Biological effects of polyphenol-rich extract and fractions from an oenological oak-derived tannin on in vitro swine sperm capacitation and fertilizing ability

BIOLOGICAL EFFECTS OF POLYPHENOL-RICH EXTRACT AND FRACTIONS FROM AN OENOLOGICAL OAK-DERIVED TANNIN ON IN VITRO SWINE SPERM CAPACITATION AND FERTILIZING ABILITY

Marcella Spinaci[1], Vera Muccilli[2], Diego Bucci[1], Nunzio Cardullo[2], Beatrice Gadani[1], Corrado Tringali[2], Carlo Tamanini[1], Giovanna Galeati[1]

[1]*Dipartimento di Scienze Mediche Veterinarie, Università di Bologna, Bologna, Italy*

[2]*Dipartimento di Scienze Chimiche, Università di Catania, Catania, Italy*

Dedicated to the memory of Carmela Spatafora

Corresponding author: Marcella Spinaci

Via Tolara di Sopra, 50, 40064 Ozzano dell'Emilia-Bologna, University of Bologna, Italy

e-mail address: marcella.spinaci@unibo.it

Abstract

Although excessive ROS levels induce sperm damage, sperm capacitation is an oxidative event that requires low amounts of ROS. As the antioxidant activity of the ethanol extract (TRE) of a commercial oenological tannin (*Quercus robur* toasted oak wood, Tan'Activ R®) and its four fractions (FA, FB, FC, FD) has been recently reported, the present study was set up to investigate the biological effects of TRE and its fractions in an in vitro model of sperm capacitation and fertilization. Boar sperm capacitation or gamete coincubation were performed in presence of TRE or its fractions (0, 1, 10, 100 μg/ml). TRE at the concentration of 10 μg/ml (TRE10) stimulated sperm capacitation, as it increased ($p<0.001$) the percentage of spermatozoa with tyrosine-phosphorylated protein positivity in the tail principal piece (B pattern) (67.0 ± 10.6 vs. 48.6 ± 9.0, mean ± SD for TRE10 vs. Ctr respectively). Moreover T10 significantly ($p<0.001$) increased oocyte fertilization rate (91.9 ± 4.0 vs. 69.0 ± 14.8, TRE10 vs. Ctr respectively). An opposite effect of TRE at the concentration of 100 μg/ml (TRE100) on both sperm capacitation (B pattern cell percentage 33.3 ± 29.2) and fertilizing ability (fertilization rate 4.9 ± 8.3), associated with a higher sperm viability (67.9 ± 9.3 vs. 35.4 ± 10.8, TRE100 vs. Ctr respectively) ($p<0.001$), was recorded. The potency of the TRE fractions seems to be highest in FB followed by FC, faint in FD and nearly absent in FA. Our results show that TRE and its fractions, in a different extent, exert a powerful biological effect in finely modulating capacitation and sperm fertilizing ability.

Keywords: antioxidant, IVF, pig sperm, polyphenols, tannins

1. Introduction

Mammalian sperm capacitation is the process by which spermatozoa acquire the ability to fertilize the mature oocyte. It is a complex process that normally occurs in the oviduct and involves biochemical and morphological changes that make the spermatozoon competent to bind to the zona pellucida that surrounds the oocyte, penetrate it and finally fuse with the oolemma [1]. Various cellular changes occur during sperm capacitation including the activation of adenylyl cyclase producing cAMP, influx of Ca^{2+} ions, generation of reactive oxygen species (ROS), cholesterol efflux from the plasma membrane leading to an increase in its fluidity, increase of intracellular pH, and activation of protein kinases resulting in protein phosphorylation of numerous proteins on tyrosine residues among others [1-3]. An accumulating body of evidence indicates that a mild and controlled generation of ROS plays a physiological role during capacitation and acquisition of sperm fertilizing ability while ROS-specific scavengers inhibit the process [4-6]. However, when ROS levels exceed the fine balance between ROS production and scavenging going over the threshold inducing correct triggering of sperm capacitation, oxidative stress generated by the excess of ROS causes adverse effects on the sperm plasma membrane, DNA, and physiological processes, producing irreparable alterations leading to cell death [7-8].

Plants polyphenols currently are among the most studied phytochemicals because of their biological functions and health-promoting effects. An important but often neglected group of natural polyphenols are tannins. Tannins have been demonstrated to have various health benefit activities, especially antioxidant, anticarcinogenic, cardioprotective, antiinflammatory [9-12].

Oak barrels, which are widely used in winemaking, provide polyphenols to wines and are thought to play crucial roles during wine aging, a critical process not only in improving sensory properties of wines, such as flavor and aroma, but also in acquiring health protective properties [12,13].

Commercial oenological tannins, often added to wine to improve its organoleptic properties, have different origins and one of the main source is oak wood [14].

Some of us recently reported a study on the ethanol extract (TRE) of a commercial oenological tannin (*Quercus robur* toasted oak wood, Tan'Activ R®, TR) and its four fractions (FA, FB, FC, FD), obtained by fractionation on Amberlite XAD-16, including data about their antioxidant activity [15].

As sperm capacitation is a physiological process considered an oxidative event [4-6], the present study was undertaken to get an insight into the biological effects of TRE and its four fractions in a well defined in vitro model of pig sperm capacitation and fertilization [16-20].

2. Materials and Methods

Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich (Milan, Italy).

The ethanol extract of the commercial Tan'Activ R® (TRE) was obtained as previously reported (QR2E extract) [15]. The four fractions of TRE (FA, FB, FC, FD) were obtained by Amberlite XAD-16 fractionation as previously described (respectively for fractions X2A, X2B, X2C, X2D) [15].

The study was performed with the same batch of TRE and its fractions. The powder of ethanol extract TRE and of the four fractions (FA, FB, FC, FD) was stored at -20°C until resuspension in DMSO (100mg/ml).

2.1. In vitro maturation of porcine oocytes (IVM)

Porcine cumulus–oocyte complexes (COCs) were aspirated using a 18 gauge needle attached to a 10 mL disposable syringe from 4 to 6 mm follicles of ovaries collected at a local abattoir and transported to the laboratory within 1 h. Under a stereomicroscope, intact COCs were selected and transferred into a petri dish (35 mm, Nunclon,Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA (Sigma-Aldrich A3311). After three washes in NCSU 37 [21]

supplemented with 5.0 µg/mL insulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 50 mM ß-mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 µL of the same medium per well and cultured at 39 °C in a humidified atmosphere of 5% CO_2 in air. For the first 22 h of in vitro maturation the medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL, eCG (Folligon, Intervet, Boxmeer, The Netherlands) and 10 IU/mL hCG (Corulon, Intervet). For the last 22 h COCs were transferred to fresh maturation medium [22]. At the end of the maturation period the oocytes were denuded by gentle repeated pipetting.

2.2. Semen collection and preparation

Sperm-rich fraction of ejaculates were collected by gloved-hand technique from three mature boars (Large White × Landrace × Duroc) of proven fertility aged between 2 and 3 years and extended in equal volume of Androhep TM (Minitub, Tiefenbach, Germany). Only ejaculates with sperm viability higher than 80% were used in the experiments. To balancing the sperm contribution of each male and eliminate variability between the evaluated semen samples, the sperm-rich fractions were pooled in this study.

Fresh semen was centrifuged at 900 x g for 2 min. After removal of supernatant, the pellet was resuspended with PBS supplemented with 0.4% BSA and centrifuged again at the same condition. The final pellet was resuspended with Brackett and Oliphant's medium [23] supplemented with 12 % heat inactivated fetal calf serum (Gibco, Invitrogen, Italy)(FCS) and 0.7 mg/ml caffeine (Sigma-Aldrich C0750) (IVF medium).

2.3. Effect of TRE on in vitro induced sperm capacitation

Sperm cells were incubated for 1h at 39 °C in a humidified atmosphere of 5% CO_2 in air in Nunc 4-well multidish at a final concentration of $30x10^6$ sperm/ml in IVF medium in presence of different concentrations of TRE (0, 1, 10, 100 µg/ml; TRE1, TRE10, TRE100 respectively). At the end of incubation period, spermatozoa were subjected to the below described evaluations.

2.3.1. Evaluation of plasma membrane integrity and acrosome status

For the evaluation of sperm viability, twenty five microliters of semen were incubated with 2 µl of a 300 µM propidium iodide (PI) stock solution and 2 µl of a 10 µM SYBR green-14 stock solution, both obtained from the live/dead sperm viability kit (Molecular Probes, Inc., Eugene, OR, USA) for 5 min at 37°C in the darkness. At least 200 spermatozoa per sample were scored with a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, The Netherlands) by the same observer, blinded to the experimental group of each sample. Spermatozoa stained with SYBR green-14 and not stained with PI were considered as viable. Spermatozoa SYBR and PI positive and those SYBR negative / PI positive were considered as cells with non-intact membrane or dead.

Acrosome integrity was evaluated by using a FITC-conjugated lectin from *Pisum sativum* (FITC-PSA) which label acrosomal matrix glycoproteins. Spermatozoa were washed twice in PBS by centrifugation, resuspended in ethanol 95% and fixed/permeabilized at 4°C for at least 30 min. Aliquots of sperm suspensions were dried on heated slides and incubated with FITC-PSA solution (5 µg PSA-FITC/1 mL H_2O) for 20 min in darkness. After staining, slides were washed in PBS and mounted with Vectashield mounting medium containing PI counterstain for DNA (Vector Laboratories, Burlingame, CA, USA). The slides were then observed with the above described epifluorescence microscope by the same observer, blinded to the experimental group of each sample. The presence of a green acrosomal fluorescence was considered indicative of an intact acrosome, whereas a partial or total absence of fluorescence was considered indicative of acrosome disruption or acrosome reaction (see supplementary file, panel A).

2.3.2. Immunolocalization of tyrosine-phosphorylated proteins

All the procedures were carried out at room temperature unless otherwise specified.

Aliquots of sperm cells from the different experimental groups were spotted onto poly-L-lysine-coated slides and fixed with methanol at -20° C for 15 min and with acetone for 30 sec. The slides were then washed with PBS and blocked with 10% (v/v) FCS (Gibco) in PBS (blocking solution) for at least 30 min. Antibody dilutions were performed in blocking solution. Monoclonal antiphosphotyrosine antibody (clone 4G10, Merck Millipore, Darmstadt, Germany) was added at the dilution 1:150 . Incubation was carried out overnight at 4° C. After extensive washing with PBS, sperm cells were incubated with a sheep-anti-mouse FITC-conjugated secondary antibody (BioFX Laboratories, Maryland, USA) for 1 h in the dark. Slides were washed with PBS and mounted with Vectashield mounting medium with PI (Vector Laboratories). Control slides were treated similarly with the omission of primary antiserum. Spermatozoa were evaluated with the above described epifluorescence microscope.

Each sample was analyzed by counting at least 200 cells in order to evaluate the different positivity patterns by the same observer, blinded to the experimental group of each sample.

Four different patterns were considered on the basis of what assessed by Bucci et al. [18] (see supplementary file, panel B):

A: positivity in the Equatorial Subsegment (EqSS) and acrosome;

B: positivity in the acrosome, EqSS and principal piece of the tail;

C: positivity in the tail and (not constant) in the EqSS.

NEG: spermatozoa with no positive signal.

2.4. Effect of TRE on in vitro fertilization (IVF)

For in vitro fertilization trials, spermatozoa were diluted in IVF medium to obtain 750×10^3 spermatozoa/ml and 500 µL of the sperm suspension were placed to each well of Nunc 4-well multidish. Then 45 to 50 *in vitro* matured oocytes were transferred to each well and sperm-oocyte coculture was performed for 1 h in presence of TRE at the concentration of 0, 1, 10, 100 µg/ml at 39 °C in a humidified atmosphere of 5% CO_2 in air. The oocytes from each group were then washed three times in fresh IVF medium to remove spermatozoa not bound to the zona, transferred to the same medium (containing no spermatozoa) and cultured for 19 h until mounting on microscope slides and fixing in acetic acid/ethanol (1 : 3) for 24 h. After staining with Lacmoid the oocytes were observed under a phase-contrast microscope (total number of IVF oocytes evaluated = 665).

Fertilization rate (number of oocytes fertilized/ number of inseminated oocytes) and monospermy rate (number of monospermic oocytes/number of fertilized oocytes) were recorded.

2.5. Effect of TRE fractions on in vitro induced sperm capacitation and IVF

In order to investigate the power of the fractions FA, FB, FC, FD obtained from TRE by Amberlite fractionation, in vitro sperm capacitation and gamete coincubation during IVF were performed in presence of different concentrations (0, 1, 10, 100 µg/ml) of FA, FB, FC, FD.

Sperm viability, acrosome integrity and tyrosine-phosphorylation of sperm proteins were evaluated as above described on spermatozoa after 1 h of capacitation in presence of different concentrations of the four fractions of TRE.

For in vitro fertilization assay, fertilization parameters were evaluated as above described on oocytes coincubated 1h with sperm in presence of different concentrations of the four fractions of TRE and then cultured 19h in fresh IVF medium until fixation (total number of IVF oocytes evaluated = 3036).

2.6 Statistical analysis

Data were analyzed using R version 3.4.0. [24] Significance was set at $p < 0.05$ unless otherwise specified. Results are expressed as mean ± standard deviation. Shapiro Wilk test was applied to test normality and Levene test was used to assay homogeneity of the variances for the arcsin transformed variables (percentage of viable cells, percentage of acrosome integer cells; sperm tyrosine phosphorylation patterns) One-way ANOVA and Tukey post hoc test were performed to assess the difference between treatments. In vitro fertilization data were analyzed by a generalized linear model (GLM) and a Tukey post hoc test was used to assess difference between treatments.

3. Results

3.1. Effect of TRE on in vitro induced sperm capacitation and IVF

When in vitro induced sperm capacitation was performed in presence of TRE100, sperm viability was significantly higher compared with control ($p < 0.001$), TRE1 ($p < 0.001$) and TRE10 ($p < 0.05$) (Fig. 1 A). Moreover, in presence of TRE100 the percentage of acrosome intact cells was higher compared with control ($p < 0.05$) (Fig.1 B).

Tyrosine-phosphorylation of sperm proteins was significantly ($p < 0.001$) influenced by TRE: TRE10 induced a decrease of the percentage of cells displaying A pattern (non capacitated cells) and a parallel increase of B pattern (capacitated cells) while an opposite effect of TRE100 was recorded (Fig.1 C).

When gamete coincubation was performed in presence of TRE10 the penetration rate (number of oocytes fertilized/ number of inseminated oocytes) was significantly increased ($p < 0.001$) while it dramatically dropped in presence of TRE100 ($p < 0.001$). The monospermy rate (number of monospermic oocytes/number of fertilized oocytes) was significantly decreased in TRE10 ($p < 0.001$).

3.2. Effect of TRE fractions on in vitro induced sperm capacitation and IVF

After 1 h incubation in capacitating condition, while neither FA nor FD significantly affected sperm viability (Fig. 2 A, D), the percentage of viable sperm in presence of FB at the higher concentration tested (100 µg/ml) was significantly ($p < 0.05$) higher compared with control and FB1 (Fig. 2 B). In presence of FC100 sperm viability resulted significantly higher compared with control and FB at lower concentration tested ($p < 0.05$) (Fig. 2 C)

Sperm acrosome integrity was not influenced by both FA and FD at all the concentration tested. In presence of FB100 the percentage of acrosome intact cells was higher compared with both control and FB1 ($p < 0.05$) while a significant difference between FC 100 and control was recorded ($p < 0.05$) (Fig. 3).

Tyrosine-phosphorylation of sperm proteins was influenced by the four fractions (Fig. 4). FA and FD induced a decrease of the percentage of cells displaying A pattern (non capacitated cells) and a parallel increase of B pattern (capacitated cells) at the higher concentration tested (100 µg/ml) ($p < 0.001$) (Fig. 4 A,D). FB 10 significantly ($p < 0.001$) decreased the percentage of A pattern sperm and increased the percentage of B pattern sperm while FB 100 showed an opposite effect significantly decreasing the percentage of spermatozoa displaying B pattern ($p < 0.001$) (Fig. 4 B).

FC1 and FC100 slightly but significantly decreased the percentage of cells displaying A pattern and increased the percentage of B pattern sperm ($p < 0.05$) while FC10 effect resulted more evident ($p < 0.001$) (Fig.4 c).

The results on the effect of the four different TRE fractions during gamete coincubation on fertilization parameters are reported in Figure 5.

Fraction FA did not influence fertilization parameters at all the dosages tested. FB and FC exerted a diphasic effect on fertilization rate that was significantly increased at the concentration of 10 µg/ml

239 and significantly decreased at 100 μg/ml compared with control ($p < 0.001$). The monospermy rate
240 was significantly decreased in both FB10 ($p < 0.001$) and FC10 ($p < 0.05$) groups. Fraction FD was
241 effective in increasing the fertilization rate only at the concentration of 100 μg/ml.
242

243 ## 4. Discussion

244 Recently some of us reported the antioxidant activity of an ethanol extract (TRE) of a commercial
245 oenological tannin (*Quercus robur* toasted oak wood, Tan'Activ R®,TR) and its four fractions
246 obtained by fractionation on Amberlite XAD-16 [15]. It is worth noting here that in this work the
247 QR2E ethanol extract, corresponding to TRE, showed good antioxidant activity, with a SC_{50} value of
248 6.96 μg/mL in the DPPH scavenging test. Among the XAD-16 fractions, fraction X2B, corresponding
249 to FB, showed the highest antioxidant activity, with a SC_{50} value of 3.95 μg/mL; X2C and X2D,
250 corresponding respectively to FC and FD, showed an antioxidant activity comparable to that of the
251 QR2E extract, whereas X2A, corresponding to FA, had the lowest antioxidant activity, with a SC_{50}
252 value of 16.48 μg/mL.
253 As a first step of this study we investigated the biological effect of the ethanol extract TRE on sperm
254 capacitation, oxidative process by which male gametes acquire the ability to fertilize oocytes
255 [1,4,25]. One of the early events occurring during sperm capacitation is the production of low levels
256 ROS that exert a positive role in the molecular mechanism regulating the process of capacitation
257 [3,6].
258 The treatment of spermatozoa with TRE at the higher concentration tested (100 μg/ml) during 1 h
259 culture under in vitro capacitating conditions exerted an inhibitory effect on sperm capacitation on
260 the basis of the results obtained on tyrosine phosphorylation of sperm proteins, process shown to
261 be highly related with the capacitation process [18,26]. The inhibitory effect induced by TRE100

agree well with the observation that tyrosine phosphorylation events associated with sperm capacitation could be reversed by antioxidants such as catalase and ascorbic acid [2,27-29].

Interestingly TRE at the concentration of 10 μg/ml stimulated the process of capacitation as it increased the percentage of spermatozoa with a positivity of tail principal piece (B pattern) [18]. It has been demonstrated that moderate and controlled amounts of ROS exert a positive influence on tyrosine phosphorylation during capacitation through their ability to enhance intracellular levels of cAMP and inhibit phosphatase activity [2,25,28,30-32]. Therefore we can hypothesize that during in vitro induction of sperm capacitation TRE at low concentration (10 μg/ml) was able to maintain the amount of ROS at low levels leading to a positive influence on tyrosine phosphorylation while higher concentration of TRE, reducing ROS production, prevented phosphorylation events associated with sperm capacitation.

TRE, only at the higher concentration tested (100 μg/ml), exerted a positive effect on the percentage of live cells after 1 h culture in capacitating conditions. It has to be taken into account that a decrease in sperm viability is normally associated with in vitro sperm capacitation process [33] and that capacitation and apoptosis are considered part of a redox-regulated continuum [34]. In fact the fine balance between ROS production and scavenging as well as the right timing for ROS production are of fundamental importance for the acquisition of fertilizing ability by spermatozoa as when the intrinsic generation of ROS overwhelms the limited capacity of these cells to protect themselves against oxidative attack, oxidative stress cause sperm senescence leading to cell death [7,25,34]. In this view TRE100, by inhibiting the capacitation process, reduced the physiological drop in sperm viability under capacitating condition. In our experiment in fact the percentage of viable cells decreased from 83,4% in fresh semen to 35,4% after 1h in vitro capacitation in control group while TRE100 maintained the percentage of sperm with intact plasma membrane to higher levels (66,9%).

285 The inhibitory effect of TRE100 on sperm capacitation is further confirmed by the reduction of
286 spontaneous acrosomal loss naturally occurring in a low rate during in vitro capacitation [35].
287 As the capacitation is the process that enables spermatozoa to gain the ability to bind to the zona
288 pellucida, penetrate it and finally fuse with the oocyte, in order to fully evaluate the biological effect
289 of TRE on sperm functions, in vitro fertilization tests in presence of different concentrations of TRE
290 were performed. According with the results obtained on capacitation parameters, TRE10 increased
291 oocyte fertilization rate that was, on the contrary, strongly inhibited by TRE100 confirming the
292 powerful biological ability of TRE to modulate in a dose dependent biphasic manner the capacitation
293 process.
294 The reduction of monospermy rate (number of monospermic oocytes/number of fertilized oocytes)
295 recorded in TRE10 was due to well known reduced ability of in vitro matured pig oocyte to efficiently
296 block polyspermy [36]: TRE10, stimulating sperm capacitation, increased the penetration rate
297 parallelly increasing polyspermy in fertilized oocytes, 77% of whom contained more than one
298 sperm head/male pronucleus.
299 The four fraction of TRE obtained by Amberlite XAD-16 fractionation (FA, FB, FC, FD) were also
300 tested in order to assess their effect on boar sperm capacitation and fertilizing ability.
301 The results obtained on tyrosine phosphorylation of sperm protein suggest that FB, the fraction with
302 the highest antioxidant activity [15], is the most potent fraction exerting, similarly to TRE, a
303 stimulatory effect of sperm capacitation at 10 μg/ml and an inhibitory effect at 100 μg/ml. FB
304 resulted less powerful as it stimulated capacitation at 10 μg/ml but did not reduce the percentage
305 of capacitated sperm compared to control at the highest dose tested (100 μg/ml). FD and, in a lesser
306 extent, FA were effective in stimulating capacitation only at the concentration of 100 μg/ml. The
307 inhibiting effect on sperm capacitation of FB100 was confirmed, for the reasons previously
308 discussed, by the higher viability and acrosome integrity observed when in vitro sperm capacitation

was performed in presence of this faction at the higher concentration. The results of in vitro fertilization assay confirm the different activity of the four fractions: FB and FC exerted a biphasic effect (stimulatory at 10 μg/ml and inhibitory at 100 μg/ml) on oocyte fertilization rate, FD increased the fertilization rate only at the higher concentration tested (100 μg/ml) while FA was ineffective. On these bases, the potency of the four fractions seems to be highest in FB followed by FC, faint in FD and nearly absent in FA. Our results agree well with those reported by Muccilli et al. [15] for the radical scavenging activity of the four fractions (see above), supporting the hypothesis that their different antioxidant activity have a role in their action on sperm capacitation. In fact, for example, Muccilli et al. [15] observed the highest antioxidant activity in fraction FB.

To our knowledge, the results of this study represent the first evaluation of biological activities of an ethanol extract of an oenological commercial oak-derived tannin and its fractions during the redox-regulated process of capacitation that make the spermatozoon competent of fertilizing the oocyte. TRE and its fraction (in a different extent) exerted a powerful biological effect in finely modulating capacitation and in turn sperm fertilizing ability. Which of constituents is responsible for the biological activity recorded has not yet been established, although Muccilli et al. [15] identified known polyphenols as major constituents of fraction X2B (FB in the present work), namely a monogalloyl glucose isomer (MW = 332.2), glucose esterified by hexahydroxydiphenic acid (HHDP) (MW = 482.2), gallic acid (MW = 170.1), and the ellagitannins castalin (MW = 632.4), vescalagin (MW = 934.6) and grandinin (or its isomer roburin E) (MW = 1066.7). However, the effects exerted appear to be of interest and worth of a deeper investigation.

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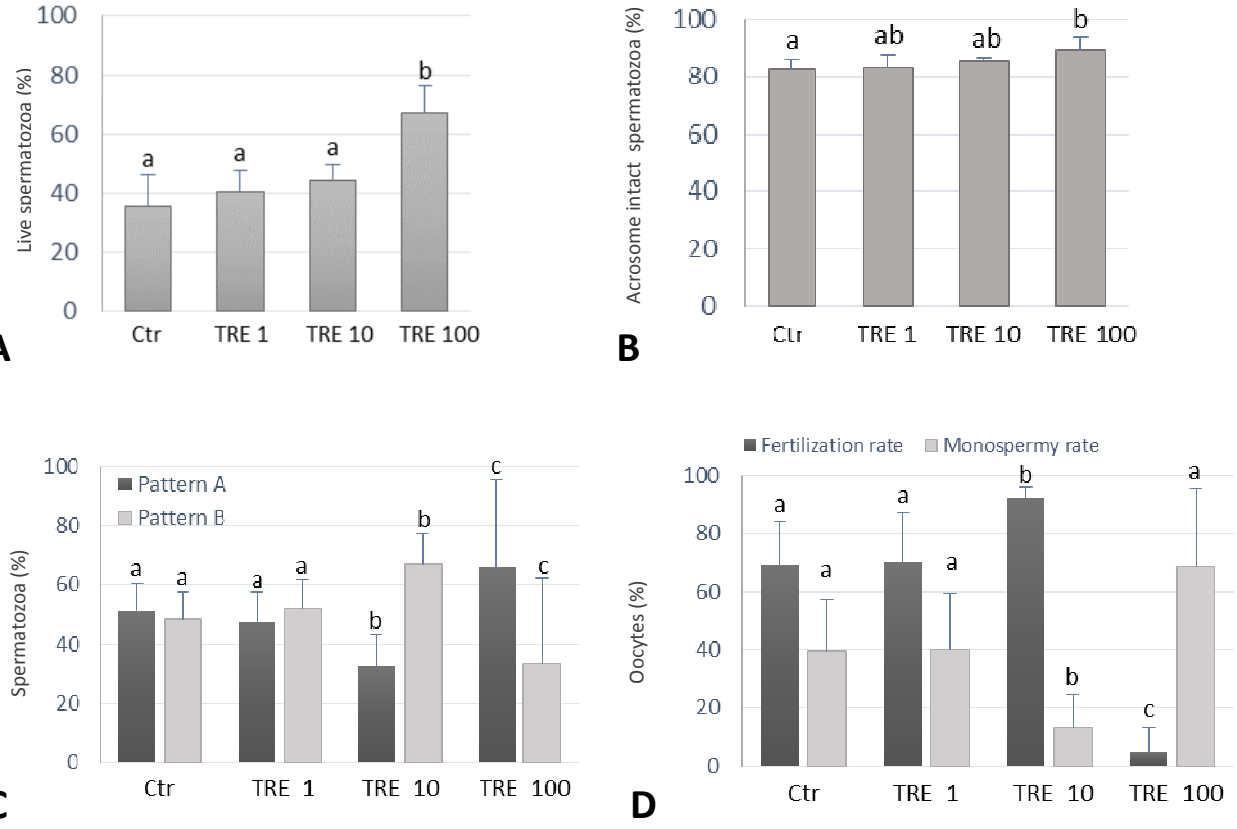
Fig. 1. Effect of TRE at concentrations of 0, 1, 10, 100 µg/ml during capacitation and gamete coincubation. (A) Sperm viability, (B) percentage of acrosome intact spermatozoa and (C) percentage of sperm cells displaying tyrosine-phosphorylation of sperm proteins typical of either non capacitated cells (pattern A) or capacitated cells (pattern B) after 1 h sperm incubation in capacitating condition in presence of different concentrations of TRE. (D) Fertilization rate (number of oocytes fertilized/ number of inseminated oocytes) and monospermy spermy rate (number of monospermic oocytes/number of fertilized oocytes) after 1h gamete coincubation in presence of different concentrations of TRE. Data represent the mean ± SD of 5 replicates for sperm viability, acrosome integrity and IVF and 4 replicates for immunolocalization of sperm phosphotyrosine, repeated in different experiments. Different letters on the same bar type represent significant difference for $P < 0.05$ between treatments.



442 Fig. 2. Effect of fractions FA (panel A), FB (panel B), FC (panel C), FD (panel D) at concentrations of

443 0, 1, 10, 100 µg/ml during 1 h sperm incubation in capacitating condition on sperm viability.

444 Data represent the mean ± SD of 5 replicates repeated in different experiments. Different letters

445 represent significant difference for $P < 0.05$ between treatments.

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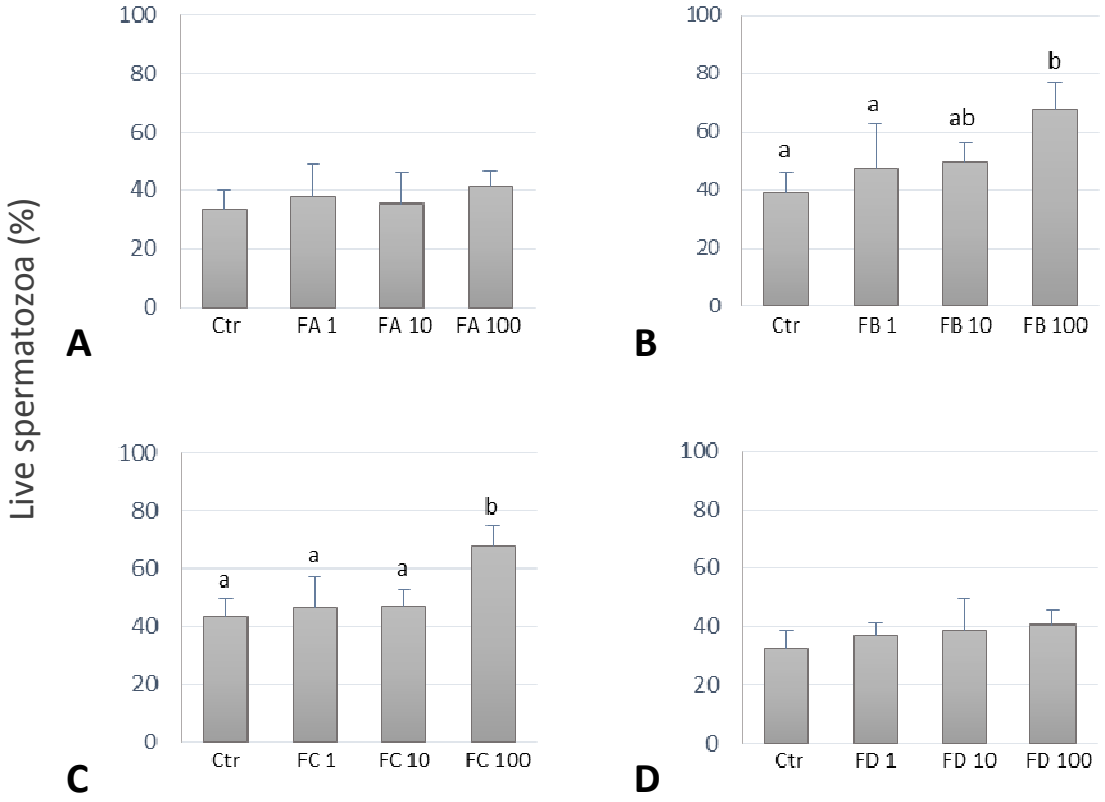

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449 Fig. 3. Effect of fractions FA (panel A), FB (panel B), FC (panel C), FD (panel D) at concentrations of

450 0, 1, 10, 100 µg/ml during 1 h sperm incubation in capacitating condition on sperm acrosome

451 integrity.

452 Data represent the mean ± SD of 5 replicates repeated in different experiments. Different letters

453 represent significant difference for $P < 0.05$ between treatments.

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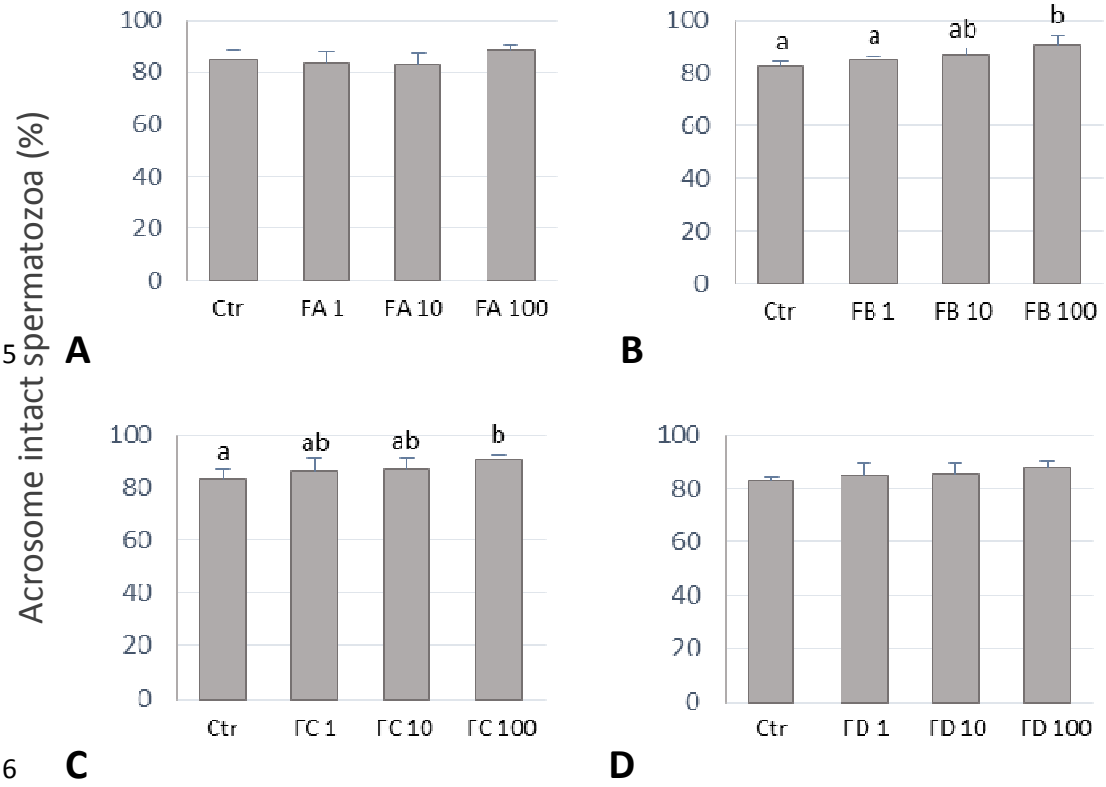

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Fig.4. Effect of fractions FA (panel A), FB (panel B), FC (panel C), FD (panel D) at concentrations of 0, 1, 10, 100 µg/ml during 1 h sperm incubation in capacitating condition on tyrosine-phosphorylation of sperm proteins: pattern A typical of non capacitated cells (positivity in the EqSS and acrosome); pattern B typical of capacitated cells (positivity in the acrosome, EqSS and principal piece of the tail).

Data represent the mean ± SD of 4 replicates repeated in different experiments.

Different letters on the same bar type represent significant difference for $P < 0.05$ between treatments.

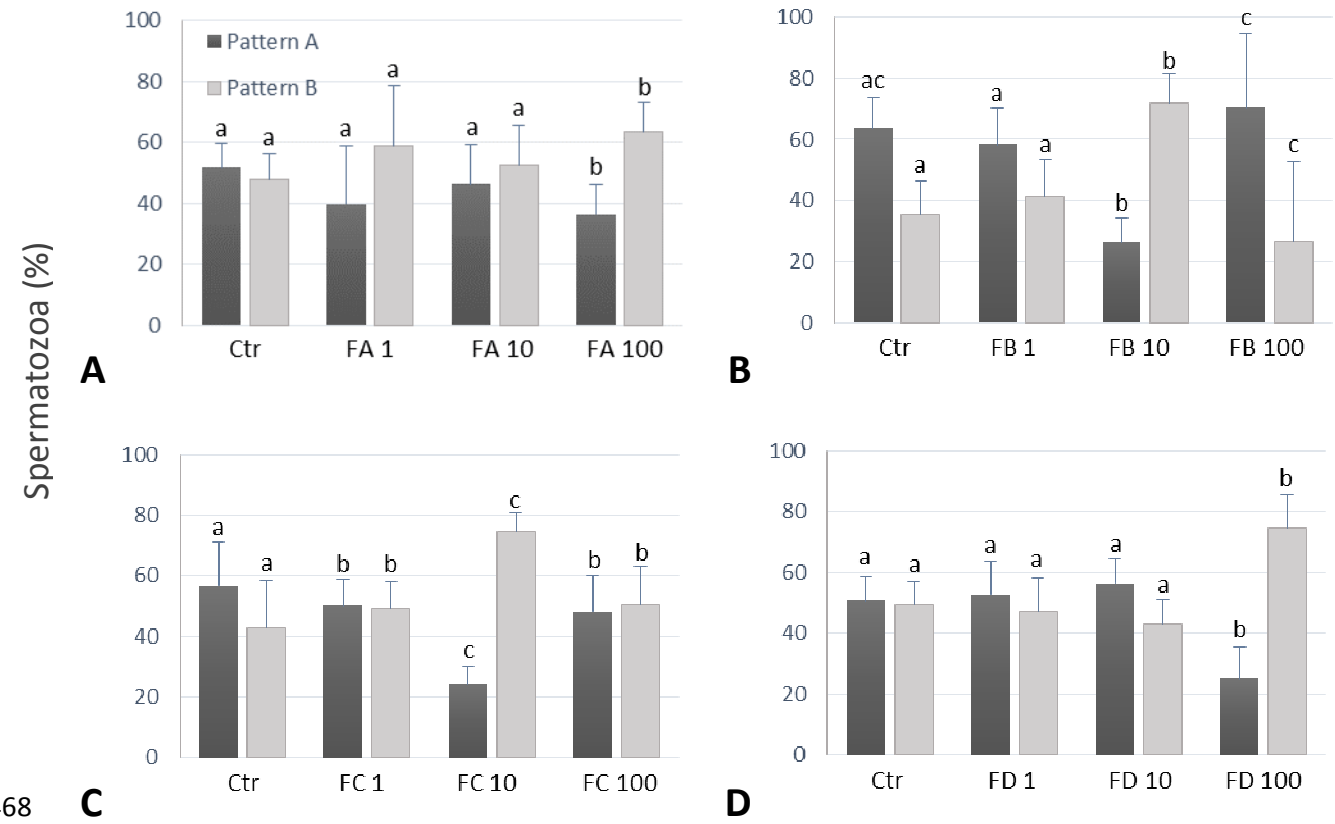

Fig. 5. Effect fractions FA (panel A), FB (panel B), FC (panel C), FD (panel D) at concentrations of 0, 1, 10, 100 µg/ml during 1h gamete coincubation on fertilizazion parameters: fertilization rate (number of oocytes fertilized/ number of inseminated oocytes) and monospermy rate (number of monospermic oocytes/number of fertilized oocytes).

Data represent the mean ± SD of 5 replicates repeated in different experiments.

Different letters on the same bar type represent significant difference for $P < 0.05$ between treatments.