OPN enhances sperm capacitation and in vitro fertilization efficiency in boars

Authors

Yun Chen[1], Kai Wang[2], Shouquan Zhang[2]*

Affiliations

[1]Shaoguan University, Shaoguan, 512000, China

[2]National Engineering Research Center for Breeding Swine Industry, Guangdong Provincial Key Lab of Agro-Animal Genomics and Molecular Breeding, College of Animal Science, South China Agricultural University, Guangzhou, 510642, China

***Corresponding author: sqzhang@scau.edu.cn**

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ORCID	Yun Chen (https://orcid.org/0000-0003-2610-4003) Kai Wang (https://orcid.org/0000-0001-6049-1970) Shouquan Zhang (https://orcid.org/0000-0001-6757-9465)
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Abstract

In this study, we used more reliable experimental materials and methods to detect the effects of OPN on boar sperm in vitro capacitation, acrosome reaction, and fertilization efficiency. We reorganized and obtained the OPN protein of the porcine source. Immunofluorescence and Western blot show the localization and expression of the OPN protein before and after sperm capacitation. To determine whether OPN can affect sperm during sperm capacitation, we examined cAMP concentrations after sperm capacitation, and the results showed that OPN significantly increased the cAMP concentration in sperm ($P<0.05$). Flow cytometry showed that 0.1 μg/ml OPN-treated sperm had better acrosome reaction ability. IVF showed that 0.1 μg/ml OPN significantly increased the rate of embryo division. In conclusion, this study found that 0.1 μg/ml porcine OPN protein can significantly improve porcine capacitated sperm motility, cAMP concentration after capacitation sperm, acrosome reaction ability, and embryo division during IVF and provides new clues to explore the mechanism of OPN's function on sperm.

Keywords: Osteopontin, In vitro fertilization, Sperm, Acrosome reaction, capacitation.

Introduction

In the worldwide animal husbandry industry, pigs account for a large proportion and are the primary source of energy and protein in developing countries [1]. Farms generally screen for boars with better fertility for breeding to enhance genetic value and fertility, but collecting fertility data about boars is time-consuming and labor-intensive [2,3]. The best approach is to use new technology to assist in reproduction. In vitro maturation (IVM) and in vitro fertilization (IVF) are techniques for the completion of fertilization in mammalian sperm and eggs in a manually controlled environment [4,5]. Although porcine embryos produced by in vitro maturation (IVM) and in vitro fertilization (IVF) develop to the blastocyst stage, the low incidence of cleavage rate remains a significant impediment [6].

The OPN protein, which has been identified as an indicator of human male fertility, promotes fertilization and preimplantation embryo development in mice. OPN has been shown to improve sperm capacitation and in vitro fertilization (IVF) efficiency in bovines [7,8]. Furthermore, when polyclonal anti-OPN antibodies were incubated with bovine sperm/oocytes in a culture medium, a reduction in sperm-egg binding and fertilization in vitro was observed [9]. The OPN concentration of high fertility bulls was four times that of low fertility bulls[10]. The expression level of OPN is positively correlated with sperm motility and the survival rate of piglets as determined by genotyping analysis in boars [11]. The addition of rat OPN to a modified Tris-buffered medium (mTBM) IVF system reduced polyspermy, improved porcine embryo development, and reduced apoptosis [12,13]. It has been reported that the concentration of OPN is related to the percentage of inactive sperm and litter rate to a certain extent[14]. These findings indicate to varying degrees that OPN plays an irreplaceable role in the development of

mammalian gametes.

At present, the literature has agreed that OPN can increase the rate of division of mammalian embryos. Nevertheless, different results have been obtained regarding whether it affects sperm capacitation and the acrosome reaction. Given these additional results, this study's objectives are to observe the effect of porcine OPN protein before and after capacitation on sperm motility, acrosome reaction, and embryo division by using more reliable experimental materials and methods.

Materials and methods

Ethics statement

All procedures involving animals met the guidelines of "The Instructive Notions Concerning Caring for Laboratory Animals" issued by the Ministry of Science and Technology of China, with approval (Approval number SCAU#0025) by the Institutional Animal Care and Use Committee of South China Agricultural University (Guangzhou, China). All efforts were made to minimize animal suffering.

Production and purification of porcine OPN protein

The samples were porcine spermatogonial stem cells (SSCs), sperm, 293FT cells, and CHO-K1 cells. According to the manufacturer's protocol, total cellular RNA was isolated from samples using the TRIzol method (TIANGEN, Guangzhou, China) and reverse transcribed to cDNA (Vyzame, Nanjing, China). DNase I was used to remove genomic DNA, and first-strand cDNA was made from 1 μg of total RNA. Full-length coding sequences of porcine OPN (see Table S1, Additional File) were amplified from SSCs. The coding sequence of OPN with a 6His-tag (see Table S2, c) was amplified and cloned into the PCDH-CMV-HSA-MCS-6His-EF1-EGFP+Puro

vector by directional cloning using HindIII and BamHI sites. The PCDH-CMV-HSA-6His-OPN-6His-EF1-EGFP+Puro plasmid was transfected into 293FT cells. Reverse transcription-polymerase chain reaction (RT–PCR) was performed with primers for OPN and β-actin (Table 1) as confirmation. See the appraisal results for details in Figure S3 and S4, Table S3, and Additional File.

293FT cells and CHO-K1 cells were purchased from the American Type Culture Collection (ATCC). The lentiviral vectors included PCDH-CMV-MCS-EF1-EGFP+Puro plasmid and two lentivirus packaging plasmids (psPAX2 and pMD2G) (SBI, Los Angels, USA). The vector used was PCDH-CMV-HSA-MCS-6His-EF1-EGFP+Puro vector (Figure S1, Additional File), modified from the PCDH-CMV-MCS-EF1-EGFP+Puro plasmid expressing HSA and 6His-tag. HSA is a signal peptide derived from a human serum protein and contains 63 bases (signal peptide sequence: MKWVTFISLLFLFASAYS). The addition of HSA dramatically increases the amount of protein secreted by CHO-K1 cells [15,16]. The 6His-tag is a tool for protein purification, and lentiviral production was performed (see Figure S2, Additional File) as previously described [17].

Porcine OPN was obtained from using the CHO cell expression system [18,19]. CHO-K1 cells were resuscitated, passaged, and plated. After culturing for 24 h, lentivirus supernatant and 10 μg/ml polybrene were added to the cells. GFP and Puro (6 μg/ml) selection were performed to select a single clone. After screening the monoclonal CHO cell line, the supernatant was collected, and the cell debris was removed by centrifugation. After concentration, porcine OPN protein was purified by 6His-tag and stored in freeze-dried powder.

OPN protein, anti-OPN antibody, and regular rabbit IgG solution preparation

Recombinant porcine OPN (1 mg/ml, in PBS; 92% pure) was produced in house. The OPN

protein was diluted to 0.1 μg/ml in IVF medium (113.1 mM NaCl, 3.0 mM KCl, 7.5 mM CaCl2-H_2O, 20.0 mM tris, 11.0 mM D-glucose 5.0 mM sodium pyruvate, 2 mg/ml BSA and 2 mM caffeine). The rabbit polyclonal antibody against OPN (82 mg/ml) was purchased from Abcam (ab8448, Cambridge, MA) and diluted (1:50, 1:200, 1:500, 1:1000 and 1:2000) in IVF medium. Normal rabbit IgG (1 mg/ml, in PBS) was purchased from Abcam (ab37415; Cambridge, MA) and diluted (1:50, 1:200, 1:500, 1:1000 and 1:2000) in IVF medium. A blank control (only IVF medium) was also prepared.

Sperm assay

Fresh ejaculates were collected from 6 Landrace 12-month boars according to the "gloved-hand" technique [20], diluted and stored in 17℃ fantastic boxes, and then brought to the laboratory within 1 h of collection. For the sperm assay, the sperm samples were divided into four groups.

The first group was for the location and expression of OPN protein before and after capacitation sperm.

The second group was used for the cAMP assay after capacitation of sperm.

The third group was for acrosome reactions in sperm. In this experiment, sperm samples were divided into 4 groups according to different treatments: blank control (untreated), normal IgG treated (negative control), anti-OPN treated, and OPN protein treated incubation for 4 h.

The fourth group was for IVF. In this experiment, sperm samples were collected and capacitated for evaluating penetration, polyspermy and embryo division during IVF.

Sperm capacitation

The semen was washed three times with sperm-washing medium (Dulbecco's phosphate-buffered saline supplemented with 0.1% BSA) and resuspended with mTBM [12]. The sperm resuspension was subjected to a discontinuous Percoll gradient (90% [v/v] and 45% [v/v]) to remove dead sperm and seminal plasma [21] and then centrifuged at 300 × g for 20 min. The concentrated sperm of each sample was adjusted to 55×10^6 spz/ml and added to the bottom of a tube with 2 ml of mTBM containing 100 μg/ml heparin sodium. The sperm were then incubated (38.5°C, 5% CO2) for sperm capacitation for 30 min with the sperm swimming-up method to reduce polyspermy during in vitro fertilization [22,23]. The washed sperm samples were divided into 2 groups: before capacitation and after capacitation. The concentrated sperm of each sample was added to the bottom of a tube with 2 ml of mTBM containing 100 μg/ml heparin sodium. Tyrosine phosphorylation protein is a marker of capacitation changes observed in boar sperm, and its expression significantly increases after capacitation [24].

Immunofluorescence Analysis

In addition to Hoechst 33342 (1:100; Life Technologies; H3570), the antibodies used in this experiment were as follows: anti-OPN antibody (1:500); normal rabbit IgG (1:500); fluorescein-conjugated goat against rabbit IgG (1:1000; Thermo; A11034; Alexa Fluor 488)); and fluorescein-conjugated goat against rabbit IgG (1:1000; Thermo; A11036; Alexa Fluor 568).

The OPN protein in sperm was detected by IF staining as previously described [25]. After the sperm were washed 3 times with PBS, the sperm concentration was adjusted to 55×10^6 spz/ml. Fifty microliters of sperm suspension was placed on slides and then dried for 60 min in air. The cells were cultured in a 24-well culture plate for 24 h and washed 3 times with PBS. Then, the samples were fixed with 3.7% formaldehyde at room temperature for 20 min, washed with PBS,

blocked with 10% normal rabbit serum for 1 h, and incubated with sufficient diluted primary anti-OPN overnight at 4°C. The samples were then exposed to fluorescein-conjugated secondary antibodies at 37°C for 1 h and incubated with Hoechst for 5 min to protect them from light. Each sample was supplemented with an antifading solution. OPN protein was detected by laser scanning confocal microscopy (LSM780, Zeiss, Germany).

Western blotting analysis

The prepared samples (55×10^6 cells/ml) containing cells before and after capacitation boar sperm were homogenized in ice-cold protein extraction buffer (KeyGEN BioTECH, Guangzhou, China) to collect soluble protein. The protein (20 μg lysates) was boiled for 10 min, subjected to 12% SDS–PAGE, transferred to a PVDF membrane (Roche Diagnostics), and blocked with 5% nonfat dried milk in Tris-buffered saline-Tween 20 for 1 h at room temperature (RT). Western blotting (WB) analysis was carried out overnight at 4°C for primary antibodies and at 37°C for 1 h for secondary antibodies. Immunodetection of OPN/tyrosine phosphotyrosine proteins was performed by enhanced chemiluminescence (ECL). Then, the membranes were stripped and reprobed with alpha-tubulin (α-tubulin)/beta-actin (β-actin) as loading standards.

The antibodies used were anti-OPN (1:1000; Abcam; ab8448), mouse monoclonal phospho-tyrosine antibody (clone P-Tyr-100) (diluted 1:1000; Cell Signaling Technology; 9411 s), mouse monoclonal anti-α-tubulin (clone B-5-1-2) (1:3000; Sigma; T6074), mouse monoclonal anti-β-actin (clone AC-15) (1:3000; Sigma; A1978), mouse monoclonal anti-green fluorescent protein (GFP) antibody (clone GSN149) (1:1000; Sigma; G1546), HRP AffiniPure goat anti-mouse IgG (1:3000; Earth Ox; E030110), and HRP AffiniPure goat anti-rabbit IgG (1:3000; Earth Ox; E030120).

Determination of the concentrations of cAMP in porcine sperm after capacitation

To analyze the cAMP concentration in sperm, after capacitation, sperm samples were analyzed using a cAMP direct immunoassay kit (Abcam, Cambridge, MA) [25]. Sperm samples included the OPN protein group, anti-OPN group, and control group (nothing added). Sperm samples (55x106 spz/ml) were lysed with protein lysis buffer for the cAMP assay. The OD was measured at 405 nm with an ELISA plate reader. For the porcine sperm cAMP assay, each experiment included a standard well, sample well, control well and blank well. Detailed experimental steps were described according to the manufacturer's instructions. A standard curve correlation coefficient > 0.95 was needed. Six replicates were performed for each assay. The cAMP concentration was calculated for each sample by the standard curve provided by the kit's manufacturer.

Acrosome reaction

The acrosome reaction was measured with a fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (FITC-PSA; Sigma; L0770) label labeled the acrosomal matrix glycoproteins. Propidium iodide (PI; Sigma; P4170) was used to determine the number of dead sperm. After washing twice in PBS, after 4 h of capacitation, sperm (55x106 spz/ml) were incubated with PI (5 μg PI/1 ml PBS) for 10 min at room temperature. Samples were incubated with a FITC-PSA solution (10 μg FITC-PSA/1 ml PBS) at 37℃ for 30 min in the dark and analyzed. Acrosome-intact sperm were stained with weak fluorescence. The presence of green acrosomal fluorescence on the top of the sperm head or equator indicated an acrosome reaction. Immunofluorescent staining was analyzed by a BD FACSCalibur flow cytometer (FCM) using CellQuest software

188 (BD Biosciences, San Jose, USA). All flow cytometry analyses were conducted within 1 h of PSA
189 staining.
190
191 **IVF assay**
192 Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO),
193 and the media were purchased from Gibco (Thermo Fisher Scientific). The IVF procedure was
194 based on a previous study with some modifications [26]. Oocyte maturation medium (TCM199
195 Gibco BRL) was supplemented with 0.1 mg/ml L-cysteine, 10 ng/ml epidermal growth factor
196 (EGF), 10% (v:v) fetal bovine serum (FBS), 10% (v:v) porcine follicular fluid (PFF), and
197 hormones (10 IU/ml hCG and 10 IU/ml PMSG). L-cysteine, EGF, hCG, and PMSG were
198 dissolved in TCM199 medium in a concentrated dilution, filtered through 0.22 μm filters, and
199 stored at -20°C until use. PFF was obtained from 3-6 mm diameter follicles and centrifuged at
200 1900×g and 4°C for 30 min. The supernatant fluid was filtered through 0.45 μm and 0.22 μm
201 filters and stored at -20°C until use.
202 Ovaries were collected within 2 h from slaughtered prepubertal pigs and stored in 0.9%
203 sodium chloride (NaCl) solution at 37°C. Cumulus-oocyte complexes (COCs) were selected
204 from ovarian follicles (3-6 mm in diameter) and precipitated three times in DPBS-PVA solution.
205 Fifty COCs were transferred to oocyte maturation medium for the first 22 h in a four-well Nunclon
206 plate (176740; Gibco; Thermo Fisher Scientific) containing 500 μl of oocyte maturation medium
207 at 38.5°C (5% CO2) and then cultured in oocyte maturation medium without hormones for
208 another 22 h[22,27]. Each oocyte maturation medium was previously equilibrated at 38.5 C and
209 5% CO2 in air for 3 h.
210 The sperm cells were treated as described above (sperm assay). Oocytes that showed the first

polar body and homogeneous cytoplasm were placed into IVF medium. Approximately 10 oocytes were placed into a droplet of 40 µl IVF medium covered with mineral oil that had been equilibrated for 6 h. Ten microliters of sperm solution at a concentration of $5x10^6$ spz/ml was added to the oocyte-containing IVF medium to obtain a sperm concentration of $1x10^6$ spz/ml. Fifty oocytes were cultured in 500 µl of porcine zygote medium (PZM) in a four-well Nunclon plate at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2[12], and penetration, polyspermy and the cleavage rate of the embryos were determined.

Statistical Analysis

The experimental data of the percentages for the AR and the cAMP measurements were analyzed with the Statistical Package for the Social Science (SPSS version 18.0, SPSS, Chicago, IL, United States) software. Data were tested for normal distribution and homogeneity of variance. cAMP significance testing was performed using the T test followed by the least-significant difference (LSD) test. P values < 0.05 were considered significant, and the data are expressed as the standard error of the mean (SEM) derived from three independent experiments. Experiments were repeated nine times. One-way repeated-measures ANOVA was used to analyze the percentages for each response category (AR and acrosome-intact). All dependent variables were examined for normality using the Wilcoxon test (SAS Institute, Cary, NC). The penetration rate and polysaccharide fertilization rate were arcsine transformed. The sperm number penetrated in oocytes was logarithmically transformed to approach a normal distribution. Significance testing was performed using a t test and one-way ANOVA, and the data are expressed as the mean ± SEM. Experiments were repeated at least three times, and the data were pooled for analysis.

Results

Location and Expression of OPN Protein in Sperm

Before sperm capacitation, OPN was located in the neck of the sperm (Figure 1B). When the sperm were capacitated, OPN was detected in the acrosome, neck, and tail of the sperm (Figure 1E). In addition, OPN expression increased after sperm capacitation (Figure 1G). Tyrosine-phosphorylated proteins were probed with an anti-phosphorylated antibody, showing increases after capacitation (Figure 1H).

Detection of sperm cAMP content

Measurement of cAMP content in sperm treated with OPN protein for 4 h. The cAMP concentration in OPN-treated sperm was significantly higher ($P<0.01$) than that in untreated control sperm (5.962±0.196 pmol vs. 5.144±0.180 pmol). For the anti-OPN-treated group, the cAMP concentration in sperm was significantly decreased ($P<0.01$) compared to the untreated sperm (4.163±0.164 pmol vs. 5.144±0.180 pmol). Data are expressed as the mean ± SEM (Table 2).

Effect of OPN Protein on the sperm acrosome reaction

There was no significant difference between the blank control and negative control (4 h). The fluorescence of the OPN protein group was significantly higher than that of the other three groups (Figure 2). The fluorescence intensity of the anti-OPN group was significantly lower than that of the other three groups. This suggests that OPN can significantly improve the acrosome reaction of sperm.

Effect of OPN Protein on the Development of Embryos In Vitro

Normal rabbit IgG, porcine OPN protein, and anti-OPN antibody were added to IVF medium separately. Based on the cleavage results, a dilution of the anti-OPN antibody of 1:1000 was used

256 ($P<0.05$) (Table 3). The IgG concentration was not related to embryo division (Table 4). The
257 difference in the cleavage between the OPN protein group and control group was significant, with
258 higher cleavage in the former ($P<0.01$); that between the anti-OPN antibody group (1:1000) and
259 the control group was also significant, with lower cleavage in the former ($P<0.05$). In addition,
260 sperm treated with 0.1 μg/ml OPN protein had significantly increased permeability and
261 polyspermy. The antibody group showed reduced penetration and cleavage and increased
262 polyspermy (Table 5).

263 **Discussion**

264 In previous studies, the literature agreed on the results of OPN's ability to promote embryo
265 cleavage, but it is controversial through which pathways work [8,12]. In this study, we found that
266 OPN protein diffused from the neck of the sperm to the head and tail before and after sperm
267 capacitation and significantly increased its expression. This suggests that OPN may be involved
268 in specific processes in the capacitation of sperm. Sperm capacitation is a prerequisite for the
269 sperm acrosome reaction and egg binding. During the capacitation period, membrane
270 permeability and intracellular signaling pathways are actively changed [28]. In a previous study,
271 OPN treatment of noncapacitated sperm did not change the motility of sperm, but it significantly
272 promoted the rate of embryonic division [12]. This may be because in IVF, sperm are subjected
273 to capacitation and will be in a state of continuous capacitation before sperm-egg binding. We
274 obtained new findings when we were incubated with OPN after sperm capacitation. This suggests
275 that OPN is likely to affect sperm performance by binding membrane proteins to activate
276 membrane signals during sperm capacitation.

277 To further explore the effects of OPN on sperm during capacitation, we tested it at the

278 molecular level. At the molecular level, capacitation involves cholesterol removal and
279 modifications of plasma membrane lipids; activation of the cyclic AMP/protein kinase A/tyrosine
280 kinase (cAMP/PKA/TK) signaling pathway that causes an increase in protein tyrosine
281 phosphorylation (Tyr phosphorylation) and protein phosphatase inactivation; increases in
282 intracellular pH and Ca^{2+} concentration; and sperm plasma membrane potential hyperpolarization,
283 actin polymerization, and cytoskeleton remodeling [29-31]. Furthermore, the synthesis and
284 activation of cAMP occur in the initiation phase of the capacitation reaction, which directly
285 affects the efficiency of the sperm acrosome reaction [32-34]. Since the subsequent influence of
286 OPN on the acrosome reaction ability of sperm was examined, we selected cAMP as a marker to
287 measure the state of capacitation. In the present study, the cAMP content in the capable processing
288 sperm of the experimental group was significantly higher than that of the control group after 4
289 hours of incubation. This suggests that OPN affects the physiological state of the cells when sperm
290 are capacitated.

291 Although the literature agrees on the results of OPN's ability to promote embryo cleavage,
292 previous studies have shown inconsistent results regarding the effects of OPN on sperm acrosome
293 reactions. Hao found that OPN could promote the cleavage rate of pig embryos but reduce the
294 acrosome reaction ability of sperm. In contrast, Monaco found that OPN enhances the ability of
295 sperm to produce acrosome reactions and increases the rate of bovine embryonic division [7,8,12].
296 In this study, we used FCM technology to test the ability of sperm to react with acrosomes and
297 found that OPN can significantly improve the acrosome reaction ability of sperm, and it is more
298 reliable than using eye observation to detect sperm state. Since Hao performed experiments on
299 porcine sperm with murine OPN, however, the cDNA sequence of OPN in pigs does not exhibit

high homology with the corresponding sequences in humans, rhesus monkeys, cattle, rats, mice, and chickens, with similarity ranging from 31.2% to 80% [35]. We used an optimized porcine OPN protein in this study. In addition to the results of the acrosome reaction, there are different results in processing time, most likely due to the effects of protein sources. In addition, we added an antibody-treated control group to IVF, which is more convincing.

Observations of the induction of sperm capacitation suggest that assessing the integration of OPN in IVF medium can increase fertilization rates and improve IVF efficiency. Compared to previous studies, we added antibody sets to the IVF trial to better describe the effect of OPN on fertilization. The IVF results showed that OPN increased the total penetration rate and embryo division and reduced polyspermy. Notably, the recombinant porcine OPN protein had a better effect on the capacitation, acrosome reaction, and fertilization efficiency of pig sperm at the same concentration (0.1 μg/ml).

Conclusions

In previous studies, the detection of OPN protein function in porcine sperm was mainly used as a murine protein in IVF. The results were inconsistent with whether OPN promoted or inhibited the acrosome reaction. We reconstituted the porcine OPN protein and found that it can enhance the acrosome reaction and embryo division. Compared with previous studies, we used more reliable experimental materials and a more accurate IVF protocol to detect whether OPN promotes and inhibits the sperm acrosome reaction and further found that the cAMP concentration changed during sperm capacitation by OPN treatment. In conclusion, this study found that 0.1 μg/ml porcine OPN protein can significantly enhance cAMP concentration after capacitation sperm, acrosome reaction ability, and embryo division during IVF and provides new clues to explore the

mechanism of OPN's function on sperm.

Supplemental Legends: Supplementary materials can be found in additional data files.

Authors' contributions

YC designed the study, performed the experiments, analyzed the data, and wrote the manuscript; KW collected zygotes from the two groups, recorded the number of embryos at the 2-cell stage, and participated in the cell culture experiments; SZ provided guidance with regard to the experiments and analytical methods, analyzed the data, revised the manuscript, and provided administrative and financial support; SZ conceived the idea and gave final approval of the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare that they have no competing interests.

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433 **Table 1 PCR and RT–PCR primer sequences**

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Gene		Primer sequence (5’ to 3’)	Product size (bp)	Accession no.
OPN	F	ATGAGAATTGCAGTGATAGCCTTC	909	397087
	R	GTTGATCTCAGAAGACGCACTCTC		
6His-*OPN*	F	**ATGCATCATCATCATCATCAT**ATG CTTCCAGTTAAACA	885	397087
	R	GTTGATCTCAGAAGACGCACTCTC		
β-actin	F	CTGTGGCATCCACGAAACTA	254	11461
	R	ACATCTGCTGGAAGGTGGAC		

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441 **Table 2 cAMP content in sperm during capacitation**

Control(pmol)	OPN	anti-*OPN* antibody
5.144±0.180	5.962±0.196	4.163±0.164

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444 cAMP content in sperm treated with OPN protein for 4 h.

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Table 3 Effects of anti-OPN antibody at different dilution proportions on cleavage at 48 h during IVF

anti-*OPN* antibody	NO. of oocytes	Cleavage at 48 h (%)
0	207	52.54±0.90[a]
1:50	186	50.48±0.28[a]
1:200	196	49.11±0.93[a]
1:500	182	48.58±1.35[b]
1:1000	195	43.03±2.52[c]
1:2000	201	47.63±1.74[b]

Note: Percentages are expressed as the mean ± SEM.

Different superscript letters indicate significant differences ($P<0.05$).

Cell cleavage was no. of 2-cell/no. of oocytes.

The experiment was replicated four times.

459 **Table 4 Effects of IgG at different dilution proportions on cleavage at 48 h during IVF**

IgG	NO. of oocytes	Cleavage at 48 h (%)
0	232	53.21±1.20
1:50	213	52.32±1.08
1:200	182	54.65±0.43
1:500	196	51.46±1.52
1:1000	215	54.73±2.18
1:2000	233	53.92±1.74

460 Note: Percentages are expressed as the mean ± SEM.

461 Cell cleavage was no. of 2-cell/no. of oocytes.

462 The experiment was replicated four times.

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Table 5 Effect of *OPN* protein on fertilization parameters during IVF

IVF	No. of oocytes	Penetration (%)	Polyspermy (%)	Cleavage at 48 h (%)
Control	272	67.41±3.25[a]	20.31±2.41[a]	55.25±1.01[a]
IgG	265	66.52±2.43[a]	21.17±1.38[a]	56.41±1.41[a]
OPN protein	287	74.29±2.15[b]	16.41±2.17[b]	62.09±1.32[b]
Anti-*OPN* antibody	283	53.72±2.71[c]	25.35±1.27[c]	43.84±0.82[c]

Note: IgG concentration is 1 μg/ml, OPN concentration is 0.1 μg/ml and anti-*OPN* antibody concentration is 82 μg/ml (1:1000)

Percentages are expressed as the mean ± SEM.

Different superscript letters indicate significant differences (P<0.05).

2-cell cleavage was no. of 2-cell/no. of oocytes.

The experiment was replicated seven times.

Figure legends

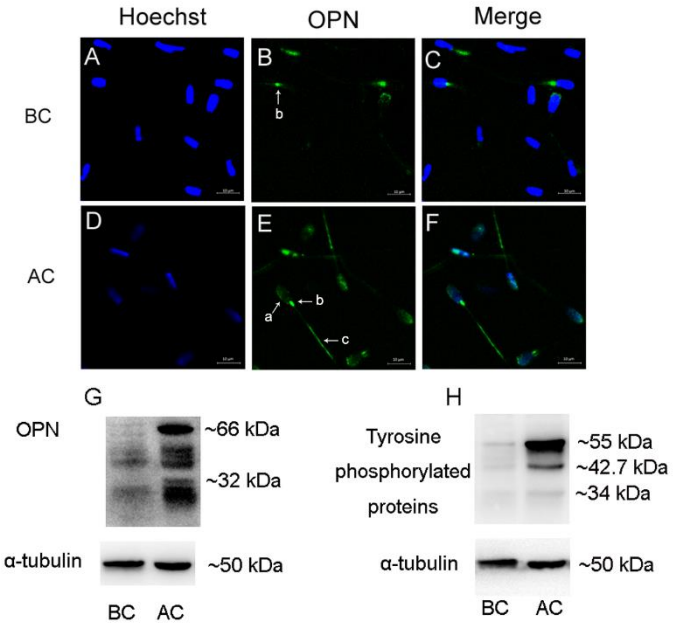

Figure 1 Changes in the localization of OPN during sperm capacitation

BC: before capacitation, AC: after capacitation. **(A and D)** Images of the nucleus before and after capacitation (Hoechst, blue). **(B and E)** Images of OPN protein expression before and after capacitation (green). a: head. b: neck. c: tail. **(C and F)** Merged images of the nucleus (Hoechst, blue) and OPN protein (green) images. The merged images were obtained using a confocal Nikon TS-1000 microscope and NIS Elements image software (Nikon, Japan). Bar=10 μm. (**G and H**) OPN protein and phosphotyrosine were probed with anti-OPN and anti-phosphotyrosine antibodies, respectively.

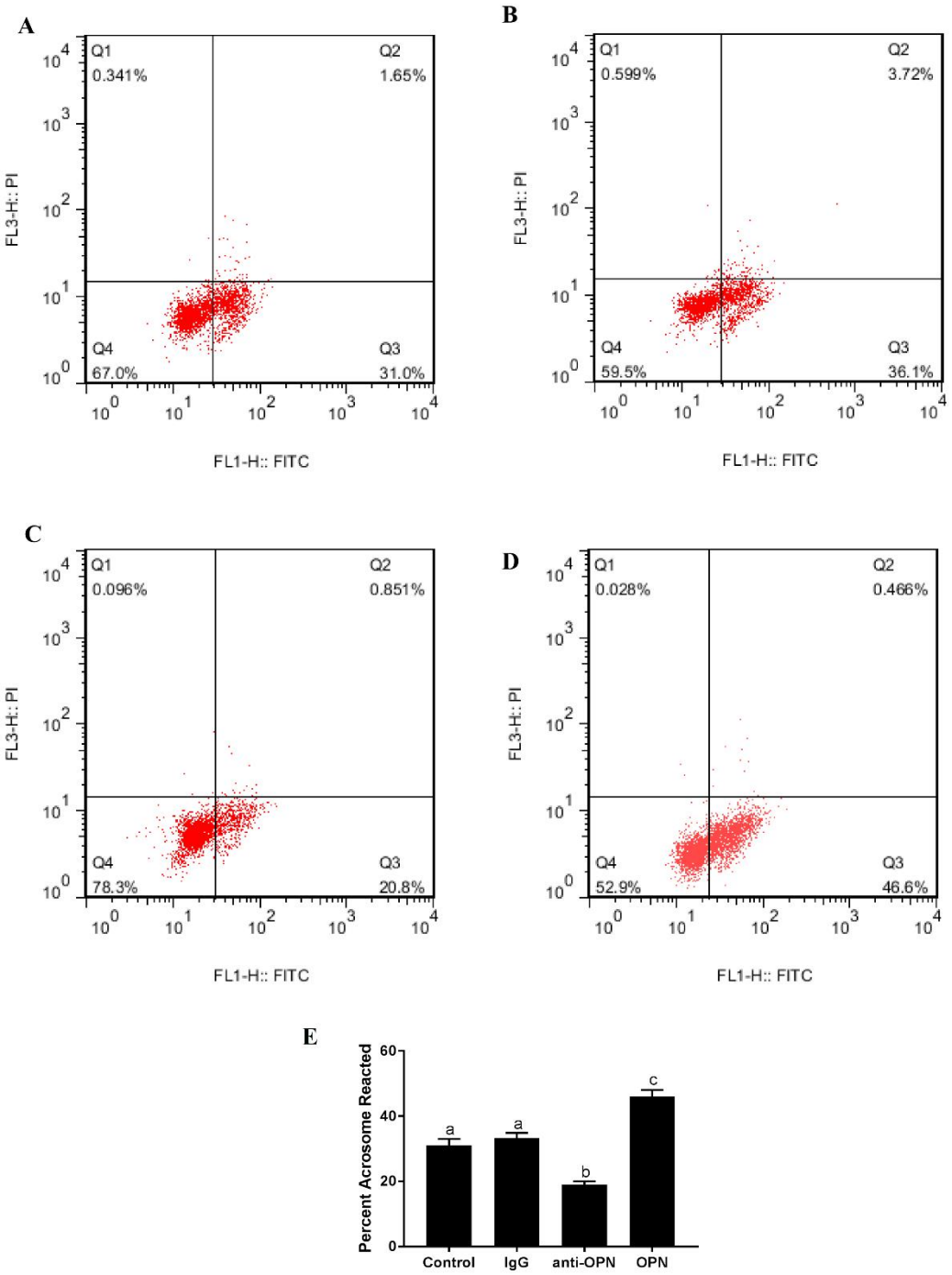

Figure 2 Effect of OPN on the acrosome reaction of sperm during IVF

(A) FITC-PSA flow diagram of the acrosome reaction in untreated sperm (4 h); (**B)** FITC-PSA flow diagram of the IgG-treated acrosome reaction (negative control); **(C)** FITC-PSA flow diagram (anti-OPN group) of the acrosome reaction after sperm incubation with the anti-OPN antibody for 4 h in culture medium; (**D)** FITC-PSA flow diagram of the acrosome reaction incubation with the OPN protein in culture medium for 4 h (OPN protein group); **Q1: dead sperm; Q2: near-dead sperm; Q3: acrosome reaction; Q4: intact acrosome.** (**E)** Percentage of acrosome reaction. Different letters represent significant differences ($P<0.05$).

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