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Sperm metabolism in pig: a role for Peroxisome Proliferator-Activated Receptor (PPAR) γ.

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Running head: PPARγ modulates metabolism in pig spermatozoa

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Footnotes: * The two authors equally contributed to this work. # Joint senior authors.

SUMMARY

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear hormone receptor expressed predominantly in adipose tissue, also implicated in energy homeostasis. In this study, by Western blotting and Immunofluorescence techniques, we demonstrated for the first time that pig spermatozoa express the PPARγ. To investigate the functional role of PPARγ in pig sperm, we evaluated its action on different events that characterize the biology of sperm cell as motility, capacitation, viability and acrosome reaction, using 15-deoxy-12,14-prostaglandin J2 (PGJ2) PPARγ-agonist. Responses to PGJ2 treatments on motility, cholesterol efflux and tyrosine phosphorylation were increased and this broadens the roles of PPARγ in the literature, since it also acts to ameliorate sperm functionality. To deepen PPARγ significance in pig sperm we focused its effects on lipid and glucose metabolism. The evaluation of the triglycerides content, lipase and acyl-CoA dehydrogenase as well as G6PDH activities, suggests that PPARγ induces energy expenditure in pig spermatozoa. These data represent a meaningful advance in the field of sperm energy metabolism since this is a new intriguing issue.

Taken together, our results demonstrated for the first time that PPARγ is expressed by pig sperm improving its functionalities such as motility, capacitation, acrosome reaction, survival and metabolism.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily. The PPARγ is a nuclear fatty acid receptor that has been implicated in energy homeostasis and in many pathological processes (Knouff and Auwerx, 2004). Particularly, it modulates lipid homeostasis in metabolically active sites, as the liver, macrophage, adipocytes, and muscle (Rangwala and Lazar, 2004; Kota et al., 2005).

PPARγ is mainly expressed in adipose tissue (Gurnell, 2005), but this receptor is also present in several cell types (Nicol et al., 2005; Wang et al., 2006; Kostadinova et al., 2005). It also acts as a regulator of the inflammation (Celinski et al., 2011) and it is activated by endogenous arachidonic acid metabolites such as 15-deoxy-12,14-prostaglandin J_2 (PGJ2) (Desvergne and Wahli 1999; Kobayashi et al., 2005).

Intensive studies and compelling evidences demonstrated a close link between energy status and reproductive function (Quandt, 1984; Moschos et al., 2002). In mice, the loss of the PPARγ gene in oocytes and granulosa cells resulted in impaired fertility (Cui et al., 2002). In our previous study, we demonstrated, that human sperm express the PPARγ and the functionality of this receptor was also investigated (Aquila et al., 2006). Many studies prove that transcriptional factors as nuclear receptors, in addition to their classic genomic action, also regulate cellular processes through their nongenomic mechanism (Cato et al., 2002). Different nuclear receptors such as progesterone receptors (Shah et al., 2005, De Amicis et al., 2011), estrogen receptor α and estrogen receptor β, androgen receptors (Aquila et al., 2004, Aquila et al., 2007; Guido et al., 2011), were found to be present in human ejaculated spermatozoa, regulating some cellular processes. Ejaculated mammalian spermatozoa are highly differentiated attractive cells showing intriguing features, particularly they go through two different physiological conditions including a quiescent metabolic state in the male genital tract and an enhanced energy metabolism to accomplish their functional maturation, known as capacitation, in the female genital tract (Rathi et al., 2001; Yanagimachi, 1994; Suarez, 2008). In the recent years, a new picture of this cell is emerging: it expresses various receptors types (Travis and Kopf, 2002; Aquila et al., 2007), and it also produces their ligands, suggesting that through an autocrine short loop it may modulate its own functions independently by the systemic regulation (Aquila et al., 2005a, 2005b). Anyways, sperm need to have a finely regulation of metabolism to affect the signaling pathways changes during their life. However, the mechanisms underlying the signaling events associated with the change in sperm energy metabolism are to date poorly understood.

In the current study we showed for the first time that pig spermatozoa express the PPARγ broadening the role of this nuclear receptor since it is also able to act on sperm physiology.

MATERIALS AND METHODS

Chemicals

BSA protein standard, Laemmli sample buffer, prestained molecular weight markers, Percoll (colloidal PVP coated silica for cell separation), Sodium bicarbonate, Sodium lactate, Sodium pyruvate, Dimethyl Sulfoxide (DMSO), 15-deoxy-12,14-prostaglandin J_2 (PGJ2), the irreversible PPARγ antagonist GW9662 (GW), phosphate buffered saline (PBS) and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Earle´s Balanced Buffered Solution (BBS) (Product number: C4228.0500) without calcium, without magnesium, without phenol red, without NaHCO3 was from Genaxxon bioscience (Milan, Italy) (unsupplemented Earle´s medium). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). Gel band purification kit, ECL Plus Western blotting detection system, Hybond™ ECL™, Hepes Sodium Salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Cholesterol-oxidase (CHOD) - peroxidase (POD) enzymatic colorimetric assay, triglycerides assay, acyl-CoA dehydrogenase assay, lipase activity, glucose-6-phosphate dehydrogenase (G6PDH) were from Inter-Medical (Biogemina Italia Srl, Catania, Italy). Goat polyclonal actin antibody (Ab) (1-19), polyclonal rabbit anti-PPARγ Ab, rabbit anti-p-Akt1/Akt2/Akt3 S473 (AKTS) Ab, rabbit anti-p-Akt1/Akt2/Akt3 T408 (AKTT) Ab, total anti-Akt1/Akt2 Ab, rabbit anti-p-MAPK 42/44 Ab, total anti-MAPK 42/44 Ab, rabbit anti-p-Bcl2 Ab, total anti-Bcl2 Ab, were from Cell Signaling (Milan, Italy), polyclonal rabbit anti-phosphotyrosine (PY99) Ab, peroxidase-coupled anti-rabbit and anti-goat, anti-rabbit IgG FITC conjugated, Protein A/G-agarose plus were from Santa Cruz Biotechnology (Heidelberg, Germany).

Semen samples and spermatozoa preparations

The investigation has been conducted on semen from eight fertile male pigs (Sus scrofa domestica, Large White) kept at 'Swine Artificial Insemination Centre' (Rende, Cosenza, Italy). The animals were 20 to 30 month-old and their weights were from 260 to 330 kg. Individual fresh ejaculates were collected using the gloved hand method and filtered immediately by Universal Semen bags (Minitub, Tiefenbech, Germany). Individual sperm rich ejaculates were collected. Semen analysis showed normal sperm parameters in all the ejaculates (vol : 200-300ml, total spermatozoa per ejaculate: 30-60 x 10^9 ,progressive sperm motility 70-90%, sperm abnormalities (clumping, abnormal tails, abnormal acrosome, etc): 20-30% . Semen was transported within half an hour to the laboratory. It

was diluted 1:10 with Tris-buffered saline (TBS) buffer and centrifuged on a discontinuous Percoll density gradient (72 % / 90 %) to remove eventual bacteria and debris (Kuster et al., 2004). Then, samples were centrifuged at 700g for 30 minutes and the upper phase was used to determinate the cholesterol levels, while the pellet containing sperm was lysed to perform western blots, triglycerides assay, acyl-CoA dehydrogenase assay, and G6PDH and lipase activity. At the begin, prior the centrifugation several aliquots were used to perform sperm motility and viability. Spermatozoa preparations were performed as previously described (Aquila et al., 2002).

Processing of ejaculated sperm

Percoll-purified sperm were once washed with unsupplemented Earle´s medium for 10 min at 800 x g, and then incubated in the same medium for 30 minutes (min) at 39 °C and 5 % CO_2, without (control) or with treatments (experimental). Some samples were incubated in Earle's BBS medium supplemented with 25 mM NaHCO3, 2 mM CaCl2, 0.6% BSA, and 1 mM pyruvate (pH 7.4) (capacitating medium), used only for the capacitated sample in the immunofluorescence assay. When the cells were treated with an inhibitor a pre-treatment of 15 min was performed and subsequently the sperm were incubated with the substances reported in the manuscript for 30 min.

To evaluate pig sperm viability three different sets of experiments were performed: in freshly ejaculated sperm, before any incubation (without or with increasing PGJ2) at time 0 min; after being selected by percoll treated without or with PGJ2 at time 0 min; percolled-purified sperm incubated without or upon increasing PGJ2 at 30 min as abovementioned.

Evaluation of sperm motility and viability

Sperm motility was assessed by means of light microscopy examining an aliquot of each sperm sample in absence (NC) or in the presence of increasing PGJ2 concentrations (1 to 20 μM) and/or 10 μM GW alone or combined with 10 μM PGJ2. Sperm motility was expressed as percentage of total motile sperm. An independent observer scored at least 200 cells.

Viability of pig spermatozoa was assessed using the DNA specific fluorochrome propidium iodide (PI). Sperm suspension ($1x10^9$ ml) was exposed to PI (12 μM) for 5 min at room temperature. Then spermatozoa were fixed by adding 1 ml of 12.5 % (w / v) paraformaldehyde in 0.5 M Tris (pH 7.4) and the slides were immediately examined under an epifluorescence microscope (Olympus BX41, Olympus Italia Srl, Segrate, Milano, Italy) observing a minimum of 200 spermatozoa per slide (100x objective).

Western blot analysis of sperm proteins

Western blot analysis was used to identify PPARγ in pig spermatozoa. Sperm samples, washed twice with unsupplemented Earle´s medium for 10 min at 800 x g, were incubated without or with the

indicated treatments, and then centrifuged for 5 min at 5000 x g. The pellet was resuspended in lysis buffer as previously described (Aquila et al., 2002). Equal amounts of protein (80 µg) were boiled for 5 min, separated by 10 % polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and probed with an appropriate dilution of the indicated Ab. The bound of the secondary Ab was revealed with the ECL Plus Western blotting detection system according to the manufacturer's instructions. The negative control was performed using a sperm lysate that was immunodepleted of PPARγ (Aquila et al., 2004) (i.e. preincubate lysate with anti- PPARγ Ab, 1 hour at room temperature, and immunoprecipitate with Protein A/G-agarose). To further verify the specificity of our Ab anti- PPARγ, the blot was probed with the normal rabbit serum instead of the primary Ab. As internal control, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) of the first Ab and reprobed with anti-β actin. The protein bands were quantified by scanning densitometry (Imaging Densitometer GS-700 BIO–RAD USA). Western blot analysis was also performed to evaluate protein tyrosine phosphorylations.

Immunofluorescence assay

Following Percoll separation, sperm cells were rinsed three times with 0.5 mM Tris-HCl buffer, pH 7.5 and fixed with absolute methanol for 7 min at –20 °C. After methanol removal, sperm cells were washed in TBS, containing 0.1% Triton X-100 and were treated for immunofluorescence PPARγ staining was carried out, after blocking with normal horse serum (10 %), using a rabbit polyclonal anti-human PPARγ as primary Ab and an anti-rabbit IgG FITC conjugated (1:100) as secondary Ab. Sperm cells incubated with normal rabbit serum instead of the primary Ab was utilized as the negative control. The slides were examined under a fluorescence microscope (Olympus BX41, Milan Italy), and a minimum of 200 spermatozoa for slide were scored.

Measurement of cholesterol in the sperm culture medium

Cholesterol was measured in duplicate by a CHOD - POD enzymatic colorimetric method according to manufacturer's instructions in the incubation medium from human spermatozoa. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) or in capacitating medium for 30 min at 39 °C and 5 % CO_2. Some samples were incubated in the presence of increasing PGJ2 concentrations (1 to 20 µM). Other samples were incubate in the presence of 10 µM GW alone or combined with 10 µM PGJ2. At the end of the sperm incubation the culture media were recovered by centrifugation, lyophilized and subsequently dissolved in 1 ml of buffer reaction. The samples were incubated for 10 min at room temperature, then the cholesterol content was measured with the spectrophotometer at 505 nm. Cholesterol standard used was 200 mg/dl. The limit of sensitivity for the assay was 0.03 mg/dl. Inter- and intraassay variations were

0,03 % and 0,02 % respectively. Results are presented as cholesterol amount (mg ⁄ dl) in culture medium from 10 x 10^6 sperm and are given as mean ± SEM.

Triglycerides Assay

Triglycerides were measured in duplicate by a GPO-POD enzymatic colorimetric method according to manufacturer's instructions in sperm lysates and as previously described (Aquila et al., 2006; Aquila et al., 2009). Data are presented as mg/10^9 sperm.

Lipase activity assay

Lipase activity was evaluated, by the method of Panteghini et al.(Panteghini *et al.* 2001) based on the use of 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester (DGGR) as substrate, as previously described (Aquila et al., 2006; Aquila et al., 2009).

Assay of acyl-CoA dehydrogenase activity

Acyl-CoA dehydrogenases are a class of enzymes which function to catalyze the initial step in each cycle of fatty acid β-oxidation. Assay of acyl-CoA dehydrogenase was performed on sperm, using a modification of the method described by Lehman et al. (Lehman et al., 1990) as previously described (Aquila et al., 2006).

Glucose-6-phosphate dehydrogenase (G6PDH) activity

Assay of G6PDH activity was performed on sperm lysates in the same experimental conditions above mentioned. The conversion of NADP+ to NADPH, catalyzed by G6PDH, was measured by the increase of absorbance at 340 nm, as previously described (Aquila et al., 2005b). The conversion of $NADP^+$ to NADPH, catalyzed by G6PDH, was measured by the increase of absorbance at 340 nm, as previously described (Aquila et al., 2006; Aquila et al., 2009).

Acrosome reaction

The evaluation of the acrosome reaction was performed by using FITC-PNA. At the end of incubation, sperm cells were washed three times with 0.5 mmol/liter Tris-HCl buffer (pH 7.5) and were allowed to settle onto slides. Smears, dried in air, were dipped in absolute methanol for 15 min and left at room temperature. The samples were then incubated with a solution of FITC-PNA in a humid chamber at room temperature. After 30 min, the slides were washed with PBS to remove the excess label. Scoring of the staining was immediately assessed, by an epifluorescence microscope (Olympus BX41) according to a published scoring system (Cheng FP et al, 1996). A minimum of 200 live sperm were examined for each treatment as previously reported (Aquila S et al, 2011). Values are expressed as percentage of acrosome-reacted cells.

STATISTICAL ANALYSIS

The experiments for Western blot and Immunofluorescence analysis were performed in at least three independent experiments. The data obtained from motility, viability, CHOD-POD enzymatic

colorimetric method, triglycerides assay, acyl-CoA dehydrogenase activity, G6PDH and lipase activities, were performed in at least six replicate experiments using duplicate determinations and were presented as the mean ± SEM. The differences in mean values were calculated using analysis of variance (ANOVA) with a significance level of $P \leq .05$.

RESULTS

Pig sperm contains the PPARγ

Using an antibody (Ab) raised against the carboxyl-terminus of the human PPARγ protein, by Western blot, we have explored the presence of PPARγ in pig ejaculated sperm. A band, corresponding to the molecular mass values of 70 kDa (Fig. 1A), was evidenced in pig sperm extracts such as in the human sperm used as positive control. The band was not detected in the negative control (lane 2), performed as previously described (Aquila et al., 2007) and when the non-immune rabbit serum (Fig. 1B) instead the primary Ab was used, further confirming the specificity of the Ab for PPARγ.

PPARγ is located prevalently in the midpiece

In the majority of sperm population, by the immunofluorescence assay with the same Ab used for western blot, the PPARγ was found in the apical region of the head, in the subacrosomial region and prevalently in the midpiece, while the signalling was almost absent in the tail (Fig. 2A). In Fig. 2B capacitated sperm were shown, and it appears that the location of the receptor mirrors that observed in uncapacitated sperm. No fluorescent signal was obtained when the normal rabbit IgG was used instead of the primary Ab (Fig. 2C).

PGJ2 influence motility through PPARγ in pig sperm.

Sperm motility indicates the ability of sperm to move properly towards an egg. In all the experiments the natural PPARγ-ligand, PGJ2 (1, 10 and 20 μM) induced a increase on sperm motility that was significant at the 10 μM concentration. Interestingly, the effect was reduced by the specific GW antagonist, suggesting a PPARγ-mediated action (Fig. 3).

PGJ2 induces capacitation in pig sperm.

Successively, we investigated whether the PPARγ-agonist was able to influence the sperm functional maturation evaluating its action on capacitation. This unique process has been correlated with functional and biochemical changes in sperm, including cholesterol efflux (Travis and Kopf 2002) and tyrosine phosphorylation of sperm proteins (Visconti et al., 1995). Washed sperm were treated with increasing concentration of PGJ2 (1, 10 and 20 μM). At the end of incubation the samples were centrifuged, the upper phase was employed to determinate cholesterol levels, while the pellet (sperm) was lysed to study protein tyrosine phosphorylation. The outcomes indicates that cholesterol efflux

increased from 1 to 10 µM PGJ2 treatment, while the 20 µM didn't give further effect (Fig. 4A). Similar results were obtained for tyrosine phosphorylations of the sperm proteins (Fig. 4B). Both processes were attenuated by using the specific PPARγ-antagonist GW suggesting an involvement of this receptor.

PPARγ influences viability by inducing AKT, BCL2 and MAPK 42/44 phosphorylations in pig spermatozoa

The sperm survival is an important aspect of ejaculate quality that determines fertilization success. From our results it emerges that PPARγ increase the sperm viability from 1µM to 10 µM PGJ2, while 20 µM did not induce further effect (Fig. 5A). These effects were obtained in the samples after incubation, while in freshly ejaculated sperm or after being selected by percoll, both before any incubation did not give substantial variations.

To deep this sperm activity from a molecular point of view, we evaluated PGJ2 action exploring the most important signalings involved in cell survival and previously investigated in human and pig sperm, the PI3K/Akt pathway (Fisher et al., 1998; Aquila et al., 2004) and p-MAPK 42/44 (Almog and Naor 2008). As shown in Fig. 5 increasing doses of PGJ2 (1, 10 and 20 µM) resulted in a significant increase in the AKTS (Fig. 5, panel B) and AKTT (Fig. 5, panel C) phosphorylations. AKT, the major downstream PI3K signal transducer, is full activated when both S473 and T308 phosphorylations were enhanced (Aquila et al., 2007). In the same vein PPARγ-agonist induced MAPK 42/44 phosphorylation (Fig. 5, panel D). Bcl-2 a key protein in survival signaling (Ito et al., 1997) phosphorylated at Serine 70, the physiologically relevant phosphorylation site, necessary for its full survival function, is enhanced upon the exposure to PGJ2 (Fig. 5, panel E). All these events were reduced by GW confirming a PPARγ involvement.

PPARγ modulates triglycerides, lipase activity and β-oxidation of the fatty acids in pig sperm

PPARγ main role is on lipid metabolism, therefore we evaluated the intracellular level of triglycerides, lipase activity and the β-oxidation of the fatty acids, under PPARγ–agonists treatments. Upon the abovementioned treatments, sperm triglycerides levels were decreased (Fig. 6A) and concomitantly, lipase activity and β-oxidation of the fatty acids augmented (Fig. 6B e Fig. 6C). GW was able to attenuated the PPARγ–agonist effects.

PPARγ affects the G6PDH activity and acrosome reaction in pig spermatozoa

In somatic cells, a role of PPARγ in glucose metabolism has been explored (Lenhard et al., 1997). The effect of glucose on the fertilizing ability of sperm appears to be mediated by the pentose phosphate pathway (PPP) (Miraglia et al., 2010) and G6PDH is the key rate limiting enzyme in this metabolic way, which has been shown to be functional in human spermatozoa (Aquila et al., 2009).

As shown in Fig. 7A, PGJ2 activates G6PDH activity at 10 and 20 µM. Thus, our results address a regulatory role of PPARγ in sperm glucose metabolism. In Fig. 7B the effect of PGJ2 on acrosome reaction showed a similar pattern of response to that observed in our experiments and accordingly to that previously reported (Joyce CL et al, 1987).

DISCUSSION

PPARγ, a nuclear hormone receptor, have an important role in the control of energy, lipid and glucose homeostasis (Rangwala and Lazar, 2004, Kota et al., 2005). Sperm energy management is currently a nebulous topic that need to be examined in depth given the peculiarities of this cell type. In the present study, we showed for the first time that PPARγ is expressed in pig spermatozoa. Intriguingly, the PPARγ action was investigated on motility, survival, capacitation and sperm metabolism.

Using an Ab raised against the carboxyl-terminus of the human PPARγ protein, by Western blot, we showed a positive signal for PPARγ at the same molecular weight as it was obtained in human spermatozoa used as positive control (Aquila et al., 2006). The immune-histochemical assays demonstrated that PPARγ protein was detectable in pig sperm, with specific signals being located in the apical region of the head, in the subacrosomial region and in the middle-piece, while a weak label was obtained through the tail. These results, are consistent with that observed in human sperm (Aquila et al., 2006). Furthermore, we evaluated the signaling in the capacitated sperm and it was not substantially different from that obtained in uncapacitated spermatozoa.

As it concerns the motility our results are in concordance with recent studies on human spermatozoa where prostaglandins are reported to enhance this process (Aitken et al., 1986; Aitken and Kelly 1985), and with our previous study where we showed that the effects of these compounds are PPARγ –mediated (Aquila et al., 2006).

During its life, sperm go through two different physiological conditions: uncapacitated, during which sperm remain in a quiescent metabolic state, when it is in the male genital tract or upon ejaculation, accumulating and / or economizing energy substrates; capacitated, when sperm travel through the female genital tract, increasing the metabolic rate to acquire the competence to fertilize the oocyte.

In this study, upon PGJ2 we observed a significant increase in cholesterol efflux and tyrosine phosphorylation of sperm proteins. All these effects were PPARγ-mediated since they were reversed by the irreversible PPARγ antagonist, GW. Data presented herein also demonstrated that PGJ2, by inducing the phosphorylation of classical key survival proteins, such as MAPK 42/44, PI3K, AKT, and BCL2, is involved in the pig sperm survival.

A key role for PPARγ in energy metabolism in somatic cells has been established and during the switch from quiescent to capacitated state, the sperm show an increased metabolic rate probably needful to the changes in sperm signalings related to the capacitation process. The connection between the events of capacitation and the variations in sperm energy metabolism is poorly known. Our data on the analyses of lipid metabolism suggest a lipolytic effect of PPARγ, accordingly to the results showed for human sperm. Therefore, we may hypothesize that during capacitation when energy expenditure increases, PPARγ works to mobilize lipid reserve, providing additional metabolic fuel to sustain capacitation process. The effect of glucose on the fertilizing ability of spermatozoa appears to be mediated by its metabolism through the PPP (De Amicis et al., 2011). Interestingly, our data showed that PPARγ was able to modulate in a dose-dependent way the G6PDH activity, the key rate-limiting enzyme in the PPP. It is important to point out that in all our experiments, we obtained different responses by using low or high PGJ2 concentrations; in fact 1 μM and 10 μM were stimulatory, whereas the higher PGJ2 concentration (20 μM) didn't give further effect with respect to that obtained with 10 μM. Differences in ligand levels may result in a diverse response as it was demonstrated in somatic cells (Castoria et al., 2003) as well as in human sperm (Aquila et al., 2005b).

Elevated levels of prostaglandins are reported in seminal plasma (Templeton et al., 1978; Rodríguez-Martínez et al., 2009), in cervical mucus (Charbonnel et al., 1982) and also sperm synthesize prostaglandins (Rodríguez-Martínez et al., 2009, Roy and Ratnam SS**,** 1992). Different studies showed that these substances enhance sperm fertilizing ability (Aitken et al., 1986; Aitken and Kelly 1985; Joyce CL et al., 1987) and our results on the acrosome reaction confirmed this role of prostaglandins. The autonomous capability of sperm to release PGs suggests that they through an autocrine short loop may act on PPARγ to regulate energy management during capacitation process (Aquila et al., 2006).

Altogether, PPARγ influences pig sperm biology and physiology by regulating motility, capacitation, acrosome reaction, survival and metabolism.

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.

ABBREVIATIONS: 15-deoxy-12,14-prostaglandin J2 (PGJ2), Peroxisome proliferator-activated receptors (PPARs), Glucose-6-phosphate dehydrogenase (G6PDH), antibody (Ab), irreversible PPARγ antagonist GW9662 (GW), pentose phosphate pathway (PPP), Mitogen-activated protein kinase (MAPK) 42/44, Phosphoinositide 3-kinase (PI3K), RAC-PK-alpha (AKT), and B-cell lymphoma 2 (BCL2), fluorescein isothiocyanate (FITC).

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FIGURE LEGENDS

FIG. 1 Pig ejaculated spermatozoa contains PPARγ

A: Western blot of PPARγ protein in pig spermatozoa, expression in two samples of ejaculated spermatozoa from normal pigs (P1, P2). Human sperm extract was used as control (HS). The negative control (see Materials and Methods), is represented in lane 2 (N), β-actin was used as loading control. **B:** To further verify the specificity of our Ab anti- PPARγ, the blot was probed with the normal rabbit serum instead of the primary Ab. The autoradiographs of the figure show the results of one representative experiment.

FIG. 2 PPARγ compartmentalization in pig ejaculated spermatozoa

A: PPARγ immunolocalization in uncapacitated sperm; **B**: PPARγ immunolocalization in capacitated sperm; **C**: Sperm cells incubated replacing the anti-PPARγ Ab by normal rabbit serum were utilized as negative control. The pictures shown are representative examples of experiments that were performed at least three times with reproducible results.

FIG. 3 PGJ2 effects on sperm motility are PPARγ-mediated.

Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 39°C and 5% CO_2, in the absence (NC) or in the presence of increasing concentration of PGJ2 (1, 10, 20 μM) or with 10 μM GW alone or combined with 10 μM PGJ2. **A**: Sperm motility was expressed as percentage of total motile sperm. Columns are mean ± SEM of six independent experiments performed in duplicate. $*P < 0.05$ versus control.

FIG. 4 Cholesterol efflux and tyrosine phosphorylation of sperm proteins increase upon PGJ2.

Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 39°C and 5% CO_2, in the absence (NC) or in the presence of increasing concentration of PGJ2 (1, 10, 20 μM) or with 10 μM GW alone or combined with 10 μM PGJ2. **A:** Cholesterol in culture medium from pig ejaculated spermatozoa was measured by enzymatic colorimetric assay. Columns are mean ± SEM of six independent experiments performed in duplicate. Results are presented as cholesterol amount (mg / dl) in culture medium from 10 x 10^6 sperm and are given as mean ± SEM. **B:** 80 μg of sperm lysates were used for western blot analysis of protein tyrosine phosphorylations. On the left side quantitative representation after densitometry on the 50 kDa band. Columns are mean ± SEM of six independent experiments. The autoradiograph of the figure shows a representative experiment. $*P < 0.02$ versus control, $**P < 0.01$ versus control.

FIG. 5 PGJ2 increases survival as well as AKT, MAPK 42/44 and Bcl2 phosphorylations in pig sperm.

Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 39 °C and 5% CO_2, and treated as indicated. **A:** Pig sperm viability upon increasing PGJ2, in freshly ejaculated sperm and after being selected by percoll, before any incubation (with or without PGJ2) and in incubated sperm. ⊓ freshly ejaculated, ❑ percolled, ■ incubated. **B:** Exactly 80 μg of sperm lysates were used for Western blot analysis of AktS, AktT, MAPK 42/44, BCL2 phosphorylations. Loading controls: total AKT (AKT Tot), total MAPK 42/44, total BCL2 (BCL2 Tot). The arrows indicate the proteins molecular weight. **C:** On the bottom is reported the densitometry evaluation of the proteins above mentioned. The autoradiographs presented are representative examples of experiments that were performed at least six times with repetitive results. **P* < 0.05 versus control, ***P* < 0.01 versus control. ⊓ pAKTT, ❑ pAKTS, ❑ pBCL2, ■ pMAPK.

FIG. 6 PGJ2 modulates lipid metabolism in pig sperm

Sperm samples, washed twice with uncapacitating medium were incubated in the same medium (NC) for 30 min at 39 °C and 5 % CO_2 and treated as indicated. Assay of triglycerides (**A**), Lipase activity (**B)** and acyl-CoA dehydrogenase activity (**C**) were performed as described in Materials and Methods. Columns are mean ± SEM of six independent experiments performed in duplicate. **P* < 0.05 versus control; ** P<0.01 versus control.

FIG. 7 G6PDH activity and acrosome reaction increased upon PGJ2

Sperm were washed with the unsupplemented Earle's medium and were treated as indicated. **A:** G6PDH activity was performed as described in Materials and Methods. Columns are mean ± SEM of six independent experiments performed in duplicate. Data are expressed as nanomoles per minute per 10^9 sperm. * *P* < 0.05. **B**: Acrosome reaction was determined as described in Methods and the values are expressed as percentage of acrosome-reacted cells. Columns represent mean ± SEM of four independent experiments each done in duplicate. **P* <0.05 and ***P* = 0.02 versus control.

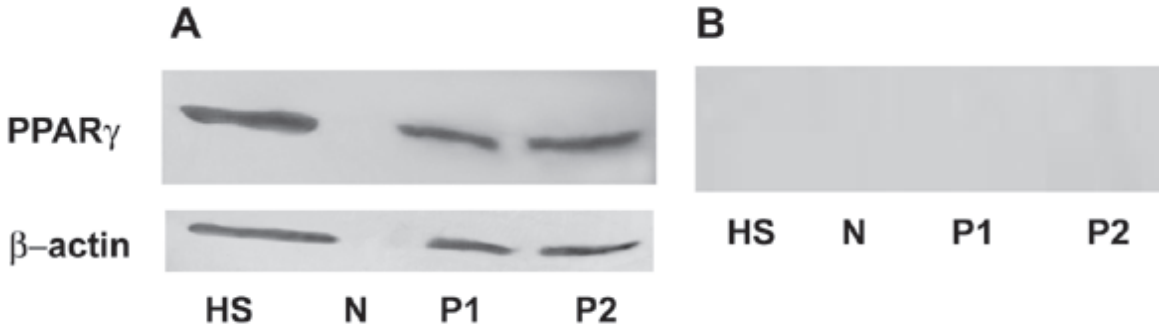


Fig. 1

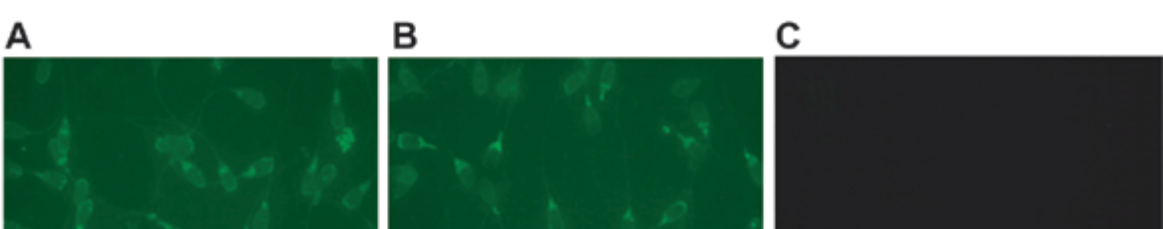


Fig. 2

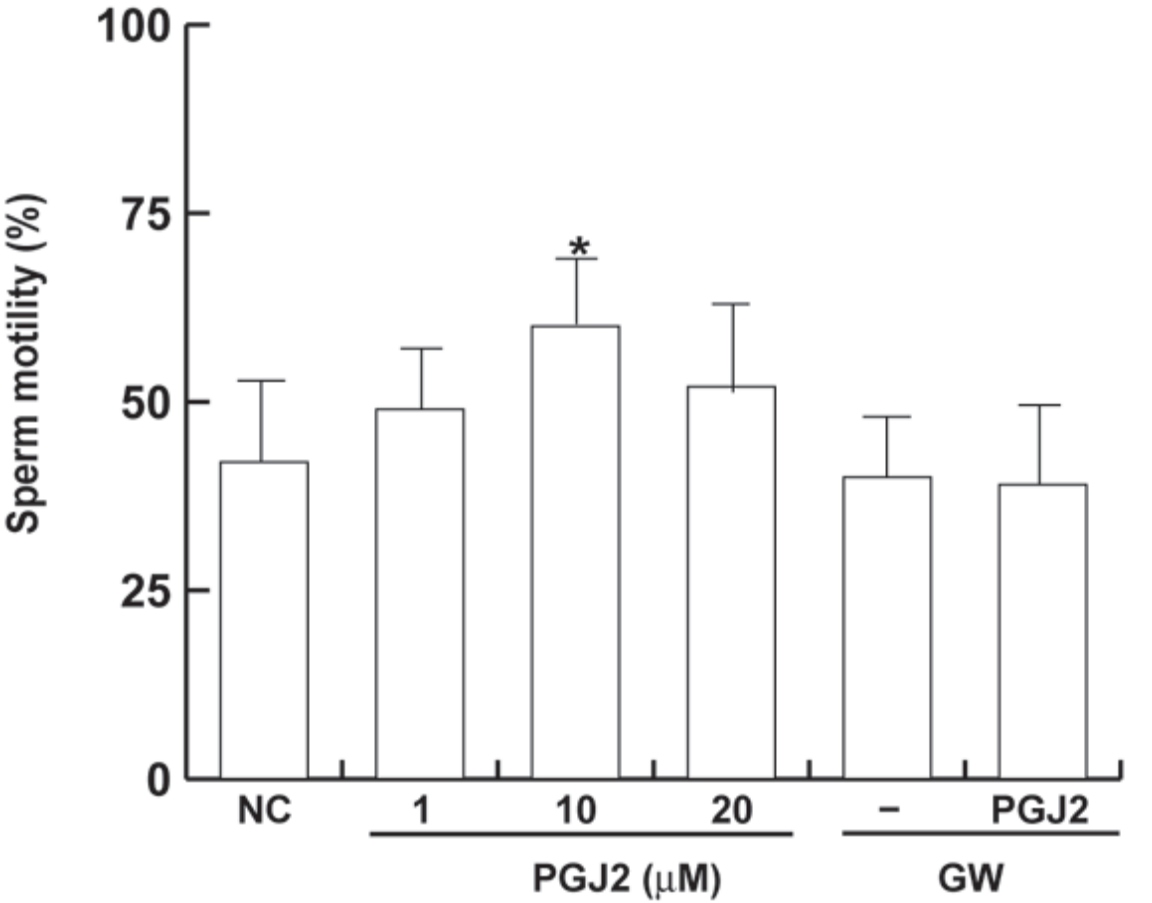


FIG. 3

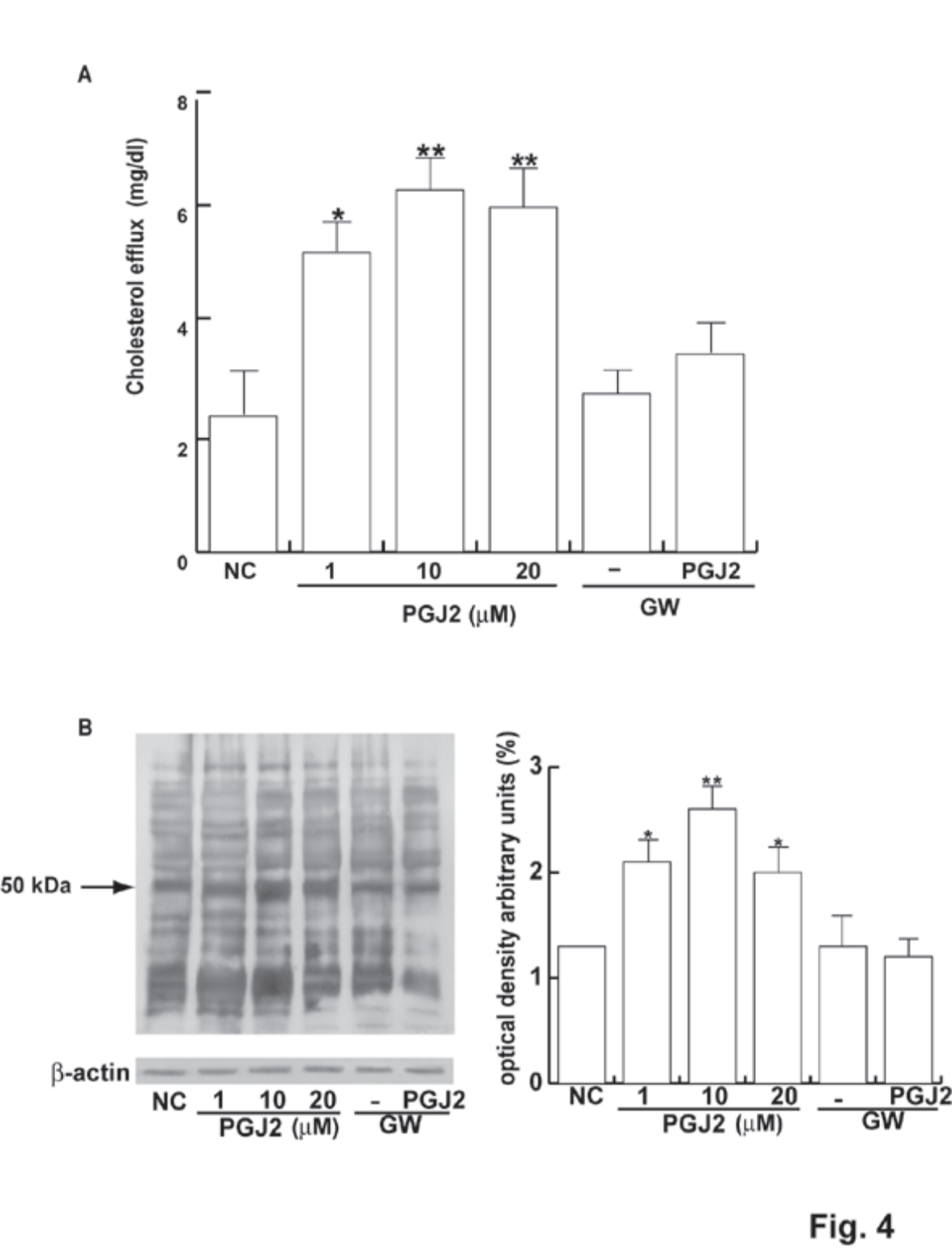

A
Cholesterol efflux (mg/dl)
8
6
4
2
0
*
**
**
NC
1
10
20
PGJ2 (μM)
–
PGJ2
GW
B
50 kDa
β-actin
NC
1
10
20
PGJ2 (μM)
–
PGJ2
GW
optical density arbitrary units (%)
3
2
1
0
*
**
*
NC
1
10
20
PGJ2 (μM)
–
PGJ2
GW

Fig. 4

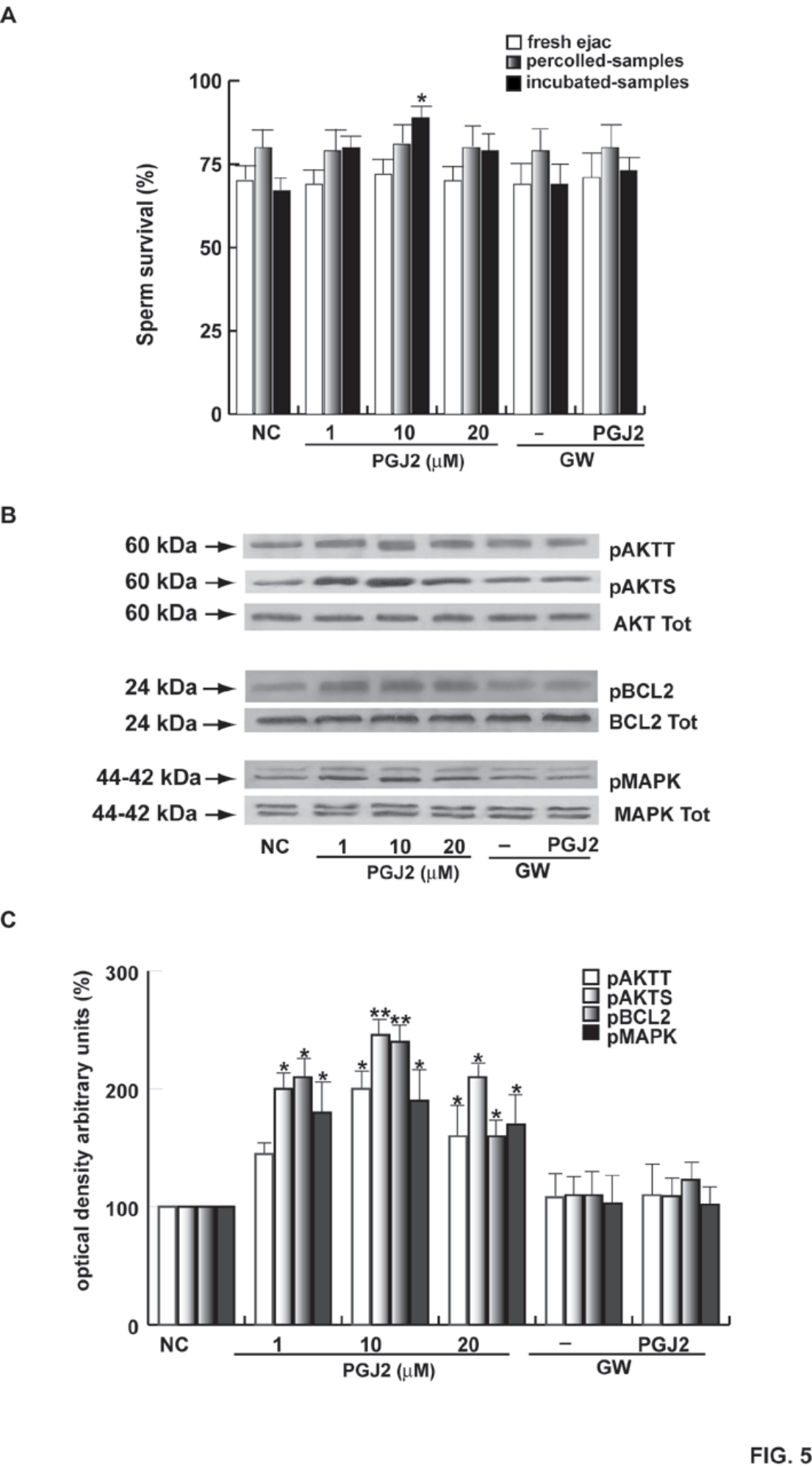

A
fresh ejac
percolled-samples
incubated-samples
Sperm survival (%)
100
75
50
25
0
*
NC
1
10
20
–
PGJ2
PGJ2 (μM)
GW
B
60 kDa
pAKTT
60 kDa
pAKTS
60 kDa
AKT Tot
24 kDa
pBCL2
24 kDa
BCL2 Tot
44-42 kDa
pMAPK
44-42 kDa
MAPK Tot
NC
1
10
20
–
PGJ2
PGJ2 (μM)
GW
C
optical density arbitrary units (%)
300
200
100
0
pAKTT
pAKTS
pBCL2
pMAPK
NC
1
10
20
–
PGJ2
PGJ2 (μM)
GW

FIG. 5

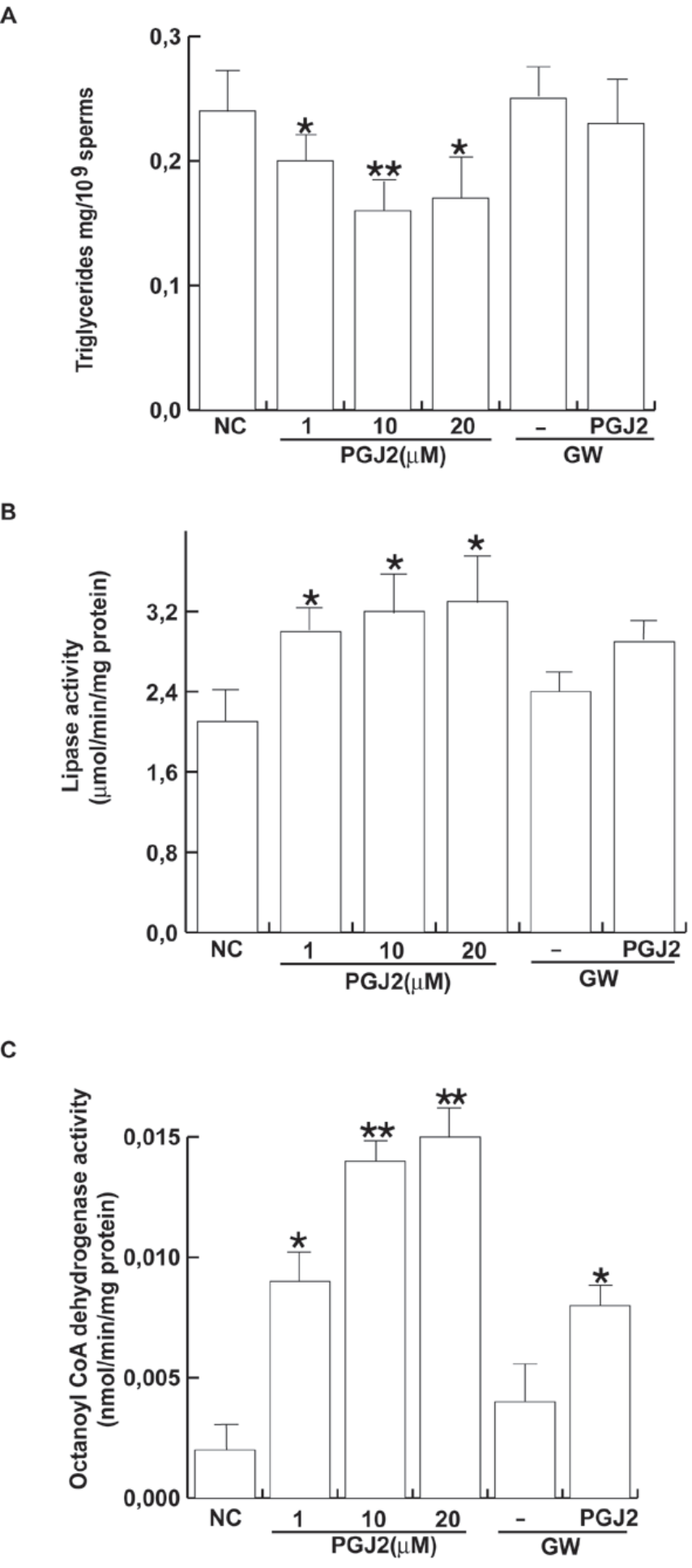
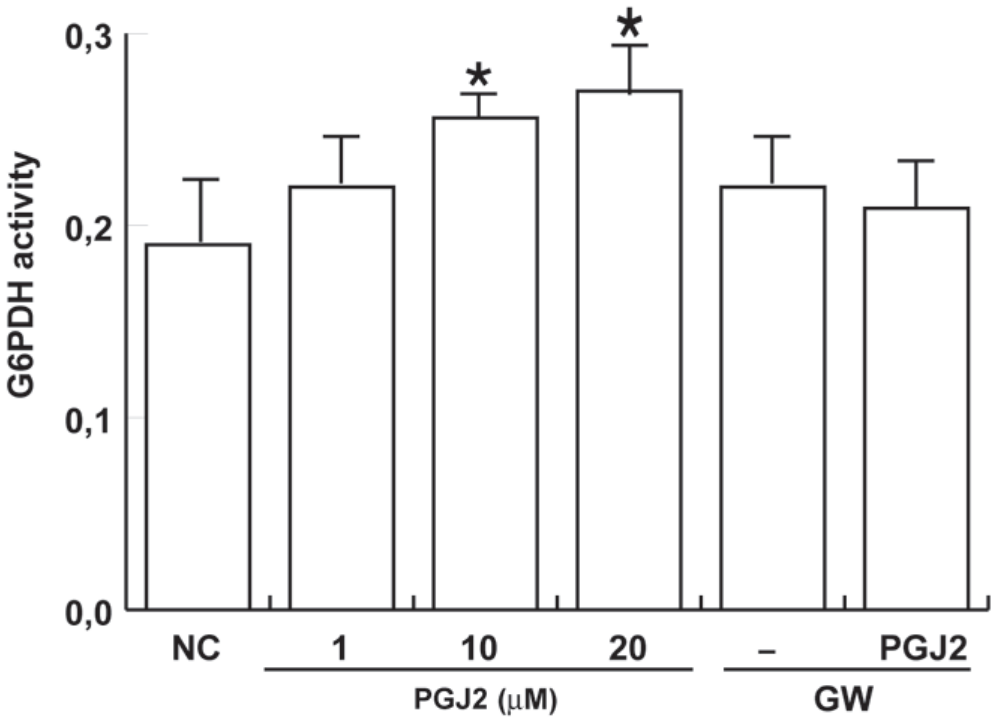

A
Triglycerides mg/10 9 sperms
0,3
0,2
0,1
0,0
*
**
*
NC
1
10
20
PGJ2(μM)
–
PGJ2
GW
B
Lipase activity
(μmol/min/mg protein)
3,2
2,4
1,6
0,8
0,0
*
*
*
NC
1
10
20
PGJ2(μM)
–
PGJ2
GW
C
Octanoyl CoA dehydrogenase activity
(nmol/min/mg protein)
0,015
0,010
0,005
0,000
*
**
**
*
NC
1
10
20
PGJ2(μM)
–
PGJ2
GW

FIG. 6

A


G6PDH activity
0,3
0,2
0,1
0,0
*
*
NC
1
10
20
–
PGJ2
PGJ2 (μM)
GW

B

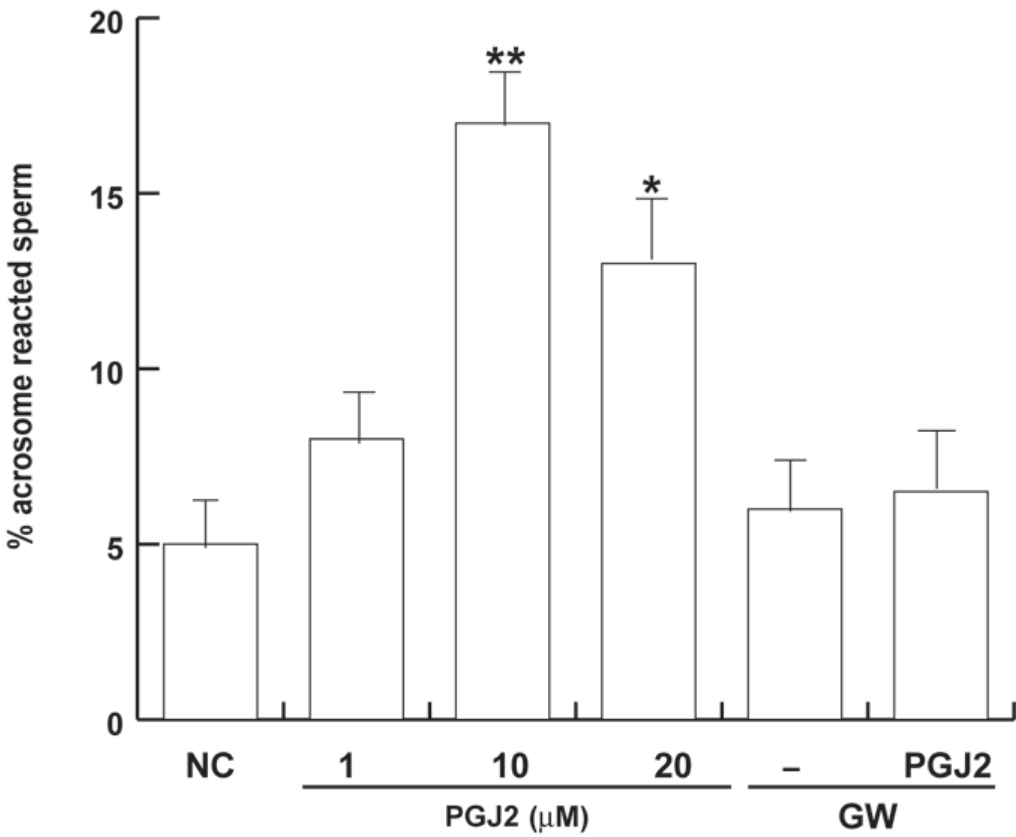

% acrosome reacted sperm
20
15
10
5
0
**
*
NC
1
10
20
–
PGJ2
PGJ2 (μM)
GW

FIG. 7