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Octylphenol induces changes in glycosylation pattern, calcium level and ultrastructure of bank vole spermatozoa *in vitro*

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ABSTRACT

Our previous studies revealed that in bank vole testicular cells octylphenol (OP) action is related either to its binding to estrogen receptors or increasing cAMP level that modulates spermatozoa motility and acrosome reaction (AR), (Kotula-Balak et al., 2011, 2014). To better understand the mechanisms underlying these changes, in the present study we aimed at evaluating the glycosylation pattern, calcium (Ca^{2+}) level and ultrastructure of OP-treated vole spermatozoa. Glycans were recognized by lectins and localized for the first time on the surface of acrosomal cap and tail of vole spermatozoa. Expression and localization of glycans were determined histochemically and analyzed quantitatively. Following OP the expression of glycans markedly changed with concomitant increase of intracellular Ca^{2+} concentration. Altered Ca^{2+} signaling pathway and ultrastructural changes in sperm acrosome region were revealed by immunoenzymatic assay and electron microscope analysis together with hypo-osmotic swelling test, respectively. In detail, AR advancement reflected by the presence of small vesicles in the close vicinity to the sperm head was observed, while tail membrane integrity remained intact.

Our study provides a new insight on OP direct effects on precocious AR of vole spermatozoa through modulation of the glycan expression and its distribution concomitantly with changes in Ca^{2+} signaling pathway and acrosome ultrastructure *in vitro*.

1. Introduction

Current epidemiological reports on declining male reproductive capability led to the "estrogen thesis" that a high level of estrogen may disturb the endocrine control of the male testis. An increase in human and animal exposure to xenoestrogens during the last decades resulted in affected fertility (Sharpe and Skakkebaek, 1993). Clinically, the most common features of testicular dysgenesis syndrome are reduced spermatozoa quantity and quality. There may be absence of spermatozoa (azoospermia), a decrease in the number (oligozoospermia), alterations in morphology (teratozoospermia) and motility (astenozoospermia), and/or changes in the vitality (necrospermia).

Octylphenol (OP) is the estrogen mimicking chemical. It is an important industrial agent that represents potentially harmful effects to both the ecosystem and human health (for review see ECSHA SVHC Support Document, 2011). This chemical is used in detergents, paints and pesticides and as a result of usage detected in rivers, sewage treatment plants and drinking water. No human data are available, although in adult laboratory rodents (e.g. mice, rats, and bank voles) OP has been shown to affect morphology and function of their reproductive systems (Blake and Boockfor, 1997; Hejmej et al., 2011, 2013; Raychoudhury et al., 1999; vom Saal et al., 1998). In contrast generational studies reported no OP-related effects on reproductive measurements including measurements of sperm and reproductive organs in three generations (Tyl et al., 1999).

Multiple studies have confirmed that spermatozoa provide themselves a persisting local source of estrogens, concomitantly being exposed to estrogens in female genital tract (Aquila et al., 2002; Carreau et al., 2012; Kotula-Balak et al., 2004; Lambard et al., 2004; Levallet et al., 1998). It is well documented that estrogens stimulate spermatogenic cell maturation as well as various sperm functions including motility, capacitation, and acrosome reaction (AR), (Adeoya-Osiguwa et al., 2003; Aquila et al., 2003; Idaomar et al., 1989). Moreover, the biochemical changes during capacitation and AR occur rapidly, addressing the nongenomic action of estrogens via estrogen receptors (ERs), (Pedram et al., 2007).

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During epididymal sperm maturation, glycosylation is one of the most important post- translational modifications of sperm surface proteins, involving several intracellular and extracellular biochemical changes in the spermatozoon correlating with acquisition of fertilizing capacity (Srivastava and Olson, 1991; Tulsiani, 2006). Alterations in the plasma membrane accompanying *in vitro* capacitation and the AR, such as removal or appearance of specific proteins or glycoproteins, have been studied using lectins in various mammals (Brandelli et al., 1994; Loeser and Tulsiani, 1999; Miller et al., 1993). Plant and animal lectins, that bind specifically to carbohydrate residues, can serve in easy way to identify the presence of all monosaccharides in surface glycoconjugates. Fucosylated oligosaccharides are detected mainly using *Aleuria aurantia* agglutinin (AAA). The expression of sialic acid (SA) linked α2-6 to terminal galactose (Gal) is often monitored by *Sambucus nigra* agglutinin (SNA) staining while *Triticum vulgare* agglutinin (WGA) binds to SA and/or *N*-acetylglucosaminyl (GlcNAc) residues. Studies by Schroter et al. (1999) and Ma et al. (2012) showed that the distribution and binding of AAA, SNA and WGA binding to the sperm membrane change dramatically during sperm development. In addition, various environmental factors have been shown to modify the expression of lectin-recognized carbohydrate moieties (Kim et al., 2008). According to a recent report by Ferens-Sieczkowska et al. (2013) the loss of sperm fertilization ability is associated with some changes in the glycosylation profile.

Intracellular calcium ions (Ca^{2+}) play a vital role in maintaining spermatozoa motility and AR. In humans, however, an apparently paradoxical effect of Ca^{2+} on spermatozoa motility has been reported (Hong et al., 1984). In the epididymis, Ca^{2+} stimulates motility of immature spermatozoa, whereas in ejaculated sperm motility is inhibited. The systems which regulate intracellular Ca^{2+} concentration in spermatozoa involve the mitochondria, the plasma membrane ATP-dependent Ca^{2+} pump, Na^{+}, Ca^{2+} antiport, and Ca^{2+} channel (Jimenez-Gonzalez et al., 2006). Although, the type of the Ca^{2+} membrane channel involved in its influx during capacitation and AR, together with the mechanism by which this channel is regulated by the steroid agonist, are still unclear (Revelli et al., 1998). It is worth adding that during AR spermatozoon releases acidic material, a signaling event that presumably involves the opening of Ca^{2+} channels and the influx of Ca^{2+} into the sperm cell heads.

Recent study from this laboratory demonstrated that OP affects bank vole sperm motility in relation to reproductive status of the animals (Kotula-Balak et al., 2014). These alterations were associated with the increase in cAMP level and number of spermatozoa with reacted acrosome. The latter have been assessed by histochemical visualization of acrosomal contents. In order to extend the study on bank vole spermatozoa our efforts have been focused on direct effects of OP on the modulation of glycosylation profile and regulation of Ca^{2+} level within the sperm cells. Since the presence of oligosaccharide residues has been reported as important for sperm maturation and the acquisition of the ability to fertilize an ovum (Benoff et al., 2003; Schroter et al., 1999) we determined the expression and distribution pattern of SA, fucose, and GlcNAc oligosaccharides. Finally, to provide new information on a direct OP effect at the subcellular level, the ultrastructural analysis of OP-treated and control (untreated) spermatozoa was performed with the use of a transmission electron microscope.

2. Materials and methods

2.1. Animals

Bank vole (*Myodes glareolus*; formerly *Clethrionomys glareolus*, Schreber) is a unique and useful model to investigate mechanisms of hormonal regulation of the reproductive system in seasonally breeding rodents (Kmicikiewicz and Bilinska, 1997; Tahka et al., 1997). In this laboratory, bank vole colonies have been bred over twenty years (Bilinska et al., 1996; Kmicikiewicz and Bilinska, 1997; Galas et al., 2002). These rodents are long day-breeders. During winter their reproductive system is regressed. Changes in both steroidogenic and spermatogenic functions that appear in spring and autumn control active or regressive phases of the vole reproductive system (Bilinska et al., 2000, 2001; Tahka et al., 1982). In the laboratory, seasonal changes of the photoperiod throughout the year can be mimicked by special light adjustment thus bank voles show similar reproductive characteristics as wild voles. Each generation consists of at least 20 breeding pairs; the male and female in each mating pair do not have common parents or grandparents. This breeding system ensures the heterogeneity of the colony.

Thirty ($n = 30$) mature bank vole males bred in separate cages without females under 18 h light: 6 h dark cycles for more than 20 generations were used. The animal rooms were maintained at a temperature of 18 °C and a relative humidity of 55 ± 5%. Voles were housed in polyethylene cages (42 cm × 27 cm × 18 cm) furnished with sawdust and wood shavings for bedding. A standard pelleted diet (LSM diet, Agropol, Motycz, Poland; total isoflavone content below 450 mg/kg diet) supplemented with seeds of wheat or oat, red beet, apples, and water was provided *ad libitum*. Spermatozoa were separated from both epididymides, diluted 1:20 in M2 medium (Sigma–Aldrich) and counted under hemocytometer. The average of two sperm counts was used to estimate sperm concentration of each individual. Only males with similar sperm concentration ($\sim 60 \times 10^4$ cells/ml) that is characteristic for 2–3 month-old voles were used for the study. Individual samples (from each male) with the final concentration 2×10^6 cells/ml were used for analyses.

Fresh spermatozoa were used for determination of Ca^{2+} levels and tail membrane integrity or were fixed in mixture of glutaraldehyde and formaldehyde for electron microscopy analysis. Air dried sperm smears were processed for examination of glycan expression.

2.2. Ethics of experimentation

Experiments were performed in accordance with Polish legal requirements, under the license given by the Local Ethics Committee at the Jagiellonian University, Krakow, Poland (No. 88/IV/2010).

2.3. Preparation of 4-tert-octylphenol

Stock solutions of 10^{-2} M 4-*tert*-octylphenol (Sigma–Aldrich, St Louis, MO, USA) and 1000 μM 17-β-Estradiol (E2), (Sigma–Aldrich) was prepared in absolute ethanol and stored at −20 °C. Working solutions of OP (10^{-4} M) and E2 (10 μM) and were daily prepared using basic *in vitro* fertilization medium (IVF; ORIGO, Måløv, Denmark) as a diluent and added directly to the cells.

2.4. Isolation and treatment of spermatozoa

Caudal epididymides from each individual were examined and minced in 1 ml of IVF medium in the 60 mm × 15 mm IVF non-pyrogenic, polystyrene cell culture dish (Corning Inc., Corning, NY, USA). Next, the tissues were disrupted with a needle, and spermatozoa were allowed to disperse for 10 min on a warming tray at 37 °C. Afterwards, spermatozoa were incubated for 45 min with 10^{-4} M OP in atmosphere of 5% CO_2 at 37 °C. OP concentration and exposition time were based on previous experiments (Kotula-Balak et al., 2011, 2014). Untreated cells and these treated with less than 0.1% ethanol were served as controls. As a positive control E2 (10 μM, to show clear effect) treatment was used.