µM over a 24-hr time course. Gene expression was quantitated by qPCR and data is presented as ZIP1/GAPDH as % control ±SEM. There are no statistically significant differences (p>0.05) among treatment groups at any time point.

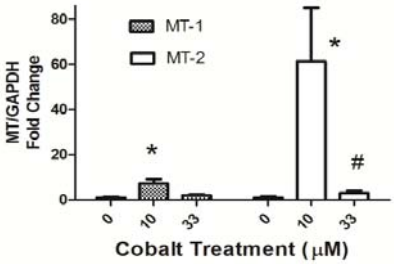

Figure 3: Effect of cobalt treatment on MT-1 and MT-2 gene expression. TM4 Sertoli cells were treated for 24 hours *in vitro* with 0, 10 or 33µM Co and gene expression was measured by qPCR. Data is presented as MT 1 or MT 2 normalized to GAPDH gene expression, and shown as fold change over control ±SEM. *Significantly different expression from control, # significantly different from Co10µM, p ≤0.05.

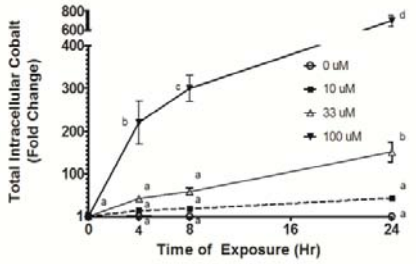

Figure 4: Effect of *in vitro* cobalt exposure on total intracellular cobalt concentration. TM4 Sertoli cells were treated *in vitro* with cobalt (0, 10, 33 or 100μM) and harvested at 0, 4, 8, and 24 hours. Total intracellular concentrations of cobalt were measure by mass spectrometry. Data is expressed as fold-change over control ±SEM. Different letters designate statistically significantly differences, $p \leq 0.05$.

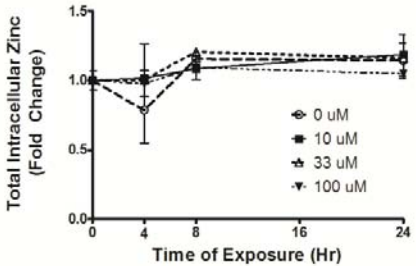

Figure 5: Effect of *in vitro* cobalt exposure on total intracellular zinc concentration. TM4 Sertoli cells were treated *in vitro* with cobalt (0, 10, 33 or 100μM) and harvested at 0, 4, 8 and 24 hours. Total intracellular concentrations of cobalt were quantitated by mass spectrometry. Data is expressed as fold-change over control ±SEM. No significant differences were observed, $p > 0.05$.

binding proteins MT1 and MT2 were significantly increased after 24 hours of Co treatment (10 μM) to 7.2 or 61-fold, respectively, as compared with controls, but not with the higher Co treatment (33 μM) (Figure 3).

Total cellular Co levels were significantly elevated in a concentration and time dependent manner at 33 and 100 μM, as measured by mass spectrometry and expressed as fold-change over control (Figure 4). However, at 24 hours of Co treatment, TM4 cells at the 100 μM concentration showed signs of damage, including membrane disruption and death. So the lower concentration of Co (33 μM) which significantly elevated total cellular Co concentration was chosen for the subsequent 24 hour treatment of TM4 cells for micro array analysis. There were no significant differences in control or 10 μM Co groups over the 24-hour time course, or from each other at any time measured (Figure 5). Effects were statistically significant at $p \leq 0.05$. Total cellular Zn levels were unchanged over 24-hour time course of Co treatment (0- 100 μM).

Discussion

Decreased gene expression of zinc efflux transporters ZNT 4, 5 and 7, and ZIP 9 and 13 influx transporters suggests that cobalt's toxic effects on the testis may be related to disruption of Zn homeostasis in Sertoli cells. ZNT4 is associated with the membranes of secretory vesicle/granules, and is responsible for deposition of Zn into vesicles/granules for exocytosis [19]. ZNT 5 and 7 are localized on membranes of Golgi apparatus and cytoplasmic vesicles, so they sequester and supply Zn for Zn-requiring proteins for their structure or function [22,23]. Therefore, the significant decrease in the gene expression of these Zn transporters with cobalt treatment may have a net negative effect on intracellular Zn availability for numerous Zn-dependent proteins in the Sertoli cell, which could ultimately affect both Zn-requiring protein expression and function.

The reduction of gene expression of *slc39a9* and *a13* may result in decreased Zn movement into the cytoplasm from Golgi apparatus, intracellular vesicles and lysosomes. Also, the lack of change in gene expression of *slc39a1* (ZIP1) suggests that Zn movement across the cell membrane into the Sertoli cell cytoplasm is not affected by Co exposure [14]. Coupled with the changes in expression of ZNT efflux transporters, these data suggest a shift in Zn distribution among different intracellular compartments, which is consistent with the lack of changes in total intracellular Zn. (Figure 5). Potentially, disruption of Zn-associated signaling pathways may play a key role in cobalt's toxicity on spermatogenesis. Specific localization studies of zinc within intracellular compartments are needed to determine whether alterations in zinc homeostasis are involved in cobalt's reproductive toxicity

The significant elevation of Co measured by mass spectrometry in TM4 Sertoli cells exposed *in vitro* to Co 33 μM for 24 hours (Figure 2) supports the conclusion that Co accumulates in these cells and that Co entry into the seminiferous tubules is regulated through the Sertoli cell *in vivo*. This is consistent with the elevation of Co previously seen in the testis *in vivo* after Co treatment [6]. Together these results suggest a direct testicular toxic effect of Co on reproduction in male mice, and it's likely that Co negatively influences the regulation of spermatogenesis via effects on Sertoli cell function. However, it is also possible that Sertoli cells may allow movement of Co into the adluminal compartment of the seminiferous tubule, directly affecting the developing germinal epithelium. This would be consistent with the lack of down regulation by Co in TM4 Sertoli cells on gene expression of the ZNT-1 transporter, which is associated with luminal spermatozoa and Sertoli cells in the mouse testis [24]. The requirement for Zn in spermatogenesis and the effect of *in vitro* Co treatment on Zn transporters together suggest that Co toxicity may be mediated by disruption of normal Zn trafficking within the Sertoli cell. Co may disrupt the necessary cross-talk between developing germ cells and Sertoli cells by affecting various Zn-requiring proteins which are crucial in the Sertoli cell regulation of the process of spermatogenesis.

Co treatment of Sertoli cells *in vitro* also significantly increased the gene expression of the metal binding proteins MT 1 and 2 at the lower (10 μM) , but not at the higher concentration (33 μM) (Figure 3). These proteins have a protective effect against metal toxicity in many tissues, including the testis [17,25]. MT 1 and 2 bind metals including Cd, Zn, Co, mercury, and iron [26,27]. Induction of MTs by metals is mediated by the MTF-1 (MRE-binding transcription factor-1) which is a zinc-finger protein [28]. In the absence of Zn, MTF-1 does not bind to the Metal Responsive Elements (MRE), preventing transcription of MTs [29,30]. In the presence of Co (33 μM), the Zn concentration in the nucleus of TM4 Sertoli cells may be insufficient to allow Zn activation of MTF-1 there by preventing transcription of MTs. This is consistent with the repression of *slc30a5* and *slc30a7* (ZNT 5 and 7) at the 33 μM Co, which normally supply

Zn for Zn-dependent proteins, such as MTF-1 [22,23].

The effect of Zn mitigation of Co toxicity seen *in vivo* in an earlier study [7] may be explained in part due to induction of MT 1 and 2 levels in Sertoli cells. This agrees with other studies which show pretreatment with Zn, selenium or low dose Cd for 24 hours has a protective effect against Cd toxicity in the testis by induction of MTs [31]. Greater induction of MT 2 compared with MT 1 has also been shown previously in Sertoli cells after Cd treatment [17]. Further, the dose-dependence in the induction pattern of MT 1 and 2 gene expression seen in cobalt-treated TM4 cells may be indicative of disruption of metal ion homeostasis within these cells. Cobalt induction of MTs at the 10 μM, but not the 33 μM concentration, may also contribute to the fact that the significant repression of *slc39* and *slc30* gene expression was seen at the higher Co dose (33 μM), but not the lower dose (10 μM).

Further evaluation of Co-induced changes in gene expression and ultimately protein translation in Sertoli cells is needed to elucidate the direct mechanism of cobalt's reproductive toxicity in males. Determination of cobalt effects on intracellular distribution of zinc would be required before we can conclude a direct role for disruption of zinc homeostasis in cobalt's mechanism of male reproductive toxicity.

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