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Original Article

The Impact of NADPH Oxidase 5 Activity and Reactive Oxygen Species on Capacitated Human Sperm

Mohammad Hojjati Far, Sara Keshtgar [*], Narges Karbalaei

- Department of Physiology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Background: Progesterone (P4) activates sperm calcium channels (CatSper), allowing calcium to enter the cell, which activates NADPH Oxidase-5 (NOX5) and produces reactive oxygen species (ROS). While calcium and ROS are essential for sperm capacitation, the role of NOX5 in capacitated sperm is unclear. This study investigated NOX5 activity in capacitated human sperm.

Methods: Forty semen samples from fertile men were processed, with motile sperm separated and divided into nine groups: control (Ham's F-10), solvent (DMSO), progesterone, diphenyleneiodonium chloride (DPI, NOX5 inhibitor), phorbol-12-myristate 13-acetate (PMA, NOX5 activator), P4+DPI, P4+PMA, Trolox, and P4+Trolox. Sperm kinematics, membrane integrity, survival rate, and ROS production was evaluated. Data were analyzed using ANOVA and Kruskal-Wallis tests, $p \leq 0.05$ considered statistically significant.

Results: Progressive motility significantly decreased with DPI (56.2±2.1%) and PMA (56.5±2.1%), both alone and combined with progesterone (58.0±2.0% and 57.4±2.2%), compared to the progesterone group (66.0±1.9%). No significant change was observed in the Trolox groups. Progesterone, alone or combined with DPI, PMA, and Trolox, significantly reduced sperm linearity from 0.6±0.01 to 0.5±0.01%. Straight-line velocity decreased in P4+PMA and P4+Trolox groups (88.2±4.4 and 89.7±3.9 *μm/s*) compared to the control group (105.0±5.5 *μm/s*). Trolox reduced ROS content, while other treatments had no effect on ROS levels.

Conclusion: NOX5 does not play a prominent role in capacitated sperm. The negative effects of PMA and DPI on sperm motility appear independent of their actions on NOX5 and ROS production. Trolox did not affect sperm motility and survival, indicating that capacitated sperm require little or no ROS.

** Corresponding Author:*
Sara Keshtgar, Department of Physiology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
E-mail:
keshtgar@sums.ac.ir,
keshtgars@yahoo.com

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Introduction

Sperm capacitation refers to the changes that enable sperm to fertilize the egg. During capacitation, sperm undergoes a drastic change in its movement pattern, which is called hyperactivity (1). After sperm hyperactivation, the relatively linear and progressive swimming pattern of sperm in seminal plasma turns into a non-progressive, intense, whiplash, irregular, and high-amplitude motility pattern. This movement pattern change mainly occurs due to increased membrane fluidity and intracellular calcium (2). However, one of the first steps in sperm capacitation and hyperactivation is increasing intracellular pH (3). During capacitation, the alkalinization of human sperm occurs due to the production or entry of bicarbonate into the sperm and the outward flow

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of protons through Hv1 channels (4). The increase of bicarbonate inside the cell activates the enzyme adenylyl cyclase 10 (ADCY10), increases the concentration of cyclic adenosine monophosphate (cAMP), and activates protein kinase A (PKA) which leads to the phosphorylation of intracellular proteins and sperm activation (3).

Alkalization and the presence of progesterone activate sperm calcium channels-known as CatSper-and cause an increase in intracellular calcium $[Ca^{2+}]_I$ (3, 5). Calcium can directly bind to membrane phospholipids and numerous enzymes and cause changes in membrane properties and sperm enzyme activity. Calcium binding to calmodulin causes structural changes, and the calcium-calmodulin complex modulates the activity of adenylyl cyclase, phosphatases, and kinases (6). Sperm-specific ADCY10 is calcium-dependent, which shows that calcium regulates capacitation in sperm in various ways (7).

P4 is one of the activating agents of ion channels in sperm, and some evidence shows that the effects of P4 on sperm are mediated by receptors located on the plasma membrane. Although the exact nature of these receptors is unclear, experimental evidence suggests that there may be at least three types of receptors: a plasma membrane calcium channel, a plasma membrane chloride channel, and a membrane-associated protein tyrosine kinase (8).

Among these, the primary mechanism through which P4 modulates sperm function is increasing the free intracellular calcium concentration. Indeed, following exposure to P4, the calcium ion concentration increases rapidly (within seconds), followed by a low to moderate increase for several minutes that requires the influx of extracellular calcium (9).

Many other mechanisms are activated by P4, including the stimulation of protein kinase C (PKC) activity and increment of the amount of intracellular cAMP levels. These are considered to be among the most important effects of P4 on sperm function (10). Besides these stimulating mechanisms, the role of ROS is not fully understood in the process of sperm capacitation. However, low levels of H_2O_2 can induce sperm capacitation (11) through elevation in cAMP and increment in tyrosine phosphorylation levels (12). ROS are generated by the mitochondria as a byproduct of the electron transport chain activity in the inner membrane (13). In addition, sperm produces ROS through specialized membrane-bound enzyme complex called NADPH oxidase, specifically the NOX5 isoform (14) .Therefore, NOX5-dependent ROS production likely contributes to the capacitation process (11).

Calcium binding to NOX5 is one of the key activating factors of this enzyme (15). A calcium ionophore A23187 increases ROS generation by human sperm (16). It has been shown that the binding of the catalytic domain of NOX5 to calcium-bound calmodulin can increase the sensitivity of NOX5 to free calcium (17). On the other hand, PKC-mediated phosphorylation increases the sensitivity of this enzyme to calcium and leads to NOX5 activation at lower intracellular calcium concentrations (18).

Altogether, an increase in intracellular calcium is necessary to activate NOX5, and the essential calcium channel responsible for this calcium influx in human sperm is the specialized sperm-specific cation channel known as CatSper (19, 20). Nanomolar concentrations of P4 induce currents of calcium ions through CatSper in human sperm (21). Considering the role of calcium in activating NOX5, the entry of calcium into the sperm through this channel could be one of the factors leading to NOX5 activation (16, 22). The importance of NOX5 enzymatic activity in sperm function remains a topic of ongoing investigation and debate. However, previous studies have emphasized the role of NOX5 activity when un-capacitated sperm are incubated with P4 (23).

This study was conducted to determine the role of NOX5 in capacitated sperm in the presence or absence of P4. Meanwhile, the importance of ROS on the function of capacitated sperm was investigated.

Methods

Chemicals and culture medium: In this study, progesterone (Sigma Aldrich, USA), phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, USA), diphenyleneiodonium chloride (DPI) (Sigma Aldrich, USA), dimethyl sulphoxide (DMSO) (Sigma Aldrich, USA), Trolox (Sigma Aldrich, USA), eosin Y (Sigma Aldrich, USA), luminol (Sigma Aldrich, USA), and horseradish peroxidase (Sigma Aldrich, USA) were used. The capacitation medium was prepared by Ham's F-10 (Biowest, France), enriched by HSA 3.5% (Biotest, Germany), and 24 *mM* of sodium bicarbonate (Sigma Aldrich, USA).

Sample collection and sperm preparation: Semen samples (n=40) were obtained from healthy, fertile men aged 20-40 years who were referred to Shiraz Fertility Center after 2-7 days of sexual abstinence. The volunteers did not have specific diseases at the time of enrollment and did not take medication, specific dietary supplements, or vitamins. The criteria for normal fertility according to World Health Organization (WHO) guidelines are: semen volume of at least 1.5 *ml*; total sperm number of at least 39 million per ejaculate; sperm concentration of at least 15 million *per ml*; vitality of more than 58% live sperm; progressive motility of at least 32%; total (progressive and non-progressive) motility of at least 40%; and morphologically normal forms of at least 4% (22). The samples included in the study met all criteria of fertility based on WHO; the average pH and semen volume of samples were 7.5±0.1 and 3.5±0.2 *ml*, respectively. The total sperm count of the semen samples was 306 million per ejaculate, the mean± SE of sperm concentration was 87.45±5.7 million *per/ml* 10^6/*ml*, and the percentage of progressive motile sperm was 46.5±1.6. Sperm viability was 70.8±2%, and viscosity of all samples was normal.

For every milliliter of semen, 1.5 *ml* of Ham's F-10 was slowly added. The samples were centrifuged at 1200 *rpm* for 10 *min*. After that, the supernatant was discarded, and 0.5 *ml* of Ham's F-10 containing 24 *mM* sodium bicarbonate and 3.5% HSA was added gently.

The tube containing samples and capacitating medium was placed inside the incubator at 37°*C* and 5% CO_2, positioned at an angle of 45 degrees, and incubated for one *hr*. After the incubation period, the tube was slowly returned to the vertical position, and the supernatant containing live and motile sperm was carefully separated. To evaluate sperm kinematics, membrane integrity, and viability, 25 semen samples were diluted to a sperm concentration of 10×10^6/*ml*. Increasing the sperm concentration *per/ml* can help improve the accuracy of ROS measurement. To evaluate the ROS production by sperm, the additional 15 samples were diluted to a concentration of 20×10^6/*ml*.

Experimental groups: The diluted samples were divided into different experimental groups: control (Ham's F-10 medium), solvent (DMSO at 0.1% concentration), 10 *µM* progesterone (P4), 1 *µM* DPI, 100 *nM* PMA, P4+DPI, P4+PMA, 200 *µM* Trolox, and P4+Trolox. All samples were incubated at 37°*C* and 5% CO_2 for 30 *min*. The study protocol was approved by the Research Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1400.602).

Motility assessment: Sperm motility was assessed using a sperm motility analyzer (Argus-CASA Software). At least 10 individual fields and 200 spermatozoa were randomly observed for sperm motility. The sperm were classified into 3 groups based on their motility (progressive, non-progressive, and immotile) in accordance with the criteria provided by the World Health Organization (WHO) guidelines (24).

Sperm kinematics: The motility parameters, including curvilinear velocity (VCL, *µm/s*), straight-line velocity (VSL, *µm/s*), velocity along the average path (VAP, *µm/s*), lateral head displacement (ALH, *µm*), linearity (LIN, %), and straightness (STR) were evaluated for each sample. However, only the results for VSL, VCL, ALH, and LIN were reported in this study. The analyzed data were exported to Excel software, and the immobile sperm were filtered. VCL higher than 150 *µm/s*, ALH more than 2 *µm*, and LIN less than 50% were considered as "capacitated sperm", according to the criteria established by Jokiniemi et al. (25).

Evaluation of viability and membrane integrity: The eosin Y staining technique was used to evaluate sperm viability. The eosin Y was added to each sample (1:1), and the cells were observed after 30 seconds. At least 200 spermatozoa were evaluated. The live cells did not absorb the dye, and the stained sperm were considered dead cells (24). The hypoosmotic swelling (HOS) technique was used to evaluate membrane integrity. For this purpose, a solution was prepared containing 0.735 *g* of sodium citrate dihydrate and 1.351 *g* of D-fructose, dissolved in 100 *ml* of purified water. One *ml* of the swelling solution was added to 100 *µl* of the sperm sample and mixed gently (24). It was then incubated at 37°*C* for 30 *min*. At least 200 spermatozoa were evaluated. The live sperm exhibited swelling, especially in the tail region, which was clearly visible.

ROS measurement: ROS production was monitored by the chemiluminescence assay in the control and all experimental groups. For the ROS measurement, a fresh 25 *mM* stock solution of luminol in DMSO was prepared and added to each 300 *µl* sperm suspension in phosphate-buffered saline (PBS). The final concentration of lu-

minol was 250 μM, and the suspension was supplemented with 12.4 *U* of horseradish peroxidase. A blank (PBS) and a control (PBS and luminol) were applied to evaluate background luminescence. The luminescence signal was monitored at 37°*C* for 60 *min*, with measurements taken every 5 *min*, using a microplate reader (Synergy HT; Biotek, USA) in the integrated mode. The data was reported as the relative light unit. The study was approved by the Research Ethics Committee of the Shiraz University of Medical Science (ethical code: IR.SUMS.REC.1397.806).

Statistical analysis: Statistical analysis was performed using SPSS software version 16 (IBM, USA). The normal distribution of data was evaluated by Kolmogorov-Smirnov test. Data with normal distribution were analyzed by one-way ANOVA with Duncan post-test. The Kruskal-Wallis test was applied if the data were not normally distributed, and the significant difference between every two groups was analyzed using the Mann-Whitney test with Bonferroni post hoc analysis. All data were reported as mean±standard error (SE) and $p<0.05$ was considered statistically significant.

Results

The sperm viability and membrane integrity: The sperm survival and membrane integrity were evaluated using the eosin Y and HOS methods, respectively. As expected, both tests had identical results, and approximately 70% of sperm were live with intact membranes in all experimental groups. The treatments did not affect sperm viability and membrane integrity (Table 1).

Progressive and non-progressive motility: Adding 1 μM DPI and 100 *nM* PMA significantly reduced the percentage of progressive motility and total motile sperm compared to the control and the P4-containing group (Table 1). Simultaneous incubation of sperm with P4+DPI or P4+PMA returned the progressive and total motility to the control level but not to the P4 group level. Trolox did not affect the percentage of progressive motility and the rate of total motile sperm (Table 1).

The sperm kinematic parameters: The results showed that adding 10 μM P4 alone or combined with DPI, PMA, and Trolox significantly reduced the LIN parameter compared to the control group when all motile sperm were considered. LIN of the DPI, PMA, and Trolox groups was the same as the control group (Table 2).

P4 alone had a non-significant effect on VSL. However, the combination of P4 with PMA and Trolox significantly reduced VSL compared to the control and DPI groups (Table 2). The VCL and ALH did not change under various treatments.

ROS production: ROS production was assessed in the experimental groups for 60 *min*. The relative light unit of all groups was the same except for the groups containing Trolox (Table 3). Trolox with a concentration of 200 μM successfully reduced the chemiluminescence signals ($p=0.004$).

Discussion

In this study, P4 had no significant effect on sperm survival, membrane integrity, and ROS production compared to the control group. Our study confirmed the ineffectiveness of P4 on

Table 1. Comparison of sperm viability, membrane integrity, and sperm progressive, non-progressive, and total motility incubated with P4, DPI, PMA, Trolox, and their combinations

Groups (n=25) Mean±SE	Control	DMSO	P4	DPI	PMA	P4+DPI	P4+PMA	TROLOX	P4+Trolox
Viable sperm (%)	74.0±1.5	73.2±1.1	72.7±1.5	70.3±1.9	71.8±1.6	73.0±1.6	72.2±1.7	75.2±1.3	72.2±1.8
Sperm with intact membrane (%)	76.5±1.3	73.6±1.4	72.5±1.6	71.9±1.7	73.2±1.4	74.6±1.6	73.4±1.5	77.6±0.9	72.0±1.6
Progressive motility (%)	61.8±1.8	63.5±1.7	66.0±1.9	56.2±2.1$^{\beta}$	56.5±2.1$^{\beta}$	58.0±2.0$^{\beta}$	57.4±2.2$^{\beta}$	60.4±1.7	61.4±2.2
Non-progressive motility (%)	11.0±1.0	11.2±0.8	11.7±0.8	12.0±0.9	12.2±1.1	13.0±1.1	12.2±1.3	13.3±1.3	10.5±1.0
Total motility (%)	72.9±2.8	74.7±1.9	77.2±2.7	68.2±3.0$^{\beta\alpha}$	68.8±3.3$^{\beta\alpha}$	71±3.0$^{\beta}$	69.6±3.5$^{\beta}$	73.8±3.0	72.0±3.2

The data are presented as mean ± SEM. P-values less than 0.05 are considered statistically significant. α indicates a significant difference compared to the control and DMSO group and β indicates significant difference compared to P4 group

Table 2. Comparison of sperm kinematic parameters incubated with P4, DPI, PMA, Trolox, and their combinations

	Groups (n=25) Mean±SE	Control	DMSO	P4	DPI	PMA	P4+DPI	P4+PMA	Trolox	P4+Trolox
Sperm kinematics	LIN (%)	0.6±0.01	0.6±0.01	0.5±0.01$^{\alpha}$	0.6±0.01$^{\varphi\varepsilon\theta\beta}$	0.6±0.01$^{\varphi\varepsilon\theta\beta}$	0.5±0.01$^{\alpha}$	0.5±0.01$^{\alpha}$	0.6±0.01$^{\varphi\varepsilon}$	0.5±0.01$^{\alpha}$
	VSL *(µm/s)*	105.0±5.5	102.2±4.8	92.0±4.8	105.4±4.3$^{\varepsilon}$	103.4±5.1$^{\varepsilon}$	91.0±4.0	88.2±4.4$^{\alpha}$	101.5±5.3	89.7±3.9$^{\alpha}$
	VCL (µm/s)	158.2±5.6	155.0±5.9	153.2±5.6	159.5±4.3	155.7±5.4	153.3±4.7	150.0±4.2	155.9±5.0	154.0±5.2
	ALH *(µm)*	2.0±0.07	1.9±0.07	2.0±0.07	2.0±0.08	1.9±0.08	2.0±0.07	2.0±0.07	2±0.07	2.1±0.07

The data are presented as mean±SEM. P-values less than 0.05 are considered statistically significant. The progresive motility (%) is computed by applying the Bonferroni correction to the p-value ($p<0.005$). α indicates significant difference with the control and DMSO group and β indicates significant difference with the P4 group. Also, θ indicates significant difference with the P4+DPI group, ε indicates significant difference with the P4+PMA group, and *φ* indicates significant difference with the P4+Trolox group

Table 3. Mean ROS production (quantitative luminescence signal) in the control group and the groups treated with P4, DPI, PMA, Trolox, and their combinations

Groups (n=25) Mean±SE	Control	DMSO	P4	DPI	PMA	P4+DPI	P4+PMA	Trolox	P4+Trolox
Relative light unit (ROS)	1065±346	1453±328	1394±419	1061±350	1025±359	1469±514	1221±444	-33±18$^{\gamma}$	14±11$^{\gamma}$

The data are presented as mean±SEM. γ: indicates significant difference with other groups (p-value=0.004)

sperm survival, as previously reported (22, 23, 26). P4 could elevate the ROS production in human sperm when sperm were incubated in Ham's F-10 (22). However, it was indicated that P4 did not affect the amount of ROS after incubation in the capacitating medium.

A non-significant increase in sperm motility by P4 was observed in the study. However, P4 attenuated the inhibitory effect of DPI and PMA on sperm progressive motility. Analysis of sperm kinematics revealed that LIN was reduced in P4 and all P4-containing groups (P4 plus DPI, PMA, and Trolox) (Table 2). Meanwhile, VCL and ALH in P4-containing groups did not differ significantly from the control group. It has been proven that the percentage of LIN decreases in hyperactivated sperm (26, 27). The present data suggests that P4 plays a key role in promoting sperm capacitation and hyperactivity, even after one-*hr* incubation of sperm in a capacitating medium.

Although the mechanism of progesterone's effect on sperm has not been fully understood, it has been proven that P4 causes calcium to enter human sperm through the CatSper channel (20-22). A study on human sperm identified a novel function of a P4-sensitive membrane lipid hydrolase, called α/β hydrolase domain-containing protein 2 (ABHD2), which metabolizes endocannabinoids 2-arachidonoylglycerol and 3-arachidonoylglycerol in sperm, only in the presence of P4. These arachidonoylglycerols act as endogenous inhibitors of CatSper. P4 stimulates ABHD2 activity, which then hydrolyzes these endocannabinoids. This increase in hydrolysis leads to the opening of CatSper and calcium entry (21, 28). However, the effect of P4 on calcium channels is transient; as a result, these effects may not be as pronounced in sperm that has already undergone capacitation. After one *hr* in a capacitating medium, 100 *nM* PMA did not affect sperm survival, membrane integrity, and the amount of ROS but significantly reduced the percentage of progressive and total motility compared to the control and P4 groups. PMA, in combination with P4, caused a significant decrease in the percentage of sperm with progressive and total motility compared to the group containing only P4.

PMA is known as a PKC activator, and it also increases the amount of intracellular cAMP (29). The PKC-dependent phosphorylation increases

the sensitivity of NOX5 to calcium ions and leads to activation of NOX5 at lower concentrations of calcium (18). Sperm incubation with 1 *μM* PMA produced the superoxide anion and H_2O_2 in the extracellular environment, which was stopped by the addition of PKC inhibitors (11). However, in another study, PMA with a concentration of 4 *μM* did not affect the ROS production in sperm (30). On the other hand, 2.5 to 15 *μM* of PMA did not change the percentage of sperm motility. At concentrations higher than 20 *μM*, the percentage of motility and sperm velocity significantly decreased (11). The relative levels of ROS production by NADPH oxidase in white blood cells and NOX5 enzyme in sperm were compared previously. Additionally, research was done to evaluate whether PKC is involved in NOX5 activation or not. The results showed that the level of ROS formation by NOX5 is relatively low compared to activated white blood cells. It was also found that PKC activation does not affect the production of ROS by NOX5, and NOX5 activation is independent of PKC-dependent phosphorylation (30).

A study conducted on boar sperm demonstrated that the addition of 10 *μM* PMA caused the phosphorylation of threonine 172 of AMP-activated protein kinase (AMPK), which disintegrated the sperm membrane lipids and reduced sperm motility. In the mentioned study, VCL and VAP significantly decreased after adding PMA (31). The studies also showed sperm agglutination with PMA. The sperm agglutination was higher and faster in a medium containing bicarbonate and calcium (31). The inhibitory effect of PMA on LIN was not observed in the capacitated sperm, which may be due to changes in membrane properties or intracellular signaling.

Previous studies have shown that NOX5 is one of the factors responsible for ROS production in human sperm, and the activity of this enzyme is inhibited by a flavoprotein inhibitor, DPI (7, 32). According to this evidence, DPI was used as an enzyme inhibitor in the current study to evaluate the effect of NOX5 on sperm physiological function after capacitation.

In the present study, the role of DPI was investigated. The results showed that 1 *μM* DPI did not affect sperm survival, membrane integrity, and the amount of ROS produced by sperm. However, DPI significantly reduced the percentage of total motility compared to the control and P4 groups. The progressive motility (%) in the groups treated with either DPI alone, PMA alone, or the combination of PMA and P4, showed a significant decrease (8 to 10%) compared to the P4 group. This inhibitory effect of DPI (7.5 and 10 *μM*) on human spermatozoa, without affecting NOX5, was previously reported. Different mechanisms could determine the effects of DPI on the cells. DPI, by acting on canalicular-like transporters of the cell membrane, causes the expulsion of the antioxidant glutathione from the cells (33). Furthermore, it has an inhibitory effect on the mitochondrial respiratory chain and ATP production (34).

DPI did not cause a significant change in the amount of ROS and therefore, DPI (1 *μM* for 30 *min*) did not affect the activity of NOX5. Despite the emphasis on the role of NOX5 and ROS in teratozoospermia samples (35) and the effectiveness of DPI in better preservation of sperm during the sperm cryopreservation process (36), the results showed that the function of this enzyme did not have a considerable effect on sperm after capacitation. Regarding the ineffectiveness of P4, PMA, and DPI on ROS production, it can be suggested that NOX5 activity of normal sperm incubated in the capacitating medium is not considerable. However, DPI and PMA may affect membrane properties or intracellular signaling.

In fact, future studies should investigate the dose-dependent effects of PMA and DPI, as well as examine some key intracellular signaling pathways.

Many research studies have highlighted the role of ROS in sperm physiology, as well as the relatively low antioxidant properties of sperm (37-40). In this study, Trolox was used to assess the role of ROS on human-capacitated sperm. Trolox is a water-soluble vitamin E analog that can penetrate cells and has strong antioxidant properties (41). Our results showed that adding 200 *μM* of Trolox significantly reduced the amount of ROS but did not affect the motility, survival, and health of the sperm membrane. The same results were reported using fresh human sperm samples (42). Although the antioxidant properties of sperm are deficient (37), sperm possess some antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidases (GPxs), and glutathione reductase (GR) (43). The antioxidant activity of these enzymes may increase after sperm capacitation.

Conclusion

This study revealed that the stimulation and inhibition of NOX5 by PMA and DPI, respectively,

do not affect the amount of ROS produced by sperm. These findings indicate that the role of NOX5 enzyme is not highly prominent in normal sperm when these cells are incubated in a capacitating medium for at least one *hr*. The unfavorable effects of PMA and DPI on sperm motility could be independent of their actions on NOX5, which needs further investigation. Furthermore, the use of Trolox did not affect sperm motility and survival, which indicated that sperm may need little or no ROS after capacitation.

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Conflict of Interest

The authors declare no conflicts of interest.

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