The slower the better: how sperm capacitation and acrosome reaction is modified in the presence of estrogens

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Abstract

In order for mammalian sperm to obtain a fertilizing ability, they must undergo a complex of molecular changes, called capacitation. During capacitation, steroidal compounds can exert a fast nongenomic response in sperm through their interaction with plasma membrane receptors, and activate crucial signaling pathways leading to time-dependent protein tyrosine phosphorylation (TyrP).

Estrogen receptor beta was detected in epididymal mouse sperm; therefore, the effect of 17β-estradiol, estrone, estriol, and 17A-ethynylestradiol on mouse sperm capacitation in vitro was investigated. The effect was evaluated by positive TyrP in sperm heads and in the whole sperm lysates. Simultaneously, the state of the acrosome after the calcium ionophore-induced acrosome reaction was assessed. Generally, estrogens displayed a time and concentration-dependent stimulatory effect on sperm TyrP during capacitation. In contrast, the number of sperm that underwent the acrosome reaction was lower in the experimental groups. It has been demonstrated that both natural and synthetic estrogens can modify the physiological progress of mouse sperm capacitation. The potential risk in the procapsatation effect of estrogens can also be seen in the decreased ability of sperm to undergo the acrosome reaction. In conclusion, the capacitating ability of sperm can be significantly lowered by increasing the level of estrogens in the environment.

Introduction

The ejaculated mammalian sperm are unable to fertilize the egg. The spermatozoa must undergo a maturation process that is known as capacitation, and it takes place in the female reproductive tract. Only capacitated sperm are able to successfully fertilize the egg (Austin 1952). These morphological and biochemical changes allow sperm to bind to egg zona pellucida and undergo the acrosome reaction (Yanagimachi 1994). One of the processes triggering capacitation is a spontaneous efflux of cholesterol from the sperm plasma membrane. This increases its permeability and fluidity and activates intracellular signalization pathways leading to the activation of adenylyl cyclase, cAMP, cAMP-dependent protein kinase A, protein tyrosine kinase, and consequently to protein tyrosine phosphorylation (TyrP; Visconti et al. 1995). The TyrP of proteins with a molecular weight (MW) between 40 and 120 kDa became an indicator of successfully ongoing capacitation and the ability of sperm to undergo the acrosome reaction (Visconti et al. 1995a). The other initiating process leading to the capacitation and acrosome reaction (AR) is the binding of estrogens onto estrogen receptors (ERs).

Estrogens play an important role in both the female and male reproductive systems (Free & Jaffe 1979). They regulate cell function through ERs (ERA and ERB; Lubahn et al. 1993), which belong to a superfamily of ligand-activated transcription factors (Evans et al. 1987) and they are localized in the cytoplasm and nucleus of somatic and germinal cells. The nuclear receptors activate gene transcription by binding to DNA regulatory sequences (Hall et al. 2001). However, many nuclear receptors are translocated to the sperm plasma membrane and facilitate not only the gene transcription but also the rapid nongenomic signalization pathways (Pedram et al. 2007). Beside these, in sperm G protein-coupled receptors such as GPR30 are reported that trigger not only a rapid nongenomic signalization but also act independently of ERs (Filardo & Thomas 2005). Estrogens are mediated through rapid intracellular signaling in sperm by tyrosine and ser/thr kinases, influencing capacitation and acrosome reaction by activating phosphorylation of several proteins (Naz & Rajesh 2004). Estrogens activate ERs and consequently initiate several intracellular signaling enzymes such as receptor tyrosine kinase, epidermal growth factor receptor phophatidylinositol 3-kinase, Src kinases,
mitogen-activated protein kinase (MAPK), protein kinase C, phospholipase D, and phospholipase C. This rapid pathway through MAPK is required for regulating protein phosphorylation in sperm (Aquila et al. 2004). Epididymal sperm are believed to have no transcription activity; therefore, a rapid nongenomic signalization plays the main role in this stage.

The concentration of estrogens quantitatively differs between males and females, and it is species specific, as well as the expression of steroid receptors. In males, the concentration of estrogens in the blood plasma is in a range of 2–180 pg/ml and depends on the species. In rat and mouse, the concentration of 17B-estradiol (E$_2$) is 2–25 pg/ml, and it is lower than in that rete testis fluid (250 pg/ml). The same applies to females where estrogen concentration in the ovarian fluid is at least twofold higher compared with plasma (Free & Jaffe 1979, Hess et al. 1995), and in rat and mouse it fluctuates during the estrus, e.g. for E$_2$ between 145 and 2100 pg/ml (Shaikh 1971). Besides physiologically endogenous estrogens, organisms including humans are exposed to environmental estrogens, which can display false hormone like activity. It is striking that the wastewater treatment technologies are not efficient enough to prevent a further contamination of surface water supplies (Kusk et al. 2011). There is enough evidence in literature showing a positive correlation between rising concentrations of environmental estrogens and increasing reproductive abnormalities (Storgaard et al. 2006) even their extremely low concentrations (ng/ml) can have an adverse effect on reproduction.

In this study, we localized ERB in mature mouse spermatozoa from cauda epididymis. In consequence to this finding, we aimed to look in detail, whether, selected estrogens (E$_2$, estrone, estriol (E$_3$), and 17A-ethynylestradiol) can influence sperm ability to capacitate and undergo induced acrosome reaction in vitro. Monitoring of TyrP of specific proteins (MW 40–120 kDa) in the mouse sperm head and tail was taken as a marker of successfully ongoing capacitation reflecting male reproductive fitness.

Results

**ERB is present in the epididymal mouse spermatozoa**

As shown in Fig. 1, ERB was detected by chemiluminescence in a whole sperm lysate obtained from cauda epididymis. A single band was marked on a nitrocellulose membrane corresponding to a MW of 64 kDa. ERB was also detected by immunofluorescent labeling in fixed mouse spermatozoa from distal regions of cauda epididymis. ERB was clearly localized as a thin sickle over the apical acrosomal region of the sperm head protruding as far as the apical hook (Fig. 2A). The staining pattern was markedly speckled and uniformly present in all intact spermatozoa.

**Estrogens increase protein TyrP in the sperm head during capacitation in vitro**

The positive staining for TyrP in epididymal mouse sperm was detected as a compact signal over the whole apical acrosomal region of the sperm head. There was also invariable positive labeling detected in the sperm tail, localized in clusters in the mid and principal piece (Fig. 2B). All sperm that displayed positive head TyrP displayed always a positive tail phosphorylation staining as well.

In general, using immunofluorescent labeling, there was a dose-dependent increase in the number of sperm,
positive for TyrP in the sperm head during capacitation in vitro in the presence of E$_2$, estrone, E$_3$, and 17A-ethynylestradiol (Table 1). Results are shown for E$_2$, as a linear time kinetics (Fig. 3A), and a log concentration kinetics (Fig. 3B) of positive sperm head TyrP during capacitation.

The sperm head TyrP increase in the presence of all estrogens occurred already at 30 min of capacitation (Fig. 3C) and carried to over 60 and 90 min, or even up to 120 min in case of 17A-ethynylestradiol (Table 1). In comparison with control, the two highest concentrations (20 and 200 ng/ml) of E$_2$, estrone, and 17A-ethynyles-tradiol significantly increased the protein TyrP compared to E$_3$, which was effective only at lower doses (0.02, 0.02, and 2 ng/ml; Table 1; Supplementary Figure 2C and D, see section on supplementary data given at the end of this article). Based on selected concentrations in presence of E$_2$ (Fig. 3A and B) and estrone (Supplementary Figure 1A and B, see section on supplementary data given at the end of this article), there was an increased activity of sperm signaling pathways leading to phosphorylation of specific sperm head proteins during capacitation in higher concentrations (0.2, 2, 20, and 200 ng/ml). Interestingly, compared to the natural estrogens, the stimulatory effect of synthetic 17A-ethynylestradiol on protein TyrP lasted until 120 min of capacitation, especially in the presence of the two highest concentrations (20 and 200 ng/ml; Supplementary Figure 1E and F).

**Table 1** Effect of estrogens on protein tyrosine phosphorylation in mouse sperm head during capacitation.

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
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<td></td>
<td></td>
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<td>10.719±1.929</td>
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<td>20</td>
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<td>15.769±1.697</td>
<td>14.834±1.772</td>
<td>15.229±1.656</td>
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<td></td>
<td></td>
<td></td>
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</tr>
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<td>Control</td>
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<td>11.304±0.632</td>
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<td>10.994±0.858</td>
<td>10.501±0.726</td>
<td>9.653±0.897</td>
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<td>17A-ethynylestradiol</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>12.567±0.500</td>
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<td>11.954±0.600</td>
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<td>0.02</td>
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<tr>
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<td>17.310±0.373</td>
<td>17.450±0.420</td>
<td>18.439±0.580</td>
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</tr>
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</table>

Percentage of positive sperm head tyrosine phosphorylation. Mean±S.E.M. Differences were analyzed by KW-ANOVA; post-hoc analysis was performed by multiple comparisons of mean ranks. *P<0.05, and †P<0.001, comparison with control.
which were capacitated for 30 min and then further incubated for the standard 60 min with CaI (Supplementary Figure 2B and C). The same effect as in the case of natural estrogens was also observed for 17A-ethynylestradiol for the highest concentration of 200 ng/ml after 30 min sperm capacitation with a further incubation with CaI for 5 min (Fig. 3D). Moreover, statistically significant differences between 17A-ethynylestradiol compared with the control, as well as to other natural estrogens, were persisting for 2, 20, and 200 ng/ml over 30 and 60 min capacitation with a further incubation with CaI for the standard 60 min (Supplementary Figure 2B and C). The comparison between estrogens during CaI-induced acrosome reaction is shown for the concentration of 2 ng/ml (Supplementary Figure 2D).

**Estrogens increase overall sperm protein TyrP during capacitation in vitro**

As shown in Fig. 4, natural estrogens increase the number of protein bands phosphorylated on tyrosine residues as well as the staining of certain bands. Compared with the control, all concentrations of E2, estrone, and E3 increased TyrP. 17A-ethynylestradiol, increased TyrP at the three highest concentrations (2, 20, and 200 ng/ml). The increase of TyrP occurred mainly in the key times of capacitation between 60 and 90 min.

The SDS–PAGE results from the whole sperm lysate obtained during set time points of capacitation in vitro show an increasing effect, dependent on the time of capacitation and estrogen concentration. A higher concentration of estrogens in the capacitating medium and a greater number of proteins phosphorylated on tyrosine residues were detected. If comparing the differences between the amounts of protein TyrP changed in response to selected concentrations of estrogens during capacitation in vitro, 200 ng/ml of all selected estrogens led to an increased TyrP in the sperm in capacitation times of 30, 60, and 90 min. For estrone, E3, and 17A-ethynylestradiol, the increasing level of

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**Figure 3** Effect of estrogens on mouse sperm tyrosine phosphorylation and acrosome reaction. (A) Linear time kinetics of a range of 17B-estradiol (E2) concentrations on positive sperm head TyrP. (B) Log concentration kinetics of 17B-estradiol at various time of capacitation. (C) Log concentration kinetics of the four studied estrogens at 30 min of capacitation. (D) Log concentration kinetics of the four studied estrogens at 30 min of capacitation and 5 min CaI-induced acrosome reaction. (E) Linear time kinetics of a range of 17B-estradiol concentrations on acrosome reaction up to 90 min. For all panels, data represent an average of five replicates as described in ‘Materials and Methods’ section. Mean ± S.E.M. are show in Tables 1 and 2 due to a clear transparency of all graphs. The arithmetic mean of total S.E.M. was 0.970 and the S.D. of the mean of S.E.M. was 0.553. In logarithmic graphs, the plotted value for 0.0001 ng/ml represents the zero control.
TyrP was persisting up to 120 min of capacitation. The 20 ng/ml concentration elevated the protein TyrP in both estrone and E3 at 30, 60, 90, and 120 min of capacitation. Unlike estrone and E3, 20 ng/ml E2 and 17A-ethynylestradiol triggered an increase of TyrP in 60 and 90 min. The concentration of 2 ng/ml gave similar results for all estrogens when E2 and estrone TyrP were elevated in 30, 60, and 90 min for E3 in 30 and 60 min and for 17A-ethynylestradiol in 60 and 90 min. Very similar results were obtained for the 0.2 ng/ml concentration of estrogens, where E2, estrone, and E3 significantly increased TyrP at 30 and 60 min and estrone at 60 and 90 min of capacitation, when comparing the results of the lowest 0.02 ng/ml concentration of estrogens, E2, estrone, and E3, elevated TyrP at 60 min and E3 at 30 and 60 min of capacitation. 

The results show a very similar response to selected concentrations of all four estrogens in terms of the detection of protein TyrP from the whole sperm lysate at experimental capacitation times. The general pattern of estrogen response resulting in elevated TyrP can differ slightly between capacitation times but usually the final outcome of elevated TyrP is overlapping at standard key times of mouse capacitation, which are between 60 and 90 min.

Differences between control samples and samples of sperm affected by different concentrations of estrogens were detected by gel densitometry (see ‘Materials and Methods’ section).

**Comparison between spontaneous and Cal-induced acrosome reaction in control**

The proportion of spontaneous and Cal-induced acrosome reaction in control sperm samples at all experimental times during sperm capacitation *in vitro* in shown in Fig. 5. There was a significantly increased number of acrosome-reacted sperm in the Cal-induced group (Mann–Whitney *U* test, **P**=0.0043/35 min, ***P***<0.001/90 and 120 min) compared with a group with spontaneous acrosome reaction rate for all experimental times except the time zero representing less than a minute with or without an induction of acrosome reaction.

**Discussion**

Estrogens were considered only as female hormones, but they play an important role in the male reproductive system too. Testosterone and androstenedione are...
precursors for the synthesis of $E_2$ and estrone by cytochrome P450 aromatase. Consequent changes take place in the liver, where estrone is changed to $E_3$. This implies that estrogens are also produced in males and not only in females; therefore, they influence male reproductive parameters (Broeder et al. 2000). An estrogen action and specific cellular response are triggered through binding of these hormones to complementary ERs. ERs play an important role in activating signaling pathways leading to sperm capacitation, essential for further successful fertilization. ERs have been detected in the sperm of many species, for example, human (Aquila et al. 2004, Solakidi et al. 2005), boar (Mutembei et al. 2005), rooster (Kwon et al. 1995), rat (Pellettier et al. 2000), but failed to be detected so far in mouse spermatozoa. In this study, the 64 kDa protein corresponding to ERB was detected to be present in sperm from cauda epididymis using immunoprotein detection. Moreover, localization of ERB was defined by immunofluorescent labeling as a thin sickle localized over the apical region of sperm head, covering also the apical hook region. While the presence of ERB on mature mouse epididymal sperm was confirmed, it could be considered to take part in activating signaling pathways leading to TyrP, during capacitation. GPR30 receptor could be another receptor responsible for interaction with estrogen hormones in sperm (Aquila et al. 2004); however, there is a recent work questioning its $E_2$-mediating role in mammalian reproductive organs (Otto et al. 2009).

![Figure 4 Increasing effect of estrogens on protein TyrP during capacitation in vitro. Estrogen concentrations 0.02, 0.2, 2, 20, and 200 ng/ml. Time of capacitation 0, 30, 60, 90, and 120 min. Every sample contained a protein equivalent of $10^6$ cells. Representative results shown.](image-url)
Protein TyrP that occurs during mammalian sperm capacitation is crucial for sperm to obtain the ability to undergo acrosome reaction. These events are, therefore, indicators of the sperm reproductive fitness leading to successful fertilization of the ova. TyrP is regulated by different intracellular pathways (Visconti et al. 2002) and these pathways are triggered by ligand-activated steroid and non-steroid receptors. Recently, the effect of estrogenic compounds on the reproductive potential of sperm has been studied (Breitbart & Etkovitz 2010, Carreau & Hess 2010). This paper addresses the question, whether a rising concentration of estrogens in vitro. Based on the presented results it can be concluded that these studied estrogens significantly stimulate the capacitation progress in a concentration-dependent manner. The number of sperm capable of undergoing head TyrP, as well as the overall TyrP, was generally higher compared with the control. Except E3, all other estrogens increased the TyrP mainly at higher experimental concentrations. However, interestingly with E3, this effect was observed mainly in the two lowest concentrations after 30 min of capacitation and it could be interpreted as a so-called U-shape effect. The dose–response relationships of certain hormones are often nonlinear and are characterized by the dose–response relationship displaying low-dose stimulation and high-dose inhibition (Calabrese & Baldwin 2002).

Synthetic estrogen 17A-ethynylestradiol affected TyrP in the sperm head in all experimental times and its effect is different from other estrogens. Its influence on TyrP does not weaken during the capacitation period and the effect is displayed even after 120 min. 17A-ethynylestradiol compared to natural estrogens shows great stability, thanks to three strong bonds in its molecule, which make it extremely stable, and worsen its degradation in liver.

The percentage of sperm with positive head TyrP in our control samples was 8–12% in contrast to experimental groups where TyrP increased up to 18%. These results correlate with recent studies and denote that during sperm capacitation the percentage of sperm head TyrP in control is ~9% compared to 3% TyrP in sperm heads when incubated for 90 min in non-capacitating medium (Asquith et al. 2004). However, one could expect that in the beginning of the sperm capacitation the amount of sperm positive for sperm head TyrP would be lower than that in a fully capacitated population. This was not, however, so obvious in our study. This could be explained by the fact that even at the starting time a sperm suspension was exposed to a complete capacitating medium for almost a minute; therefore, sperm were still subjected to all relevant ions and proteins. For this reason, we cannot rule out the possibility that signaling pathways could have been activated. Also, in our study, the sperm from a very distal region of cauda epididymis was used in contrast to study of Asquith et al. (2004) who used sperm from the whole cauda epididymis.

It needs to be stated that TyrP like capacitation does not take place synchronously (Stewart-Savage 1993); however, only those fewer than 15% of free-swimming population of sperm that are tyrosine phosphorylated can recognize zona pellucida.

The presented results show that estrogens in general increase sperm TyrP, however, each estrogen can trigger a response of different strength with respect to its concentration and also capacitation time. A possible explanation of a non-identical estrogenic response may be due to the fact that estrogens activate diverse types or parts of signaling pathways, or bind to and activate different receptors triggering pathways leading to sperm TyrP during capacitation.

Simultaneously the effect of estrogens on the number of acrosome-reacted sperm after Cal-induced acrosome reaction was studied. Cal is usually used for the activation of releasing lytic proteins from the acrosome and simulates the in vivo zona pellucida triggered acrosome reaction (Yamagata et al. 1998). All studied estrogens affected the onset of acrosome reaction; however, in this case, estrogens significantly reduced the percentage of sperm that completed the acrosome reaction. These results correlate with recent studies (Baldi et al. 2000, Vigil et al. 2008), which show a decreased ability of sperm to undergo the acrosome reaction after their capacitation with E2. Similarly, our results summarizing the state of the acrosome after Cal-induced acrosome reaction correlate with an elevated amount of sperm head TyrP during sperm capacitation in presence of estrogens. On the other hand, it was presented (Adeoya-Osiguwa et al. 2003) that E2
stimulated acrosome reaction in uncapacitated sperm, but in capacitated sperm it had no effect. These results, however, are not in contrary to ours, as the acrosome reaction was not initiated in uncapacitated sperm, and moreover, no changes between the experimental groups and controls were observed after 90 min of capacitation, when this is more or less complete. The observed decrease of sperm ability to undergo acrosome reaction was obtained for sperm after 30 min of capacitation. On the other hand, those sperm capacitated with natural estrogens for a longer period did not show differences to the control, suggesting either the slowing down of capacitation or an effect that is lost or compensated in fully capacitated sperm. This was not the case, however, with 17A-ethynylestradiol, where a decreased acrosome reaction persisted in sperm capacitated for 60 min, when mouse capacitation is completed. This proposes its prolonged adverse effect on the sperm-fertilizing ability.

In mice, there is a high spontaneous acrosome reaction (Johnson et al. 2007), whose rate depends on time of capacitation. The proportion of spontaneous and Cal-induced acrosome reaction gives, therefore, important information to assess the magnitude of the actual effect of estrogens on Cal-induced acrosome reaction. The decrease of sperm ability to undergo the induced acrosome reaction in presence of selected estrogens falls in early capacitating times below the spontaneous acrosome rate, which may emphasize the actual effect of estrogens on ability of sperm to undergo the acrosome reaction.

The results of TyrP from immunofluorescent analysis were confirmed by immunoprotein detection of the whole sperm samples. The characteristics of specific changes in the sperm head are crucial for judging the sperm ability to fertilize (Stewart-Savage 1993). For this reason, we have predominantly focused on the effect of different concentrations of selected estrogens on the TyrP in the sperm head. On the other hand, the status of the whole sperm considering TyrP in the flagellum could not be ignored. The SDS–PAGE results show that selected estrogens increase the overall protein TyrP in the whole sperm lysate during capacitation in vitro. In correlation with previously published results (Visconti et al. 1995a), our control protein samples showed a time-dependent increase in TyrP during mouse sperm capacitation. The results of protein phosphorylation from sperm capacitated with selected estrogens showed a significant increase in the number of proteins phosphorylated on tyrosine residues in higher concentrations for E2, estrone, and 17A-ethynylestradiol, and in a lower concentration for E3. As protein TyrP is ongoing in the sperm head and the flagellum, results from immunoprotein detection of the whole sperm lysate samples and the immunofluorescence detection of TyrP in the sperm head cannot be fully compared. Nevertheless, some correlation could be made and a parallelism can be seen between these two groups of results.

Comparing currently obtained results, it is clear that each of the selected estrogen significantly affects the two ‘markers’ of sperm capacitation such as TyrP and a consequent acrosome reaction in the opposite way. Before sperm–egg fusion, sperm undergoes molecular changes, which are indicated by the status of TyrP (Visconti et al. 1999), activation of acrosome response, and sperm binding to zona pellucida (Baldi et al. 2002). These processes strictly follow one another in a precisely set order. Among many, TyrP triggers actin polymerization, which prevents the premature fusion of the plasma and outer acrosomal membranes leading to acrosome reaction (Brener et al. 2003). If the ERs are hyperstimulated by an excessive amount of estrogens, the activity of the protein tyrosine kinase is elevated and the phosphorylation on tyrosine residues remains triggered. Therefore, the phospholipase D is constantly activated leading to a consequent polymerization of actin (Breitbart et al. 2005). During the acrosome reaction, the activation of phospholipase C leads to an increase of intracellular Ca$^{2+}$ ions causing actin depolymerization. However, if in the beginning of the acrosome reaction the phospholipase D remains constantly upregulated by protein tyrosine kinase, the activity of protein kinase C can be possibly delayed and depolymerization of actin slowed down. The timing of all the processes involved in the molecular changes leading to capacitation and acrosome reaction is very important and inaccurate time setting of consecutive events can result in a reduction in the sperm-fertilizing ability.

Spermatozoa face on their journey through the female reproductive tract under different concentrations of estrogen, similar to those selected in our in vitro experiments, depending on the phase of the cycle. Estrogens are low during the preovulatory stage and E2 is associated with sperm longevity (Mbizvo et al. 1990), but the situation changes at the time of ovulation, when the concentration of estrogens released with follicular fluid rises (Shaikh 1971). In correlation to our result, the capacitation and acrosome reaction are modulated by higher concentrations of estrogens and these can serve as a sperm-specific selection barrier where inhibiting effect of estrogens on induced acrosome reaction may have a physiological relevance (Vigil et al. 2008). Therefore, estrogens may be seen as one of many preferred cryptic female mechanisms to select the best possible sperm to fulfil the task.

In conclusion, this study shows an existence of ERB in mature mouse spermatozoa. It also provides evidence that estrogens significantly stimulate the capacitation progress in a concentration-dependent manner. On the other hand, estrogens decrease the number of acrosome-reacted mouse sperm after the induced acrosome reaction. Based on our results, it can be inferred that a raising concentration of estrogens in the environment may represent a potential risk in altering certain mechanisms contributing to the fitness of sperm fertilization.
Materials and Methods

Animals

Inbred BALB/c mice were obtained from a breeding colony of the Laboratory of Reproduction, Faculty of Science, Charles University in Prague or purchased directly from Velaz (Unetice, Czech Republic). Mice were housed in the animal facilities and food and water were supplied ad libitum. Male mice used for all experiments, were in a reproductive age of 10–12 weeks. All animal procedures were carried out in strict accordance with the Animal Scientific Procedure, Art 2010, and subjected to review by the Local Ethics Committee.

Capacitation

Sperm from the distal regions of cauda epididymis were released into M2 fertilizing medium (Sigma–Aldrich) under paraffin oil in 37 °C in 5% CO2.Released sperm were assessed for motility and viability. Sperm stock was diluted to the required concentration (5×10^4/ml) into M2 medium under paraffin oil with five different concentrations of selected estrogens (0.02, 0.2, 2, 20, and 200 ng/ml). Sperm capacitated in M2 medium without added estrogens were used as a control. 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 being added to capacitated medium, removed out of the medium, and washed. This manipulation did not exceed 1 min. These samples served as a negative control. Sperm motility and viability were assessed at every experimental time point.

Immunofluorescence detection of ERB

The distal region of mouse cauda epididymis was placed in 37 °C PBS in 5% CO2, for 10 min. Released sperm were washed in PBS, smeared onto glass slides, air-dried, and fixed with methanol for 7 min at −20 °C followed by 10 min in 0.2% Triton X-100. For immunofluorescent labeling, sperm were blocked with 3% BSA in PBS for 1 h, followed by incubation with the primary antibody BERB H150 (sc-8974, Santa Cruz Biotechnology, Heidelberg, Germany; 1:50) in PBS at 4 °C, followed by a secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H+L; A11008, Molecular Probes, Grand Island, NY, USA; 1:1000) in PBS. Slides were mounted into a Vectashield Mounting Medium with DAPI (Vector Laboratories, Peterborough, UK). Samples were examined with an Olympus IX81 fluorescent microscope and photographed with a Hamamatsu ORCA C4742-80-12AG, using Olympus Soft Imaging Solutions Software.

Immunofluorescent detection of TyrP

Sperm smears were air dried and fixed with 3.7% formaldehyde in PBS pH 7.34 at room temperature for 10 min, followed by washing in PBS, incubation with ammonium chloride (NH4Cl) 15 mM for 5 min, and with 0.1% detergent Triton X-100 for 3 min. Slides were washed with PBS and left in PBS with sodium azide (NaN3) at 4 °C. For immunofluorescent labeling, slides were blocked with 10% BSA in PBS for 1 h and incubated with primary MAB anti-phosphotyrosine (PTyr 01 112630025, Exbio Antibodies, Prague, Czech Republic) diluted 1:500 in 1% BSA in PBS for 2 h, followed with Alexa Fluor 488, donkey anti-mouse IgG (A21202, Molecular Probes) secondary antibody 1:1000 in PBS for 1 h. Irrelevant isotype-matched MABs and secondary antibodies (used without primary antibodies) served as negative controls. The MAB MM10 anti-mouse CD46 (HM1118, HyCult, Uden, The Netherlands), which recognizes an acrosome-associated antigen, was used as a positive control. Slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). Slides were examined with an epifluorescent microscope (as stated above). For every experiment we collected sperm data from 10 mice. The positive or negative signal was evaluated from a total of 200 sperm on every slide. In each group, at least five samples were analyzed. Data were analyzed statistically by Kruskal-Wallis ANOVA (KW-ANOVA); post-hoc comparison was performed by multiple comparisons of mean ranks; for details see ‘Statistical analysis’ section.

Acrosome reaction

Spermatozoa from cauda epididymis were capacitated as described earlier. During capacitation the acrosome reaction was induced by Cal (A23187, Cal, Sigma–Aldrich) at a final concentration of 5 μM. At each experimental capacitating time of 30, 60, 90, and 120 min, the Cal was added for the standard 60 min incubation. Cal was also added into parallel sperm droplets after 30 min capacitation but only for 5 min. Sperm that served as zero control and their presence in capacitating medium did not exceed 1 min were not further induced with Cal. The control samples were also evaluated for a rate of spontaneous acrosome reaction at relevant times to Cal-induced AR at 35, 90, and 120 min. All the sperm samples were incubated at 37 °C under 5% CO2. A drop of spermatozoa was placed onto a glass slide and 2.5 μM PNA lectin (peanut aglutinin lectin conjugate) (Molecular Probes) was added. The status of the acrosome was examined immediately under a fluorescent microscope. For every experiment we collect sperm data from 10 mice. A total of 200 cells were evaluated in each group and at least five samples were analyzed. Data were analyzed statistically by KW-ANOVA; post-hoc comparison was performed by multiple comparisons of mean ranks; for details see ‘Statistical analysis’ section.

SDS–PAGE immunoblotting

SDS electrophoresis and immunoblotting technique were used for the TyrP assessment and ERB detection. They were performed by protocols based on standard methods (Laemmli 1970, Towbin et al. 1979). Sperm were collected at 0, 30, 60, 90, and 120 min of capacitation in vitro. A suspension of noncapacitated sperm from a sperm stock released from cauda epididymis was used. The sperm solution was diluted with PBS and the number of sperm cells was counted using a Bürker chamber to ascertain a final concentration of 10^6 cells in the sample. Sperm pellet was resuspended in an equal volume of SDS–PAGE nonreduced sample buffer and heated at 97 °C for 3 min. Samples containing a protein equivalent of 10^6...
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 MW markers. Proteins were then transferred onto a nitrocellulose membrane. Non-specific sites on the membrane were blocked with PBS-blocking solution (5% skim milk and 0.05% Tween 20). ERB was identified by primary polyclonal antibody (H150, Santa Cruz Biotechnology) against N-terminal A/B domain, diluted 1:200 and secondary goat anti-rabbit antibody conjugated to HRP (170 5046, Bio-Rad) diluted 1:20 000. Proteins phosphorylated on tyrosine residues were identified by the primary MAB anti-phosphotyrosine (PTyr 01 112630025, Exbio Antibodies) diluted 1:500 followed by a peroxidase goat anti-mouse IgG secondary antibody (A 0168, Sigma–Aldrich) diluted 1:20 000. Protein staining was visualized by chemiluminescence (Super Signal West Dura Extended Duration Substrate, Thermo Fisher Scientific, Pardubice, 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 analyzer 4.18 (Raytest GmbH, Straubenhardt, Germany) and the relative intensity of individual signals was determined.

### Statistical analysis

Experimental data were analyzed using a program STATISTICA 6.0 (StatSoft CR s. r. o., Czech Republic). The statistical differences among compared groups (number of cells with specific sperm head status in experimental samples vs control sample in appropriate time) were analyzed by one-way analysis of covariance (KW-ANOVA). The number of cells with specific sperm head status at the beginning of the capacitation process was used as a covariate to reduce the effect of differences in the capacitation status of the sperm among individual animals, which is not the result of estrogen treatment. Post-hoc comparison was done by multiple comparisons of mean ranks. The *P* value < 0.05 (**P** < 0.01 and ***P*** = 0.001 respectively) was considered significant. The results of statistical analysis including mean ± s.e.m. are presented in Tables 1 and 2.

### Supplementary data

This is linked to the online version of the paper at [http://dx.doi.org/10.1530/REP-11-0326](http://dx.doi.org/10.1530/REP-11-0326).

### 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.

### Funding

This study was supported by Grants of the Ministry of Education of the Czech Republic VC No. 1M06011 and VZ No. 0021620828 and by the Institutional Research Support SVV263207.

### Acknowledgements

We are very thankful to Prof Ivan Lefkovits from Department of Biomedicine, Basel, Switzerland, for great help with presenting data in graphs and Timothy Paul Hort for English corrections.

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Received 31 August 2011
First decision 26 September 2011
Revised manuscript received 15 November 2011
Accepted 1 December 2011