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Reproductive toxicology of 2,4 dinitrophenol in boar sperm

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ABSTRACT

The aim of this study was to evaluate the toxic effects of 2,4 dinitrophenol (DNP) on swine spermatozoa; particularly plasma membrane fluidity and integrity, mitochondrial function and motility, including oxidative stress parameters of lipid peroxidation and production of reactive oxygen species, when cooling to 17 °C during 24 to 96 h. We analyzed 22 ejaculates exposed to extender Beltsville Thawing Solution (BTS) (control) and in the same diluent concentrations of 0.01 μM (T1), 0.1 μM (T2), 1.0 μM (T3) and 10 μM (T4) of DNP. Shapiro Wilk test indicated the data was not normally distributed and means were compared using the Kruskal-Wallis test. Statistical analysis showed that DNP does not differ from the control, regardless of storage time, for any of the variables. Finally, quality parameters among the groups did not differ.

1. Introduction

2,4-Dinitrophenol (DNP) is an organic nitro derivative compound, with the chemical formula C6H4N2O5 and whose molar mass is 184.11 g/mol. It is solid at room temperature in the form of yellow crystals and it is slightly soluble in water (The Merck Index, 1989; USEPA, 1993).

DNP was patented in 1892 and is still widely used in agriculture and other industries. At high concentrations it is toxic to humans and animals, primarily by interfering in cell metabolism, due to uncoupling of oxidative phosphorylation (De Felice and Ferreira, 2006). Its basic mechanism is to dissipate the proton gradient formed in the intermembrane space, preventing the formation of ATP (Lodish et al., 1999). The fact that the electron transport chain can proceed with no production of ATP allows increased basal metabolic rates. For this reason DNP was used extensively in the 1930s as a drug for weight loss and as obesity treatment (Parascandola, 1974). However, in 1938 the use of DNP as a drug for human use was banned in the United States by the Food and Drug Administration due to a number of deaths and the development of cataracts in some patients (Parascandola, 1974).

However, it is worrying that DNP is used in agriculture as well as in the paint and metals industries (Volk et al., 1998; Whitacre and Ware, 2004) and in car exhaust release (Nojima et al., 1983). This is because humans and other animals can be exposed to DNP through wastewater, river water, groundwater, soil and in the atmosphere (Leuenberger et al., 1988). DNP doses that can cause systemic damage and/or cause death vary considerably between species and then among individuals, depending on the weight, age and sex (Shea et al., 1983; Mwesigwa et al., 2000; Takahashi et al., 2009). The most notable effect of DNP is hyperthermia, but it is also capable of compromising liver, muscle, eye, and reproductive function. The effects of DNP on reproduction have been studied in rats by Takahashi et al. (2009) who showed that doses of 30 mg/kg of body weight per day (over about 46 days) significantly reduced the number of postnatal live pups, live births and the body weight of newborns. However, there are no reports on the effects of DNP on reproduction of other species.

To evaluate reproductive toxicity in humans, *Sus scropha domesticus* (swine) can be used as a model animal, because they present anatomical and physiological similarity to humans (Dantzer, 1986; Hughes, 1986; Kerrigan et al., 1986). In addition, several studies have shown that pigs sperm can be used efficiently as a biosensor for toxicological evaluation of microbial toxins (Andersson et al., 1998, 2007, 2009; Mikkola et al., 2004, 2007; Kruglov et al., 2009; Peltola et al., 2004) and specifically as for the mitochondria (organelles DNP target) Vicente-Carrillo et al. (2015) found that pig sperm was efficient to understand the toxicity of about 130 substances that disrupt the mitochondria such as arsenic trioxide. Sperm functionality is essential for reproductive success and sperm damage is increasingly used in

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biological tests to predict fertilization ability in ecotoxicological studies (His et al., 1999; Nipper, 2000; Losso et al., 2007; Beiras and Bellas, 2008). Sperm assessment is useful because it is quick and simple in comparison to embryo testing (Anderson et al., 1991).

The aim of this study is to determine the toxic effects of 2,4 dinitrophenol on pigs spermatozoa in different DNP concentrations (0.01; 0.1; 1.0; 10 μM), including the oxidative stress effects on spermatozoa organelles, under cooling to 17 °C for up to 96 h.

2. Materials and methods

2.1. Animals and semen collection

In this experiment we only used biological material collected from commercial animals therefore there was no need for approval by the Ethics Committee and it was only required that a notification was sent to the Ethics Committee on behalf of the Animal Use (CEUA) of the Federal University of Rio Grande. Eleven different males of Landrace and Large White breed were used, totalizing 22 ejaculates (two collections by male). They were housed in individual pens in a commercial artificial insemination center (longitude 51° 57′59″ and latitude 29° 30′07″) and semen was collected by the gloved-hand method. Immediately after collection semen was diluted 1:1 (v/v) in Beltsville Thawing Solution - BTS (Pursel and Johnson, 1975) under isothermal conditions (BTS about 37 °C/physiological temperature), and about 8 h after collection semen was sent under refrigeration (17 °C) to the Laboratory of Animal Reproduction of the Faculty of Veterinary Medicine of the Federal University of Pelotas. Samples that were analyzed presented at least 70% total motility after arrival at the laboratory.

2.2. Pretreatment evaluation and storage

The average concentration of sperm before treatment was 3.10^9 spermatozoa/mL. The samples were evaluated for parameters of motility, plasma membrane integrity, mitochondrial function, plasma membrane fluidity, reactive oxygen species production and index lipid peroxidation by thiobarbituric acid reactive substances (TBARS) to obtain the parameters before centrifugation and dilution in treatments containing DNP. The methodology for evaluation of the each parameter will be covered in detail in the item "Pre-cooling and cooling (24 to 96 h) sperm evaluations".

2.2.1. Preparation of DNP solution

DNP was used in powder form obtained from Sigma-Aldrich (product cod: 34334). DNP was weighed and diluted in BTS in order to generate a concentrated solution of 1000 μM. From this solution (1000 μM) we prepared, by adding BTS, the different concentrations used in the study: 0.01/0.1/1.0 and 10 μM.

2.3. Cooling

The control (only BTS) and treatments containing DNP concentration was 5.10^7 sperm/mL which were obtained after the following procedure: 15 mL of semen were centrifuged at 800*g* for 10 min, in duplicate. The supernatant was discarded and the pellet containing the cells resuspended in BTS. This mixture was added to the control BTS and also the other solutions of BTS plus DNP at final concentrations of: T1 = 0.01 μM, T2 = 0.1 μM, T3 = 1 μM and T4 = 10 μM. After these procedures, the semen was stored in a cooler with controlled temperature at 17 °C and the semen was evaluated from 24 up to 96 h. All chemical reagents used in this experiment were purchased from Sigma Aldrich (Saint Louis, MO).

2.4. Pre-cooling and cooling (24 to 96 h) sperm evaluations

2.4.1. Motility

The motility (percentage of motile cells) was evaluated with an optical microscope with slide and coverslip both preheated to 37 °C (Bearden and Fuquay, 1997; CBRA, 1998). The samples were previously kept in a water bath of 37 °C for 15 min.

2.4.2. Analysis by flow cytometry

Flow cytometry was performed using the Attune Acoustic Focusing Cytometer® (Applied Biosystems). We analyzed the results using the Attune Cytometric Software v 2.1. For detection of cell populations in all analysis, cells were stained with Hoechst 33342 and the population was detected by the photodetector VL1 (filter 450/40). The green fluorescence of H_2DCFDA (ROS), carboxyfluorescein diacetate (plasma membrane integrity), rhodamine 123 (mitochondria functionality) were read with a BL1 photodetector (filter 530/30), the orange fluorescence of the merocyanine 540 (plasma membrane fluidity) was read with BL2 photodetector (filter 574/26). The red fluorescence of propidium iodide (membrane integrity) was read with the photodetector BL3 (640LP filter). Ten thousand events were analyzed per sample at a flow rate of 200 cells/s. The non-spermatozoa debris were eliminated based on scatter plots (Petrunkina et al., 2005; Piehler et al., 2005).

2.4.2.1. Sample preparation for flow cytometry. For all evaluations we added an aliquot of 5 μL of semen and 5 μL of Hoechst 33342 solution (10 mg/mL) to determine spermatozoa population and fluorescent dyes specific for each evaluation. The samples remained in the dark at room temperature (24 °C) for 30 min. After this we added 500 μL of phosphate buffered saline (PBS). The samples were then homogenized and then analyzed by flow cytometry.

2.4.2.2. Plasma membrane integrity. This evaluation was performed by combining carboxyfluorescein diacetate (DCF) and propidium iodide (PI). DCF penetrates spermatozoa and is converted by esterases (enzymes that catalyze the hydrolysis of an ester bond) in viable cells in a non-permeable fluorescent compound that is retained in the cytoplasm, whereas the IP only penetrates the nucleus of cells with injured membrane. Therefore, only the gametes marked with DCF are considered to have an intact plasma membrane and those stained with PI an injured plasma membrane. The working solution used for staining of spermatozoa contained PBS, Hoechst 33342, DCF (1 μg/mL) and IP (50 μg/mL). Sperm were classified as either normal membrane/not injured (DCF+/− IP), and nonviable/dead (DCF+/+ IP/DCF−/IP+). Data was expressed as the percentage of spermatozoa in each category (Fernández-Gago et al., 2013; Gillan et al., 2005).

2.4.2.3. Plasma membrane fluidity. For this evaluation we used the hydrophobic fluorescent dye, merocyanine 540 (M540) at a concentration of 2.7 mM and Hoechst 33342 (10 mg/mL) in PBS. Cells were examined for high fluorescence (fluidity) and low fluorescence (not fluidity). Data was expressed as the percentage of spermatozoa in each category (Fernández-Gago et al., 2013).

2.4.2.4. Functionality of mitochondria. This evaluation was performed using the fluorescent dye rhodamine 123 which stains mitochondria, the more active the mitochondria (with a higher electrochemical potential), the higher the intensity of green fluorescence. Rhodamine was present in the working solution containing PBS, Hoechst 33342 (10 mg/mL) and rhodamine 123 (100 nM). Spermatozoa were classified either as those with more active mitochondria (high fluorescence, higher accumulation of rhodamine) and less active (low fluorescence, lower accumulation of rhodamine). Data was expressed as the percentage of spermatozoa in each category (Gillan et al., 2005).