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Cobalt-induced Changes in Iron Homeostasis in Skeletal Muscles of Immature Mice After Perinatal Exposure to Cobalt Chloride

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Perinatal exposure to CoCl2 induced significant time-dependent 11.3-fold and 88.9-fold increase in accumulation of the metal in the muscles of the treated immature day 18 and day 25 animals, respectively. The muscle samples of day 25 metal-exposed mice accumulated 4.44-fold higher cobalt (Co) levels compared to the day 18 experimental animals. Iron (Fe) content was also increased but the difference was significant only for day 25 mice probably due to the reduced TfR1 expression found in day 18 Co-exposed mice and increased in day 25 treated animals. Surprisingly, hepcidin showed the same expression pattern – decrease in day 18 and an increase in day 25 Co-exposed mice. Alterations in Fe homeostasis may be of significant importance for myogenesis, physical performance, skeletal muscle regeneration and in ageing may contribute to skeletal muscle atrophy.

Key words: cobalt chloride, iron, iron-regulatory proteins, skeletal muscle

Introduction

Cobalt (Co) is an essential trace element, environmental pollutant and a hypoxia-mimicking agent. Food is the main source of exposure to Co for infants, children and adults. The only known biological function of organic Co is its role as metal component of vitamin B_{12}, cyanocobalamin, whereas inorganic cobalt compounds have been described as toxic for the environment and humans following excessive exposure [14].

The effect of cobalt chloride ($CoCl_2$) as a hypoxia-mimicking agent on muscle fiber development and regeneration are controvercial. Recent studies show that *in vitro* $CoCl_2$ suppresses myoblast differentiation in a dose-dependent manner [20]. Hypoxia-induced muscle wasting is a phenomenon frequently reported in several environmental and pathological conditions, such as exposure to high altitudes, prolonged immobilization, chronic obstructive pulmonary disease, exercise, and anemia [2]. On the contrary, hypoxic preconditioning with $CoCl_2$ enhances physical performance and protects muscle from exercise-induced oxidative damage via GSH, HO-1 and MT-mediated antioxidative capacity [19]. The same authors also demonstrate an increase in mitochondrial biogenesis, glucose uptake and metabolism by aerobic respiration in skeletal muscle, which leads to increased physical performance in $CoCl_2$-preconditioned rats [20]. There are concerns about Co being misused as blood doping agent by athletes to enhance aerobic performance [15] and some energy drinks may contain high amounts of vitamin B_{12} [7]. Exercise and physical activity on the other hand reduce endogenous iron (Fe) content in the skeletal muscles. Iron homeostasis in the skeletal muscles has not been extensively characterized. The tissue contains from 10 to 15% of body iron, mainly in the form of heme-iron bound myoglobin. Fe released during muscle fiber damage must be promptly removed to limit its oxidative effect, however, iron must become available during skeletal muscle repair to allow the synthesis of myoglobin [3]. Fe is shown to accumulate in the skeletal muscles with senescence and disuse atrophy [8, 10].

There are scarcity data [11] on the *in vivo* effects of Co exposure on skeletal muscle Fe content as well as on the related risk health effects. Alterations in Fe homeostasis and specifically Fe deficiency contribute to skeletal muscle dysfunction and exercise intolerance [11]. Combined conditions of hypoxia and iron deficiency are the most detrimental for skeletal myocytes in the context of morphology alterations and expression of atrophy markers [11].

The aim of the study was to assess cobalt accumulation and iron redistribution and Fe-regulatory proteins transferrin receptor 1 (TfR1) and hepcidin expression in skeletal muscles after perinatal exposure to cobalt chloride.

Material and Methods

Animal Model

Experimental animals were purchased from the Experimental and breeding base for laboratory animals (EBBLA) – Slivnitza, Bulgaria and left to acclimatize for a week prior treatment. Pregnant ICR (Institute of Cancer Research) mice were subjected to a daily dose of 75 mg cobalt chloride/kg body weight ($CoCl_2x6H_2O$) for 2-3 days before delivery and treatment continued until day 25 after delivery. The compound was dissolved and administrated with drinking tap water. Animals were fed a standard diet and had access to food ad libitum with strong control of the feeding regime. Our previous experience showed no significant gender differences neither in body weight nor in haematological parameters and the experimental groups consisted of both male and female mice. The mice were maintained in the Institute's animal breeding facility at

23 ± 2°C and 12:12 h light/dark cycle in individual standard hard-bottom polypropylene cages. The suckling mice were sacrificed by decapitation after etherization on postnatal days 18 (n=4) and 25 (n=6). Femur muscles were excised, weighed and stored at -20°C prior to ICP-DRC-MS analysis. Age-matched mice obtaining regular tap water were used as a control group (n=5 for day 18 and n=4 for day 25 mice). The experiment was carried out in accordance with guidelines EU Directive 2010/63/EU for animal experiments. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

ICP-DRC-MS Analyses of Cobalt and Iron Content in Muscles

Prior to analysis 50-100 mg of the studied tissue samples were subjected to digestion in concentrated HNO_3 (Sigma-Aldrich, Co., USA) in the Berghof SW-4 DAP-40 microwave system (Berghof Products + Instruments GmbH, Eningen, Germany). The digested samples were transferred into 15 mL polypropylene test tubes and adjusted to the final volume of 15 mL with deionized water (18 MΩ cm, Milli-Q, Millipore, Bedford, MA, USA) and thoroughly mixed up by shaking in the closed test tubes.

Analysis of Co and Fe levels in the samples was performed using inductively-coupled plasma mass spectrometry with dynamic reaction cell technology (ICP-DRC-MS) at NexION 300D spectrometer (Perkin Elmer, USA) equipped with ESI SC-2 DX4 autosampler (Elemental Scientific Inc., Omaha, NE, USA). Co and Fe content in the studied samples was expressed as μg/g wet weight.

Calibration of the system was performed using standard solutions with different concentrations of Co and Fe prepared from Universal Data Acquisition Standards Kits (PerkinElmer Inc., Shelton, CT 06484, USA. Internal online standardization was performed using 10 μg/L Yttrium (Y) and Rhodium (Rh) Pure Single-Element Standard (PerkinElmer Inc., Shelton, CT, USA) prepared on a matrix containing 8% 1-butanol (Merck KGaA, Gernsheim, Germany), 0.8% Triton X-100 (Sigma-Aldrich Co., St. Louis, MO, USA), 0.02% tetramethylammonium hydroxide (Alfa Aesar, Ward Hill, MA, USA) and 0.02% ethylenediaminetetraacetic acid (Sigma-Aldrich Co., St. Louis, MO, USA). Laboratory quality control was performed via permanent analysis of the certified reference material (GBW09101, Shanghai Institute of Nuclear Research, Shanghai, China). The recovery rate for Co and Fe elements was within the interval of 92-104% and 95-107%, respectively.

Enzyme-linked Immunosorbent Assay (ELISA) for Transferrin Receptor 1 (TfR1) and Hepcidin

Muscle tissue homogenates were analysed using mouse TFR1 ELISA kit and Hepcidin ELISA kit (Wuhan Elabscience Biotechnology Co., Ltd, China) according to the manufacturer's instructions. The optical density was read at 450 nm on ELISA Reader GDV (GIO. DE VITA EC., Italy). The final concentrations were determined using Curve Expert 1.4 software and are expressed in ng/g for TfR1 and in pg/g for hepcidin.

Statistical Analysis

The obtained data were processed using Statistica 10.0 (Statsoft, Tulsa, OK, USA). The results from the hematological and biochemical analyses are presented as mean value ±

SD. Data on Co and Fe content in the studied samples are expressed as median and the respective 25 and 75 percentile boundaries (interquartile range). Statistical significance between the experimental groups was assessed using Mann-Whitney U-test at the level of significance of $p<0.05$.

Results

Prenatal and early postnatal exposure to $CoCl_2$ resulted in a significant metal ion accumulation in the muscles of the experimental suckling mice (**Table 1**). Day 18 Co-exposed mice accumulated 11.3-fold higher levels of Co compared to the untreated age-matched control group. The increase in 25-day-old Co-exposed animals was found to be 88.9-fold in comparison to the control values. The muscle samples of day 25 metal-exposed mice accumulated 4.44-fold higher Co content compared to the day 18 experimental animals suggesting a significant time-dependent effect. At the same time, no significant difference in muscle Co content was observed in control animals of different age.

Table 1. Co Content (μg/g) in Muscles of Day 18 and 25 Control and Co-exposed Mice

Sample	d18	d25
control	0.016 (0.010-0.049)	0.009 (0.005-0.025)
Co-treated	0.18 (0.16-0.20) *	0.80 (0.25-0.93) *

Data are presented as mean ± SD. Asterisk (*) signifies significant difference at $p<0.05$ between age-matched control and CoCl2-exposed mice.

Simultaneous analysis of Fe content in the muscle tissue also showed an increase upon Co exposure (**Table 2**). Although being insignificant, in day 18 metal-treated mice Fe was elevated by 15.8% compared to the untreated control animals. Significant increase by 39.1% was observed in day 25 $CoCl_2$-treated immature mice. The muscle tissue of day 25 metal-exposed mice contained significantly higher Fe levels by 55.2% compared to day 18 Co-treated mice which suggests Co-induced Fe accumulation in the target organ.

Table 2. Fe Content (μg/g) in Muscles of Day 18 and 25 Control and Co-exposed Mice

SampleW	d18	d25
control	12.12 (10.72-12.89)	15.65 (12.97-17.76)
Co-treated	14.03 (12.79-16.68)	21.77 (21.09-44.85) *

Data are presented as mean ± SD. Asterisk (*) signifies significant difference at $p<0.05$ between age-matched

Immunological analysis of the expression of the Fe-regulatory proteins TfR1 and hepcidin showed similar pattern. Both proteins expression was decreased in day 18

mice following $CoCl_2$ exposure and increased in day 25 metal-treated experimental animals. The elevated TfR1 concentration in muscles of day 25 Co-exposed mice (**Fig. 1**) corresponded to the significant increase in Fe content in this tissue. Surprisingly, hepcidin concentration was insignificantly (2.7%) reduced in day 18 Co-exposed mice while a 3-fold increase was observed in day 25 metal-treated animals (**Table 3**).

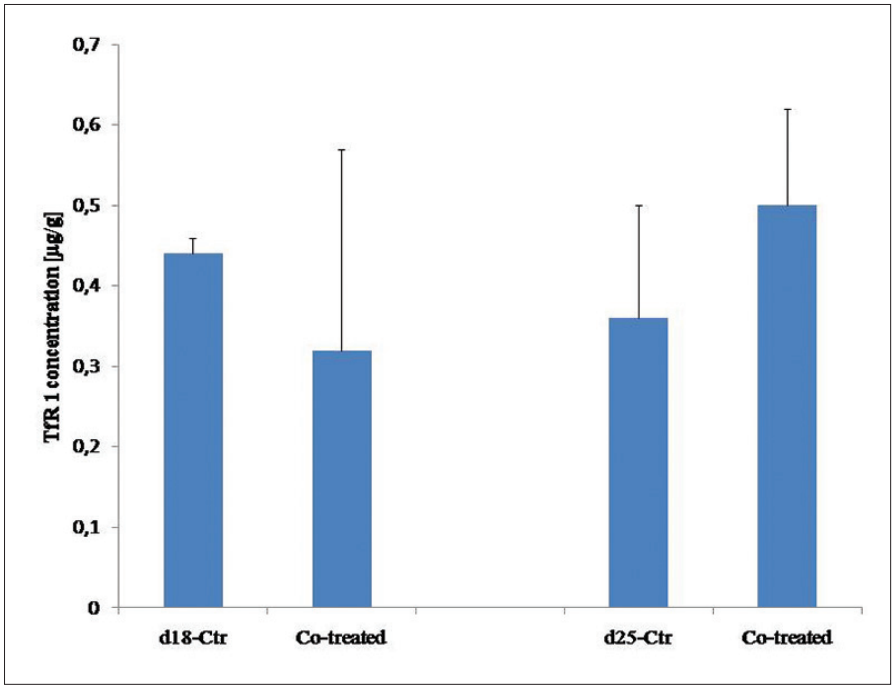

Fig. 1. TfR 1 concentration (ng/g) in skeletal muscle homogenates of day 18 and 25 control and Co-treated mice.

Table 3. Hepcidin concentration (pg/g) in Skeletal Muscle Homogenates of day 18 and 25 Control and Co-Treated Mice

Experimental groups	Control	Co-exposed	Change
Day 18	157.45±10.68	153.20±106.07	~ 2.7% ↓
Day 25	62.35±48.01	183.25±4.31	~ 3-fold ↑

Data are presented as mean ± SD.

Discussion

Prenatal and early postnatal exposure to $CoCl_2$ resulted in a significant time-dependent metal ion accumulation in the muscles of the experimental immature mice. Our results are in agreement with data reviewed by Simonsen et al. [21] showing that Co content in skeletal muscles increases with time. The elevated Co content altered endogenous muscle Fe concentration and induced changes in Fe-regulatory protein expression. Although Fe homeostasis is tightly controlled, its alterations in the skeletal muscles leading to Fe overload are not fully elucidated. Literature data show that Fe content in skeletal muscles of healthy individuals is comparable to that of the liver. High tissue Fe stores are associated with impaired muscle contractivity and muscle endocrine function, insulin resistance and Fe-dependent oxidative stress [6]. The oxidative stress induced by Fe overload results in delayed muscle regeneration with decreased in size myofibres and reduced expression of myoblast differentiation biomarkers [9]. In a mouse model of Fe overload Reardon and Allen observed reduced skeletal muscle weight and reduced exercise capacity [16]. As suggested by Hofer et al. [9] increase in Fe content possibly contributes to muscle atrophy with ageing or disuse. In our study we also find an age-dependent significant increase in Fe content in skeletal muscles of suckling mice upon perinatal exposure to $CoCl_2$. The results suggest that Co administration enhanced Fe accumulation in the target organ. Our results for increased Fe content following $CoCl_2$ exposure of immature mice correspond to our previously published experimental data for other organs [5]. The elevated intracellular Fe may be a source for free radical generation. It may contribute to the generation of hydroxyl radical via Fenton reaction, oxidative modification of proteins and lipids, lipid peroxidation, etc. [10].

Alterations in Fe homeostasis are mediated by changes in Fe-related proteins. Barrientos et al. demonstrate the critical role for TfR1 for skeletal muscle development as its inactivation alters normal muscle energy metabolism [1]. According to Corna et al. TfR1 protein levels are virtually undetectable in healthy skeletal muscle and substantially increase after injury [3]. In addition, Robach et al. show that when hemoglobin content is increased during enhanced erythropoiesis, myoglobin expression in the skeletal muscles is down regulated due to body Fe mobilization and acquisition by the erythrocytes [17]. The same authors also find significant reduction in Fe-related proteins TfR1 and ferritin. These results are in agreement with our data for the reduced TfR1 in day 18 $CoCl_2$-exposed mice. Altered muscle oxygen homeostasis as well as the increased Fe demands for muscle fiber development possibly serve as a feed back mechanism leading to increased TfR1 in day 25 Co-treated mice. As evidenced by Corna et al. [3], TfR1 expression parallels the appearance of novel fibers. This is also suppported by Kobak et al. [11] who demonstrated that in hypoxia, increased expression of Atrogin1 and MuRF1 was associated with an increased expression of TfR1, reflecting intracellular iron demand in cultured *in vitro* rat skeletal myocytes. In our study, the observed elevation of TfR1 level in day 25 $CoCl_2$-exposed mice may be responsible for significantly increased Fe content in these animals. Robach et al. [18] demonstrate increased Fe accumulation and up regulation of TfR1 and ferroportin despite significantly reduced hepcidin levels in healthy volunteers treated with recombinant erythropoietin (rhEpo) suggesting that muscle Fe homeostasis is mediated by other factors as erythropoietin. At systemic level Fe metabolism is regulated by

hepcidin. It is mainly produced in the liver but is also found in the skeletal muscles [13]. Diet and physical activity regulate both iron accumulation and hepcidin expression. The siginificant Fe storage in skeletal muscle tissue of day 25 $CoCl_2$-exposed mice in our experimental model in parallel with the increased hepcidin level suggests that Fe accumulation is possibly regulated by hepcidin-independent mechanism. A markedly increased expression of hepcidin during hypoxia is also demonstrated by Dziegala et al. [4] in cultured skeletal myocytes. Kulik-Rechberger et al. [12] find high hepcidin levels in newborns and hypothesize that it may be due to high ferritin level at that age.

Conclusion

Prenatal and early postnatal exposure to $CoCl_2$ resulted in a significant metal ion accumulation in the muscles of the experimental immature mice altering endogenous muscle Fe content and inducing changes in Fe-regulatory protein expression. The results suggest that changes in Fe homeostasis may be one of the mediators of $CoCl_2$ effects on myogenesis, physical performance, skeletal muscle regeneration and in ageing may contribute to skeletal muscle atrophy.

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