In vivo exposure to 17β-estradiol triggers premature sperm capacitation in cauda epididymis

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Abstract

Estrogens play a crucial role in spermatogenesis and estrogen receptor α knock-out male mice are infertile. It has been demonstrated that estrogens significantly increase the speed of capacitation in vitro; however this may lead to the reduction of reproductive potential due to the decreased ability of these sperm to undergo the acrosome reaction. To date the in vivo effect of estrogens on the ability of sperm to capacitate has not been investigated. Therefore, in this study, we exposed mice (n=24) to 17β-estradiol (E2) at the concentration of 20 ng/ml either during puberty from the fourth to seventh week of age (n=8), or continuously from birth for a period of 12 weeks (n=8) at which age the animals from both groups were killed. The capacitation status of epididymal and testicular sperm was analysed by tyrosine phosphorylation (TyrP) antibody (immunofluorescence and western blot) and chlortetracycline (CTC) assay. According to our results, in vivo exposure to increased E2 concentrations caused premature sperm capacitation in the epididymis. The effect of E2, however, seems reversible because after the termination of the exposure premature epididymal sperm capacitation is decreased in animals treated during puberty. Furthermore the changes in epididymal sperm capacitation status detected by TyrP and CTC positively correlate with plasma levels of E2 and the expression of the estrogen-dependent trefoil factor 1 (Tff1) gene in testicular tissue. Therefore, our data implicate that in vivo exposure to E2 under specific conditions leads to the premature capacitation of mouse sperm in epididymis with a potential negative impact on the sperm reproductive fitness in the female reproductive tract.


Introduction

Estrogens play a key role during male reproduction including spermatogenesis (Carreau et al. 2011, 2012) and sperm maturation such as capacitation (Baldi et al. 2009). Estrogen response is mediated through both genomic and non-genomic actions, where the genomic one involves binding to estrogen receptors ERs and nuclear transcription factors that activate the expression of target genes. The non-genomic estrogen action happens through transmembrane receptors such as ERA, ERB and G-protein coupled receptors (GPR30), triggering rapid intracellular signaling pathways, including the activation of serine/threonine and tyrosine phosphorylation (TyrP), leading to sperm capacitation (Kalab et al. 1998, Filardo et al. 2002). As a consequence of phosphorylation on mainly tyrosine residues, which is the key marker of successfully ongoing capacitation (Visconti et al. 1995a,b), the cytoskeleton protein network changes its dynamics, and acrosome reaction (AR) can occur (Yanagimachi 1994). Interestingly, progesterone-induced AR is inhibited by the extra amount of 17β-estradiol (E2) through interaction with a specific non-genomic estrogen receptor on the sperm plasma membrane, suggesting that E2, present at micromolar levels in follicular fluid, may act as a physiological modulator of sperm progesterone response, ensuring the appropriate timing of activation in the fertilisation process (Luconi et al. 1999). In rat and mouse males, the concentration of E2 in blood plasma is 2–25 pg/ml, and it is lower than that in rete testis fluid (250 pg/ml; Free & Jaffe 1979). On the other hand, the concentration of estrogen in ovarian fluid is much higher when compared with the plasma (Free & Jaffe 1979, Hess et al. 1995) and it reaches multiple values depending on the time of the estrus fluctuating between 145 and 2100 pg/ml in rats and mice (Shaikh 1971). Most of our knowledge regarding these processes has been obtained from in vitro studies (Adeoya-Osiguwa et al. 2003, Ded et al. 2010, Sebkova et al. 2012), and for this reason it is really important to also do in vivo experiments.

Recent studies have shown that exposure to environmental estrogens may also influence male fertilising
capability (Akingbemi 2005, Mathur & D'Cruz 2011). This includes chemicals that occur naturally in plants such as genistein and resveratrol (phytoestrogens) and also man-made chemicals such as bisphenol-A, diethylstilbestrol and vinclozolin (xenoestrogens), which can disrupt the endocrine function of animals and negatively influence spermatogenesis and sperm parameters (Peknicová et al. 2002, Kyselova et al. 2003, 2004, Elzeinova et al. 2008). These chemicals again mainly act by binding to estrogen receptors (Kuiper et al. 1998, Sohoni & Sumpter 1998, Akingbemi & Hardy 2001) and trigger a rapid non-genomic signaling response. The key estrogen receptor is ERA as its knock-out mice are infertile due to the disruption of the estrogen action within somatic cells of the reproductive system (Dupont et al. 2000). ERA activity has been demonstrated by expression of the trefoil factor 1 (Tff1) gene (Kim et al. 2000, Park et al. 2012), which is also a marker gene for the analysis of estrogenic activity (Dorosh et al. 2011).

The objective of this study was to test the in vivo effect of E2 on the level of TyrP as a marker of ongoing mouse sperm capacitation and to monitor the Tff1 gene expression as the marker for ERA activity.

Results

In vivo exposure to E2 increases the TyrP of epididymal sperm head proteins

The number of positively labelled sperm heads for TyrP (Fig. 1) at the start of capacitation (time 0) was significantly higher in both experimental groups with pubertal (A) and continuous exposure to E2 (B) compared with the control (Fig. 2). The difference between the control and experimental pubertal E2 group was on the border of significance (P ≤ 0.05). On the other hand, the difference between the control and experimental continuous E2 group was highly significant (P ≤ 0.001) despite the fact that the variance in the continuous E2 samples was higher compared with the control and pubertal E2 samples. Moreover, the difference between pubertal E2 and continuous E2 was also significant (P ≤ 0.01), thus indicating that sperm cells from animals after continual exposure to E2 (continuous E2) are highly different to those after pubertal exposure (pubertal E2) and the control.

In vivo exposure to E2 changes the distribution of chlortetracycline fluorescent patterns in epididymal sperm cells

In addition to the analysis of the sperm head TyrP, chlortetracycline (CTC) analysis was performed to evaluate changes in CTC fluorescent patterns relating to sperm capacitation (Fig. 3). Data from the CTC analysis correlate with the data from TyrP analysis. The control group had the lowest number of cells with CTC pattern B relating to the capacitated status of the sperm (Fig. 3). Similar to the data from TyrP analysis, the number of cells with CTC pattern B (Fig. 3) relating to the capacitated status was significantly higher in both experimental groups compared with the control (Fig. 4). The difference between the control and pubertal E2 was the lowest (P ≤ 0.05), and the difference between the control and continuous E2 was the highest (P ≤ 0.001). Also the difference between the two experimental groups, pubertal E2 and continuous E2, was significant. This fact indicates the significant changes in the distribution of the CTC fluorescent patterns after the termination of E2 exposure in the pubertal E2 group. Together, with the data from TyrP labelling, western blot (WB; Fig. 5) and CTC analyses provide strong evidence for the procapacitation effect of E2 based on the analysis of these two parameters. Also the correlation between the number of TyrP-positive sperm heads and the number of spermatozoa with the pattern B was high (r = 6.683, P = 0.0358).

Figure 1 Immunofluorescent detection of tyrosine phosphorylation (TyrP) in mouse epididymal sperm. (A) TyrP-positive sperm head and (B) TyrP-negative sperm head. Scale bar 20 μm.

Figure 2 Number of capacitated epididymal sperm detected by anti pY antibody (time 0). Control group, pubertal E2 and continuous E2. Bars denote arithmetical means of capacitated cells (%), whiskers denote S.E.M. and points denote individual measurements. *P < 0.05, ***P < 0.001.
The initial differences of the sperm head TyrP are propagated during in vitro capacitation

After analysis of the native epididymal sperm suspension, sperm were capacitated to assess the potential differences in the capacitation progress at individual capacitation times. At the start of the capacitation process, there exist significant differences between the control and experimental groups representing the differences in the phosphorylation of the sperm head proteins directly in the epididymis (Fig. 6). These differences were propagated into the subsequent capacitation times. After 30, 60, 90 and 120 min of capacitation, the differences between the control and experimental groups followed those from the time ‘zero’ representing a positive epididymal sperm head TyrP. The highest, but not statistically significant, difference between the capacitation progress was at 30 min of capacitation (time) for continuous E2 (Fig. 7). This difference indicates the fact that some population of the spermatozoa from animals exposed to E2 is on the border of the capacitation status and after the initial capacitation impulse, this population responds by increasing the total amount of capacitated spermatozoa in the first subsequent capacitation time (30 min;Fig. 7).

The TyrP of testicular epididymal sperm head proteins is not altered after in vivo exposure to E2

To examine the potential effect of E2 exposure on the capacitation status of testicular sperm, the TyrP of epididymal sperm head proteins was analysed (Fig. 9). In the control and both experimental groups, the percentage of TyrP-positive sperm heads was relatively low (about 1.5%) with no significant differences between the groups.

The indicators of capacitation status positively correlate with the serum levels of E2 and expression of Tff1 gene

For the analysis of the connection between the indicators of sperm capacitation status and other relevant physiological parameters of estrogen action, the measurement of E2 serum levels (Fig. 8A) and expression of the estrogen-dependent Tff1 gene (Fig. 8B) were analysed and statistically correlated with the previous results from the sperm analysis. According to the obtained results, the percentage of the TyrP-positive sperm head cells in the epididymis positively correlates both with E2 plasma levels and with the expression of the Tff1 gene in the testicular tissue. The concentration of plasma E2 in the control group and pubertal E2 group was relatively similar with the arithmetical mean around 46 pg/ml and had a s.d. of 17. On the other hand, plasma E2 levels in Continuous E2 group were much higher and reached an average concentration of 3705 pg/ml with an s.d. of 205. The correlation coefficient r between the number of TyrP-positive sperm cells and plasma levels of E2 was 0.549 (P≤0.05), which indicates a high correlation between these two parameters (Fig. 8A). Also the correlation between the expression of the Tff1 gene expressed as Cq values and the number of TyrP-positive sperm were high (r = −0.881, P ≤ 0.001) indicating a strong connection between these two parameters (Fig. 8B).
E2 was selected for this purpose. We have shown that estrogens; therefore, the most common and well-studied stimuli. This type of study required capacitation status can be altered during sperm storage it is important to know whether or not the sperm indicators of the capacitation status in the epididymis, that some of the sperm already express specific their release into the testicular tubule. Owing to the fact exposed to estrogens and various compounds with reproductive tract. On the other hand, sperm are in vivo simulating the conditions in the female reproductive tract, which shows that the effect can be reversible if the exposure is terminated. Despite this process, there are still differences between the control and experimental pubertal E2 groups in the number of cells with capacitated patterns. This situation may be related to the fact that although there are no differences in the plasma concentration of estrogens the concentration in the reproductive tract could be still higher. The higher expression of Tff1 gene in pubertal E2 supports this hypothesis. On the other hand, the molecular changes initiated by estrogens on the level of spermatogenesis and propagated into the subsequent developmental changes of the sperm cannot be excluded, as the gene expression pattern is altered weeks after the exposure and could be propagated by epigenetic mechanisms to subsequent generations. During in vivo exposure the estrogens can also influence the future sperm capacitation ability during but it has to pass through multiple biological barriers before it is able to have a possible effect on epididymal sperm. The normal serum concentration of E2 is at the range of 2–20 pg/ml (Snyder et al. 2009). There is also evidence that in the rete testis the concentration of estrogens is tenfold higher and related to the plasma levels (Free & Jaffe 1979). In our study, the extensiveness of the E2 effect on the sperm physiological parameters positively correlates with the serum levels. We also measured the expression of the Tff1 gene, the specific estrogen response gene in the testicular tissue. The correlation between the expression of this gene and physiological parameters of the spermatozoa was also positive and more significant compared with the plasma levels of E2. Therefore, the extensiveness of the molecular changes in epididymal sperm cells is largely dependent on the plasma and tissue levels of E2. After exposure the phosphorylation status and CTC patterns relating to calcium homeostasis then returned to levels more similar to the control ones, which may show that the effect can be reversible if the exposure is terminated. Despite this process, there are still differences between the control and experimental pubertal E2 groups in the number of cells with capacitated patterns. This situation may be related to the fact that although there are no differences in the plasma concentration of estrogens the concentration in the reproductive tract could be still higher. The higher expression of Tff1 gene in pubertal E2 supports this hypothesis. On the other hand, the molecular changes initiated by estrogens on the level of spermatogenesis and propagated into the subsequent developmental changes of the sperm cannot be excluded, as the gene expression pattern is altered weeks after the exposure and could be propagated by epigenetic mechanisms to subsequent generations. During in vivo exposure the estrogens can also influence the future sperm capacitation ability during

control and experimental groups and also without any significant difference between the two experimental groups (P≥0.05; Fig. 10). Owing to the fact that no significant differences among the groups were observed, the power analysis for the statistical test was performed and it was 0.227.

Discussion

Estrogens play an important role in almost all sperm physiological processes from the production of sperm in the testes to fertilisation in the female oviduct. For the last decade, the effect of estrogens on specific physiological processes in sperm has largely been studied during in vitro experiments. In these experiments, natural and synthetic estrogens have shown a procapacitation concentration-dependent effect on epididymal (mouse; Sebkova et al. 2012) and ejaculated spermatozoa (boar; Ded et al. 2010). Exposure to estrogens during in vitro capacitation provides a good model for simulating the in vivo conditions in the female reproductive tract. On the other hand, sperm are exposed to estrogens and various compounds with estrogenic activity from the time of their production to their release into the testicular tubule. Owing to the fact that some of the sperm already express specific indicators of the capacitation status in the epididymis, it is important to know whether or not the sperm capacitation status can be altered during sperm storage in the cauda epididymis in response to the estrogen stimulants. This type of study required in vivo exposure to estrogens; therefore, the most common and well-studied E2 was selected for this purpose. We have shown that molecular capacitation markers in sperm after in vivo exposure to E2 are similar to those observed in vitro. Both TyrP and CTC pattern distribution were changed in a similar way after in vitro and in vivo exposure.

Contrary to previous in vitro experiments, during in vivo exposure, E2 does not reach sperm cells directly, but it has to pass through multiple biological barriers before it is able to have a possible effect on epididymal sperm. The normal serum concentration of E2 is at the range of 2–20 pg/ml (Snyder et al. 2009). There is also evidence that in the rete testis the concentration of estrogens is tenfold higher and related to the plasma levels (Free & Jaffe 1979). In our study, the extensiveness of the E2 effect on the sperm physiological parameters positively correlates with the serum levels. We also measured the expression of the Tff1 gene, the specific estrogen response gene in the testicular tissue. The correlation between the expression of this gene and physiological parameters of the spermatozoa was also positive and more significant compared with the plasma levels of E2. Therefore, the extensiveness of the molecular changes in epididymal sperm cells is largely dependent on the plasma and tissue levels of E2. After exposure the phosphorylation status and CTC patterns relating to calcium homeostasis then returned to levels more similar to the control ones, which may show that the effect can be reversible if the exposure is terminated. Despite this process, there are still differences between the control and experimental pubertal E2 groups in the number of cells with capacitated patterns. This situation may be related to the fact that although there are no differences in the plasma concentration of estrogens the concentration in the reproductive tract could be still higher. The higher expression of Tff1 gene in pubertal E2 supports this hypothesis. On the other hand, the molecular changes initiated by estrogens on the level of spermatogenesis and propagated into the subsequent developmental changes of the sperm cannot be excluded, as the gene expression pattern is altered weeks after the exposure and could be propagated by epigenetic mechanisms to subsequent generations.

During in vivo exposure the estrogens can also influence the future sperm capacitation ability during
spermatogenesis and sperm storage. Therefore, the analysis of the capacitation status of testicular sperm has been performed in the current study. During this analysis, there were no significant differences in the number of TyrP-positive testicular spermatozoa among the control and experimental groups. Based on the results of a previous in vitro study (Sebkova et al. 2012), it can be assumed that with an elevated in vivo estrogen exposure, capacitation may start in the epididymal spermatozoa. On the other hand, it has been reported that estrogens trigger multiple phosphorylation pathways in the testicular tissue or can change their cell sensitivity (Miyaso et al. 2012). For this reason it is possible to consider that potential molecular changes in testicular sperm, and individual sperm developmental stages can lead to a higher number of capacitated sperm observed in the epididymis. As shown in this study the sensitivity (statistical power) of testicular sperm head TyrP analysis was lower compared with the epididymal one and therefore the potential differences in this parameter in testicular tissue cannot be fully excluded.

The premature molecular changes related to the capacitation process may lead to many consequences after ejaculation and physiological capacitation in the female reproductive tract. The precise timing of individual molecular processes during the capacitation is an absolute prerequisite for successful fertilisation. From the population of millions of sperm there are only tens or hundreds reaching the egg and only one can successfully fertilise it. Already at the start of capacitation, sperm cells are unequal concerning their morphology and ability to reach, penetrate and fertilise the egg. It has been recently shown that higher TyrP can lead to a decreased ability of sperm to undergo AR in the presence of calcium-ionophore (Sebkova et al. 2012). The hyperphosphorylation of sperm proteins may therefore lead to the inability of sperm to undergo AR.

On the other hand, calcium changes detected by the CTC method are prerequisite for physiological AR and egg fertilisation. In general, considering a wide range of mammalian species, including humans, the premature calcium influx induced by estrogens may lead to premature AR without the presence of zona pellucida factors. Together, with the hyperphosphorylation of sperm proteins the percentage of spermatozoa with an ideal physiological status relating to their ability to reach and fertilise the egg strongly decreases.

The population of epididymal spermatozoa is different concerning its maturation and ‘capacitation’ status. This diversity seems to be a good criterion for successful fertilisation, due to the high number of spermatozoa, as it is usual in each sperm population that there is a subpopulation of spermatozoa with ideal molecular characteristics to fertilise the egg. Estrogens and other
compounds with estrogenic activity can affect the balance of the capacitation status directly in the epididymis and through this activity they may decrease the fertilising potential of spermatozoa. After terminating the E₂ exposure, the situation more or less went back to normal, which was close to the status of the control group; therefore, this effect seems to be reversible. Based on our data, it is also clear that in vivo E₂ exposure influences the proportion of spermatozoa with the specific molecular characteristics relating to only the beginning of the capacitation. During the subsequent in vitro capacitation, the differences between the control and experimental groups remained the same as observed at the beginning. It implies that the sperm population, of the exposed animals reaching the egg could have a significantly altered capacitation status and a lower fertilising potential compared with the control ones.

The premature sperm capacitation in the cauda epididymis may be one of the factors bringing a negative effect of the endocrine-disrupting chemicals on the male reproductive functions. It has been recently shown that not only natural but also artificial estrogens significantly affect the sperm capacitation process (Sebkova et al. 2012). Although 17α-ethynylestradiol only seems to act at higher concentrations (≥2 ng/ml), the combination of all substances with potential or confirmed estrogenic activity can further misbalance the physiology of epididymal spermatozoa. Furthermore some artificial compounds with estrogenic activity have a significant effect on sperm physiology at lower doses compared with the strongest natural estrogen such as E₂ (Fraser et al. 2006, Sebkova et al. 2012).

In conclusion, our data imply that in vivo exposure to E₂ leads to premature ‘capacitation’ of mouse sperm in the cauda epididymis with a further potential negative impact on sperm reproductive fitness in the female reproductive tract. This effect is caused mainly by the hyperphosphorylation of sperm proteins and the premature calcium influx. These processes lead to a decreased ability of sperm to undergo an AR. Based on this evidence, in vivo exposure to E₂ can lead to a decrease of the fertilising potential in male mice with significant relevance to endocrine-disrupting compounds with estrogenic activity.

Materials and Methods

Animals and E₂ exposure protocol

Inbred mice (Anlab, Prague, Czech Republic) were housed in an animal facility (Institute of Molecular Genetics, ASCR, v. v. i.). Estrogen-free food and water were supplied ad libitum. The total number of animals in the experiment was 24 in each group, sperm from both epididymides (n=16) of eight animals were analysed (n=8). Male mice were exposed to E₂ (Sigma) at a 20 ng/ml concentration either during puberty from of age (Pubertal E₂) or continuously from birth to 12 weeks of age (Continuous E₂). The control group of mice was not exposed to E₂. At the age of 12 weeks, the animals from all groups were killed by cervical dislocation. All animal procedures were carried out in strict accordance with the Animal Scientific Procedure, Art 2010, and approved by the Local Ethics Committee (approval number 151/2009).

Sperm preparation and capacitation

Mouse sperm cells were released from the distal regions of cauda epididymis into M2 fertilising medium (Sigma) under paraffin oil at 37 °C in 5% CO₂. Sperm viability and motility were checked (under a light microscope). Sperm stock was diluted to the required concentration (5×10⁶/ml) into M2 medium under paraffin oil. Sperm samples were collected at 0, 30, 60, 90 and 120 min of capacitation in vitro. The time marked as 0 was the minimum time required for sperm to be added to the capacitated medium, removed from the medium and washed. This manipulation did not exceed 1 min. These samples served as a negative control. Sperm motility and viability were controlled at every experimental time point.

Immunofluorescent detection of TyrP

Samples of sperm cells were spread on microscope slides. After air-drying, sperm were fixed with 3.7% formaldehyde in PBS (pH 7.34) at room temperature for 10 min, followed by washing in PBS, incubated with 15 mM ammonium chloride (NH₄Cl)
for 5 min, and with 0.1% Triton X-100 for 3 min. Slides were washed with PBS which was followed by immunofluorescent staining. Sperm were blocked with 10% BSA in PBS for 1 h and incubated with primary MAB anti-phosphotyrosine P-Tyr-01 (Exbio, Prague, Czech Republic) diluted 1:500 in 1% BSA in PBS over night at 4°C, followed by Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes, Prague, Czech Republic) secondary antibody 1:1000 in PBS for 1 h. Slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Slides were examined with an epifluorescent microscope. For every experiment, we collected sperm data from eight mice. The positive or negative signal was evaluated from a total of 200 spermatozoa on every slide. In each group, at least two samples were analysed. Data were analysed statistically.

CTC fluorescent assay
Spermatozoa were resuspended in PBS and mixed with an equal volume (45/45 μl) of CTC solution (750 mmol/l CTC in 130 mmol/l NaCl, 5 mmol/l cysteine, 20 mmol/l Tris–HCl, pH 7.8) and incubated for 30 min. Cells were then fixed with 8 μl of 12.5% paraformaldehyde in 0.5 mol/l Tris–HCl (pH 7.4). After incubation, sperm suspension was smeared onto a glass slide and covered with a cover slip. To avoid evaporation and CTC fading, slides were kept in a wet chamber until evaluation. Samples were examined with a Nikon Labophot-2 fluorescent microscope equipped with a 40× Nikon Plan 40/0.65 objective and with a COHU 4910 CCD camera (COHU Inc. Electronics Division, San Diego, USA) with LUCIA imaging software (Laboratory Imaging Ltd., Prague, Czech Republic).

SDS–PAGE with immunoblotting
SDS electrophoresis and immunoblotting technique was used for the TyrP assessment and was carried out using protocols based on standard methods (Laemmli 1970, Zigo et al. 2011). Suspension of non-capacitated sperm from a sperm stock released from cauda epididymis was used. Sperm samples were collected at 0, 30, 60, 90 and 120 min of capacitation in vitro, diluted with PBS and a final concentration of 10⁶ sperm cells was ascertainment using a Bürker chamber. Sperm pellets were resuspended in an equal volume of SDS–PAGE non-reduced sample buffer and heated at 97°C for 3 min. Samples containing protein equivalent to 10⁶ capacitated sperm cells were run on a 5% stacking and 10% running SDS polyacrylamide gel using Precision Plus Protein All Blue standards (Bio-Rad) as molecular weight markers. After transferring proteins onto a nitrocellulose membrane, non-specific sites were blocked with PBS blocking solution (5% skimmed milk and 0.05% Tween 20). Proteins phosphorylated on tyrosine residues were identified by the primary MAB anti-phosphotyrosine P-Tyr-01 (Exbio) diluted 1:500, followed by a peroxidase goat anti-mouse IgG secondary antibody (Sigma–Aldrich) diluted 1:20 000. Protein staining was visualised by chemiluminescence Super Signal West Dura (Thermo Scientific, Prague, Czech Republic). These experiments were performed at least three times with similar results. Representative results are shown.

Gel densitometry was performed with an Aida image analyser 4.18 (Raytest GmbH, Sprockhövel, Germany) and the relative intensity of individual signals was determined.

Quantitative RT-PCR (RTqPCR) of Tff1 gene
The analysis was performed according to the protocol from our previous study (Zatecka et al. 2013). The total RNA from one testis per animal was extracted by a Tri-Reagent Kit (Sigma) according to the manufacturer’s instructions. Isolated RNA was stored at −70°C. The RNA quality and purity was measured on a spectrophotometer Helios A (Thermo Electron Corporation, Marietta, OH, USA). For the synthesis of cDNA, 5 μg purified RNA was used. Each sample was treated with 1 μl DNase I (Invitrogen), 1 μl DNase I reaction buffer (Fermentas, Burlington, ON, Canada) and H₂O to reach a volume of 10 μl. This mixture was incubated for 30 min at 37°C in a Touchgene Gradient Thermal Cycler (Techne, Burlington, NJ, USA). After incubation 1 μl EDTA (Fermentas) was added and followed by incubation at 65°C for 10 min. Then 30 μl reaction mixture (8 μl reaction buffer for M-MuLV reverse transcriptase (Fermentas), 5 μl 10 mM 4dNTP (Ferments), 0.3 μl RiboLock inhibitor (ferments), 1 μl oligo (dT) + random primers (Promega) and 15.2 μl H₂O) were added to the samples. The mixture was incubated for 60 min at 42°C followed by 10 min at 70°C and at the end maintained at 4°C. Obtained cDNA was stored at −20°C. For RT-qPCR – 5× diluted cDNA was used. The qPCR primer pair 5′-TGCCGGGATTTCCGTGGT-3′ (forward) and 5′-CCAGTGCCAGGTGAGGTG-3′ (reverse) specific for the mouse Tff1 gene was used for RT-qPCR. The product length was 131 bp. For each reaction, 2 μl 5× diluted cDNA, 10 μl SYBR Green Master Mix (Fermentas), 0.5 μl primer and 7 μl H₂O were used. All reactions were performed in duplicates in a PCR cycler (Eppendorf, Prague, Czech Republic). The relative amount of mRNA in each sample was calculated from the measured quantification cycle (Cq) values. The expression of the reference gene for ribosomal 18S was used to normalise the measured values. The qPCR primer pair 5′-CCACGCCGTT-GAACCCCATATTCCGTGGT-3′ (forward) and 5′-CCATCCATCGTGGGTTTACGGTAGTGCC-3′ (reverse) with the product length 151 bp was used.

Measurement of the E₂ serum levels
Whole blood from mice was left for 60 min at room temperature. After incubation, the blood samples were centrifuged at 1200 g for 15 min. Serum levels of E₂ were analyzed using a RIA kit (Immunorad Beckman, Prague, Czech Republic). The detection limit was <2 pg/ml and the cross reactivity of antiserum with other serum estrogens was lower than 1%.

Preparation and analysis of testicular suspensions
Testes were placed into a glass homogeniser with 2 ml PBS and homogenised manually to obtain single-cell testicular suspension. After the homogenisation procedure the suspension was filtrated by a Cell Strainer 70 μm (BD Bioscience, Prague, Czech Republic) to remove residues of tough tissue. After
filtration, single-cell testicular suspension was centrifuged (300 g), resuspended in 1 ml 4% formaldehyde in PBS (pH 7.0) and the cells were fixed at RT for 60 min. After fixation, the suspension was centrifuged at 300 g for 5 min, resuspended in 96% ethanol and refrigerated at −20 °C until the time of analysis. Samples of testicular suspensions were resuspended and washed 2× in PBS and spread on microscope slides. After this procedure, the protocol was the same as for the immunofluorescent detection of TyrP on epididymal sperm, but only 100 cells per slide were counted.

Statistical analysis

Experimental data were analysed using STATISTICA 6.0 (Statsoft, Prague, Czech Republic) and GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA). The differences between the control and experimental groups in the number of the capacitated cells (TyrP positive sperm heads and CTC pattern B, Figs 2, 4, 6 and 7) were analysed by KW-ANOVA, and post hoc analysis was performed by Dunn’s comparisons: *P<0.05, **P<0.01, ***P<0.001. The calculated Pearson coefficients (r) were tested for their significance (P≤0.05, Figs B and 9). The statistical power of the testicular sperm analysis was performed by STATISTICA 6.0 power module and was calculated for the ANOVA test.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


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