Reproductive Toxicology 59 (2016) 66–79

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Hexavalent chromium affects sperm motility by influencing protein tyrosine phosphorylation in the midpiece of boar spermatozoa

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ARTICLE INFO

Article history:
Received 18 August 2015
Received in revised form 14 October 2015
Accepted 2 November 2015
Available online 12 November 2015

Keywords:
Chromium
Boar
Sperm capacitation
Signal pathway
Protein phosphorylation

ABSTRACT

Hexavalent chromium reportedly induces reproductive toxicity and further inhibits male fertility in mammals. In this study, we investigated the molecular mechanism by which hexavalent chromium affects motility signaling in boar spermatozoa *in vitro*. The results indicated that Cr(VI) decreased sperm motility, protein phosphorylation, mitochondrial membrane potential ($\Delta\Psi m$) and metabolic enzyme activity starting at 4 μmol/mL following incubation for 1.5 h. Notably, all parameters were potently inhibited by 10 μmol/mL Cr, while supplementation with the dibutyryl-cAMP (dbcAMP) and the 3-isobutyl-1-methylxanthine (IBMX) prevented the inhibition of protein phosphorylation. Interestingly, high concentrations of Cr (>10 μmol/mL) increased the tyrosine phosphorylation of some high-molecular-weight proteins in the principle piece but decreased that in the middle piece associated with an extreme reduction of sperm motility. These results suggest that chromium affects boar sperm motility by impairing tyrosine phosphorylation in the midpiece of sperm by blocking the cAMP/PKA pathway in boar sperm *in vitro*.

1. Introduction

Chromium is well recognized as an essential trace element in the diets of domestic animals because it decreases fat and increases lean deposition in the carcass in fodder [1]. However, being a necessary trace element for animals, chromium has a role in maintaining proper carbohydrate, lipid, protein and nucleic acid metabolism by increasing the action of insulin and even has a positive role in enhancing the ability of anti-stress and immunocompetence [2–4]. Therefore, chromium is commonly added to animal feed to enhance the carcass quality, growth performance, and meat quality and to decrease the fat content [5,6]. Research has revealed that dietary supplementation with chromium picolinate throughout gestation can increase the body mass gain, the number of piglets born alive and the concentration of colostrum [7]. Elevated levels of chromium in bone, kidneys, liver and ovaries have been found in pigs fed supplemental chromium [8]. The enrichment of chromium has also been observed in testis [9]. Kidneys have been observed to contain the greatest Cr concentration, while the largest increase in Cr concentration is in the ovaries [8,9]. Importantly, the half-life of chromium, 40 months in serum and 129 months in urine, is long [10]. Therefore, long-term dietary chromium supplementation may easily cause the accumulation of chromium in domestic animal organs. In toxicological research, however, hexavalent chromium has not only been reported to produce acute and chronic toxicity, allergic dermatitis, carcinogenicity, genotoxicity, cytotoxicity, and immunotoxicity, but to also lead to general environmental toxicity [11]. Still, it is unclear whether the long-term feeding of chromium in the diet has adverse effects on livestock. However, there was a lack of negative responses in the growth performance in pigs fed with 5000 μg/kg of supplemental Cr for 75 days [8], but it was proven that tissue development and organ function were seriously affected in rats [12], aquatic animals [13] and humans [14] exposed to chromium. Therefore, the safe use of chromium in animal fodder needs further research.

Chromium could induce reproductive toxicity in the male reproductive system of humans and experimental animals, such as a reduction in sperm count and motility or an increased level of abnormal sperm [15,16]. Moreover, testosterone and gonadotropin levels were significantly altered [9]. The development, morphology and function of testis were severely affected, which significantly decreased the male reproductive capacity [17]. The altered secretion of sex hormones and oxidative stress are the two main factors underlying the reproductive toxicity induced by chromium. It is well documented that testosterone and gonadotropin hormones are responsible for the normal growth and function of accessory

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http://dx.doi.org/10.1016/j.reprotox.2015.11.001
0890-6238/

sex organs [18]. The consequent accumulation of chromium in the testis could disrupt the blood-testis and then affect the normal function of Sertoli cells, causing an increase in FSH levels and a decrease in testosterone levels, which directly regulate sperm number [19,20]. Oxidative stress caused by chromium is well established as a major factor responsible for male infertility [21,22]. Once inside the cell, Cr(VI) ultimately reduces to the Cr(III) form through the formation of reactive intermediates such as the pentavalent and tetravalent forms. High levels of ROS may lead to the generation of oxidative stress, including DNA damage, lipid peroxidation and protein modification [23]. Increased Cr in testis induces tissue damage that impacts spermatozoa formation, and it can directly cause DNA damage in spermatogenic cells and mature sperm [24]. All of these effects decrease sperm numbers, increase an abnormal sperm rate and finally inhibit male fertility [22]. Though many reports have proven that chromium is toxic to the male reproductive system, the effects on domestic animal reproductive systems and the underlying molecular mechanism are still unclear.

Sperm motility, including hyperactivation, is one of the macro-indexes of spermatozoa associated with sperm protein phosphorylation, which is key to successful fertilization and which plays a pivotal role in regulating sperm physiological function, such as sperm capacitation, hyperactivation, acrosome reaction, *etc*. [25,26]. The strengths of hyperactivation and protein phosphorylation are regarded as the two main landmarks of reproductive potential in mammalian sperm *in vitro*. To the best of our knowledge, there have been few reports on the effects on boar sperm caused by chromium *in vitro*. Especially, whether the accumulation of chromium in blood and tissues adversely impacts boar male fertility. Therefore, in the present study, sperm motility and parameters, protein phosphorylation and related enzyme activity were analyzed to explore the effects of chromium on sperm motility and protein phosphorylation in boar sperm *in vitro*. Our study will provide some guidelines for the proper use of chromium in livestock and the exploration of the mechanism underlying reproductive toxicity caused by chromium.

2. Materials and methods

2.1. Materials

Hexavalent chromium (Cr_6^+, K_2CrO_4) was purchased from national medicines (China). Molecular weight markers, *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinoline sulfonamide (H-89), 3-isobutyl-1-methylxanthine (IBMX) and dibutyryl-cAMP (dbcAMP) were acquired from Bio-Rad (USA). PVDF membranes was purchased from Millipore (Billerica, MA). Phospho-PKA substrate (RRXS*/T*) (100G7E) rabbit mAb, anti-phosphotyrosine antibody, anti-α-tubulin antibody, anti-rabbit IgG HRP-conjugated secondary antibody and anti-mouse IgG HRP-conjugated secondary antibody were purchased from Cell Signaling Technology. Alexa 555-conjugated anti-rabbit antibody, Alexa 488-conjugated anti-mouse antibody, peanut agglutinin (PNA) and propidium iodide (PI) were purchased from Molecular Probes (Invitrogen). The chemiluminescence detection kit (ECL) was from GE healthcare. Other chemical products were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Media

The basal medium was modified Whitten's (MW), and the non-capacitating (N-Cap) medium consisted of 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM NaH_2PO_4, 137 mM NaCl, 5.55 mM glucose and 2.0 mM sodium pyruvate. Medium containing 25 mM $NaHCO_3$, 2 mM $CaCl_2$ and 0.4% BSA was designated the capacitating (Cap) medium, as described by Tardif et al. [27]. The pH was adjusted to ~7.4 using 1 mM NaOH. The medium was maintained at 37 °C until the beginning of the experiment, and the pH was checked again and adjusted if necessary.

2.3. Sperm collection and incubation

Semen was routinely obtained from eight mature (2–3 year-old) and sexually mature Duroc boars of the same rearing conditions using the manual method; these boars were selected solely for high sperm quality and proven fertility (over 80% pregnancy rates). Then, the semen were counted and collected by 800 × *g* for 5 min at room temperature, and the sperm pellets were re-suspended. Non-capacitating media was used in the experiment at 37 °C inside a sterile collection recipient and transported to the lab for the experiments. An equal sperm suspension was diluted in different media to a final concentration of 2–5 × 10^7 cells/mL. To study the effects of chromium on boar sperm *in vitro*, we designed three experimental groups. Group I: Cr^{6+} was added to a final concentration of 0.1, 0.5, 1, 2, 4, 6, 8, and 10 μmol/mL in the N-Cap medium. In this group we detected sperm motility, GAPDH activity, ATP level, MMP and protein phosphorylation. Group II: Cr_6^+ was added to a final concentration of 0.1, 1, 10, 20, 50, and 100 μmol/mL in the N-Cap medium. Group III: Cr^{6+} was added to a final concentration of 0.1, 0.5, 1, 2, 4, 6, 8, and 10 μmol/mL in the Cap medium. In group II and III, we detected sperm protein phosphorylation. Group IV: Different concentrations of dbcAMP (0.1, 0.25, 0.5, 1.0 mM) and 1.0 mM IBMX added to the N-Cap medium with the addition of 10 μmol/mL Cr^{6+}, to detect the effects of dbcAMP on boar sperm protein phosphorylation Another treatment group: N-cap, Cap, 0.5, 10, 100 μmol/mL Cr in the N-Cap medium, sperm incubated in this group were used to detect the location and effects of Cr on sperm protein phosphorylation. Then, the sperm were incubated in different media at 37 °C in a humidified atmosphere for up to 90 min [27,28], and gently shake every quarter to prevent precipitation.

2.4. Measurement of sperm kinematics

At the end of incubation or treatment, the sperm kinematics were assessed using light microscopy and CASA (TOX IVOS, Hamilton Thorne Research, Inc., Beverly, MA, USA). Aliquots (5 μL) of sperm samples were pipetted into disposable counting chambers (standard count, 4-chamber, 20 micron slides, Leja, NieuwVennep, the Netherlands). The following parameters were measured: sperm motility (MOT), curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL), progressive (PRO), hyperactivation, amplitudeof lateral head displacement (ALH), beat cross of frequency (BCF), percentage of linearity (LIN), percentage of straightness (STR). Sperm with hyperactivated motility was sorted by the criteria, VCL > 97 μm/s, ALH > 3.5 μm, LIN < 32%, WOB < 71%. (WOB = VAP × 100/VCL, LIN = VSL × 100/VCL, STR = VSL × 100/VAP), established by Harald Schmidt and Günter Kamp [29]. A minimum of 9 fields per sample was evaluated, and an independent observer scored at least 200 cells for each measurement.

2.5. Measurement of GAPDH activity, ATP assay and cAMP assay

2.5.1. GAPDH activity

Boar sperm were collected after 90 min of incubation and washed with cold PBS after treatment with or without SACH, and the supernatant was discarded by aspiration. A total of 600 mL of sonication buffer consisting of 0.3% HCAPS, 150 mM NaCl, 1 mM DTT, 10 mg/mL aprotinin and 10 mg/mL leupeptin was added, and the suspension was sonicated 3 times on ice. The GAPDH enzyme reaction occurred in the mixture described by Welch et al. [30] containing 0.25 mM NAD, 3.3 mM DTT,