Pig sperm preincubation and gamete coincubation with glutamate enhance sperm-oocyte binding and in vitro fertilization

PIG SPERM PREINCUBATION AND GAMETE COINCUBATION WITH GLUTAMATE ENHANCE SPERM-OOCYTE BINDING AND IN VITRO FERTILIZATION

M. Spinaci[1], D. Bucci[1], B. Gadani[1], E. Porcu[2], C. Tamanini[1], G. Galeati[1].

[1] Department of Veterinary Medical Sciences (DIMEVET), Via Tolara di Sopra, 50, 40064 Ozzano dell'Emilia-Bologna, University of Bologna, Italy

[2] Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna, Italy.

Corresponding author: Marcella Spinaci

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

ABSTRACT

As the taste receptor for monosodium glutamate (umami) is expressed in both murine and human spermatozoa and the presence of α-gustducin and α-transducin, G proteins involved in the umami taste signaling, has been described in boar germ cells, the aim of this study was to evaluate if monosodium glutamate (MSG) would exert any effect on sperm-oocyte binding, in vitro fertilization (IVF) and sperm parameters during in vitro induced capacitation.

For sperm-zona pellucida binding assay, boar spermatozoa were preincubated for 1 h and then coincubated for 1 h with denuded in vitro matured oocytes in presence of different concentrations of MSG (0, 0.1, 1, 10 mM). MSG 1 and 10mM significantly ($P<0.05$) increased the mean number of sperm bound to ZP compared with control (12.3±9.0, 17.8±11.3, 17.6±10.8, MSG 0, 1 and 10mM respectively).

For in vitro fertilization trials, both sperm preicubation (1h) and gamete coincubation (1h) were performed in presence of different concentrations of MSG (0, 0.1, 1, 10 mM). After 19h of culture in fresh IVF medium, oocytes were fixed. MSG 1mM significantly ($P < 0.05$) increased the penetration rate compared with control (53.7±20.4 vs. 36.8±16.2).

The addition of MSG during in vitro induced capacitation of boar spermatozoa did not cause any significant difference, compared with control, on the percentage of viable cells, spermatozoa with intact acrosome and the percentage of spermatozoa displaying tyrosine-phosphorylation of sperm tail proteins.

In order to evaluate whether the effect elicited by MSG could be due to glutamate uptake in boar spermatozoa, fertilization trials were performed in presence of either 1mM MSG or 1mM MSG + 100μM DL-threo-beta-hydroxyaspartic acid (THA), a non selective inhibitor of glutamate uptake. A significant increase ($P < 0.05$) in the penetration rate in both MSG and MSG + THA groups compared to control was recorded (39.8±15.7, 53.7±22.1, 52.2±23.7, Control, MSG and MSG + THA respectively) while no difference in penetration rate between MSG and MSG+THA treatment was observed suggesting that sperm glutamate transporters are not involved in the pathway mediating this effect.

Our study demonstrates for the first time that glutamate exerts a positive effect on sperm-oocyte binding and fertilization. Further studies are needed to clarify the mechanism by which glutamate exert his effect.

Key words: monosodium glutamate, sperm-ZP binding assay, IVF, boar spermatozoa, capacitation

1. INTRODUCTION

During the transit along the female genital tract to reach the oocyte, spermatozoa encounter an environment that varies in composition. Glutamic acid was reported to be present at different concentrations in female reproductive tract fluids. In mouse glutamate concentrations decline from uterus to the ampullary region of the tube [1] and in pig the decrease is so prominent that glutamic acid is very low or undetectable in sow oviduct fluid [2,3]. However, Hong and Lee [4] reported that glutamate is one of the most abundant amino acids in pig follicular fluid (pFF) with concentrations at least two-fold higher than those of other amino acids. Follicular fluid entrapped in the cumulus oophorus after ovulation may therefore modify the microenvironment in the close vicinity of pig oocyte increasing glutamate concentration.

The two subunit of the umami receptor dimer (T1R1 and T1R3), which form the functional receptor for monosodium glutamate in tongue taste buds, are expressed in mature murine and human spermatozoa and in murine spermatids, while trancrips for sweet taste receptor (T1R2) were not detected [5,6]. Moreover we have recently demonstrated the expression α-gustducin and α-transducin, G proteins accepted as specific markers of chemosensitive cells, in boar spermatozoa [7]. The expression of α-gustducin was also demonstrated in mouse, rat, bull and human male gametes [6,8]. Both α-gustducin and α-transducin are involved in the umami taste signaling [9] in response to aminoacids and, among them, glutamate is the most extensively studied ligand for umami receptor in taste buds.

These studies suggest a specific function of umami signaling in spermatozoa that during spermatogenesis lose the majority of the cytoplasm thus eliminating what is non-useful for their final mission: the transport of male genetic material to the oocyte.

On these bases, the aim of our study was to evaluate whether monosodium glutamate (MSG) would exert any effect on in vitro sperm-oocyte binding and fertilization. The effect of MSG on sperm viability, acrosome integrity and immunolocalization of tyrosine-phosphorylated sperm proteins was also assessed.

2. MATERIALS AND METHODS

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

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. After three washes in NCSU 37 [10] supplemented with 5.0 mg/mLinsulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 50 µM 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_2/7% O_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 [11]. 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 of proven fertility and extended in equal volume of Androhep TM (Minitub, Tiefenbach, Germany**).** Only ejaculates with sperm viability higher than 85% were used in the experiments. In order to minimize the boar effect, ejaculates were pooled.

Semen was washed twice with PBS supplemented with 0.4% BSA and finally resuspended with Brackett & Oliphant's medium [12] supplemented with 12 % fetal calf serum (Gibco, Invitrogen, Italy) and 0.7 mg/ml caffeine (IVF medium) [13].

2.3. Effect of MSG sperm-zona pellucida binding and fertilization parameters

2.3.1. Effect of MSG sperm-zona pellucida binding

For the sperm-zona pellucida binding assay, spermatozoa were diluted to a concentration of 500×10^3 spermatozoa /ml and 500 µL of the sperm suspensions were preincubated for 1 h in presence or absence of different concentrations of MSG dissolved in water (0, 0.1, 1, 10 mM).

At the end of the maturation period 30-35 denuded oocytes were added into each well and after 1 h of gamete co-incubation at 38 °C in 95% humidity and 5% CO2 in air the oocytes were washed four times in PBS 0.4% BSA with a wide bore glass pipette in order to remove the spermatozoa loosely attacked to zona pellucida. The oocytes were then fixed in 4% paraformaldehyde for 15 min at room

104 temperature and then incubated with 8.9 µM Hoechst 33342 for 10 min in PBS 0.4% BSA in the dark,
105 washed twice in the same medium, and individually placed in droplets of Vectashield (Vector
106 Laboratories, Burlingame, CA, USA) on a slide, and covered with a coverslip. The number of
107 spermatozoa attached to the zona pellucida of each oocyte was assessed by using the above
108 described microscope and was expressed as mean number of spermatozoa per oocyte.
109

110 ***2.3.2. Effect of MSG fertilization parameters***

111 For in vitro fertilization trials, spermatozoa were diluted to obtain 750×10^3 spermatozoa /ml and
112 500 µL of the sperm suspensions were preincubated for 1 h in presence or absence of different
113 concentrations of MSG (0, 0.1, 1, 10 mM).
114 At the end of the preincubation period, 45 to 50 *in vitro* matured oocytes were transferred to each
115 well. After 1 h of coculture, oocytes were transferred to fresh IVF medium and cultured for 19 h
116 until fixation in acetic acid/ethanol (1 : 3) for 24 h and stained with Lacmoid.
117 The oocytes were observed under a phase-contrast microscope and the following parameters were
118 assessed:
119 (1) penetration rate (number of oocytes fertilized/ number of inseminated oocytes);
120 (2) monospermy rate (number of oocytes containing only one sperm head–male pronucleus/
121 number of penetrated oocytes);
122 (3) total efficiency of fertilization (number of monospermic oocytes/number of inseminated
123 oocytes);
124 Degenerated and immature oocytes were not counted.
125

126 **2.4. Effect of MSG addition during in vitro induced sperm capacitation**

127 Sperm cells were incubated for 2h in a Nunc 4-well multidish at a final concentration of $50x10^6$
128 sperm/ml in presence or absence of different concentrations of MSG (0, 0.1, 1, 10 mM). At the end
129 of incubation period, spermatozoa were subjected to the evaluation of the parameters below.

130 ***2.4.1. Evaluation of plasma membrane integrity and acrosome status***

131 Twenty five microliters of semen were incubated with 2 µl of a 300 µM propidium iodide (PI) stock
132 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). 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, 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 H2O) for 20 min in darkness. After staining, samples were washed in PBS and mounted with Vectashield mounting medium containing propidium iodide (PI) counterstain for DNA (Vector Laboratories). The slides were then observed with the above described epifluorescence microscope and at least 200 spermatozoa per sample were evaluated.

The presence of a green acrosomal fluorescence was considered indicative of an intact acrosome, whereas a partial or total absence of fluorescence was considered to indicate acrosome disruption or acrosome reaction.

2.4.2. 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 then with acetone for 30 sec. The slides were then washed with PBS and blocked with 10% (v/v) FCS in PBS (blocking solution) for at least 30 min. Antibody dilutions were performed in blocking solution. Monoclonal anti-phosphotyrosine antibody (clone 4G10, Merck Millipore, Darmstadt, Germany) was added at the proper dilution. 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 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 microscope.

Each sample was analyzed by counting at least 200 cells in order to evaluate the different positivity patterns.

Four different patterns were considered on the basis of what assessed by Bucci et al. [14]:

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.

2.5. Effect of MSG in presence of DL-threo-beta-hydroxyaspartic acid (THA)

In order to evaluate whether the effect elicited by MSG could be due to glutamate uptake in boar spermatozoa, fertilization trials were performed as above described in presence of either 1mM MSG or 1mM MSG + 100µM DL-threo-beta-hydroxyaspartic acid (THA), a non selective inhibitor of glutamate uptake [15]. Viability and tyrosine-phosphorylation of sperm proteins were also evaluated.

2.6. Statistical analysis

Data were analyzed using R version 3.0.3 [16]. Significance was set at $P<0.05$ unless otherwise specified. Results are expressed as mean ± standard deviation. One-way ANOVA was used to assess the difference in sperm viability, acrosome integrity and protein tyrosine phosphorylation between treatments. To assess the effect of MSG on in vitro fertilization we set a General Linear Model with binomial distribution, while for assessing the effect of MSG on binding we set a GLM with Poisson distribution. To define difference between groups a Tukey post hoc test was used.

3. RESULTS

3.1. Effect of MSG sperm-zona pellucida binding and fertilization parameters

To evaluate the effect of MSG on boar sperm ability to bind to ZP, denuded in vitro matured porcine oocytes were co-incubated for 1h with semen previously incubated 1h in presence of different concentrations of MSG. The results are summarized in Table 1. After 1 h of gamete coincubation, 1 and 10mM MSG significantly ($P<0.05$) increased the mean number of sperm bound to ZP compared with the control. No significant differences were observed in presence of glutamate 0.1 mM.

The results of in vitro fertilization trials are summarized in Table 1. The addition of MSG 1mM during sperm pre-incubation and gamete coincubation significantly ($P < 0.05$) increased the penetration rate compared to control. MSG did not induce any significant difference in both monospermy rate and total efficiency of fertilization.

3.2. Effect of MSG addition during in vitro induced sperm capacitation

The addition of MSG during in vitro capacitation of boar spermatozoa at the concentrations of 0.1, 1 and 10 mM did not induce any significant difference on the percentage of viable cells and spermatozoa with intact acrosome as compared with control (Tab. 1).

The results on immunolocalization of *t*yrosine-phosphorylated proteins of spermazotoa capacitated in presence of 0.1, 1 and 10 mM MSG are reported in Fig. 1. A not significant tendency to a decrease of the percentage of cells displaying A pattern (typical of non capacitated cells) and to a parallel increase of B pattern (typical of capacitated cells) compared with control group was recorded (Pattern A: 39.0±12.6, 33.3±11,3, 28.9±11.1, 33.4±16.2; Pattern B: 59,2±12.8, 66.7 ±11.3, 69.1±11.4, 64.2±13.7, MSG 0, 0.1, 1 and 10mM respectively).

3.3. Effect of MSG in presence of DL-threo-beta-hydroxyaspartic acid (THA)

When sperm pre-incubation and gamete coincubation were performed in presence of either 1mM MSG or 1mM MSG + 100µM THA (non selective inhibitor of glutamate uptake) a significant increase ($P < 0.05$) in the penetration rate compared to control group was recorded (Tab. 2). No difference in penetration rate between MSG and MSG+THA treatment was observed.

THA did not induce any significant change as compared to MSG group in sperm viability (46.7±14.6, vs. 46.0±9.7, MSG vs. MSG + THA respectively) and in the percentage of cells displaying the different phosphotyrosine patterns (Pattern A: 34.5±13.1 vs. 36.3±18.8; Pattern B; 65.4±17.0 vs.63.6±18.9; MSG vs. MSG + THA respectively).

4. DISCUSSION

The effect of monosodium glutamate (MSG) on pig sperm-zona pellucida binding and in vitro fertilization was studied. Our results demonstrate that glutamate influences pig sperm-oocyte interactions. MSG at concentrations 1 mM and 10mM was able to significantly increase the number of spermatozoa bound to zonae pellucidae compared with control. Moreover, MSG 1mM resulted in a significant higher percentage of penetrated oocytes.

Sperm viability and percentage of acrosome intact cells were not influenced by MSG during in vitro induced capacitation. Therefore, the positive effect on sperm oocyte-binding and fertilization does not seem to depend on any effect on plasma or acrosome membrane integrity or spontaneous

226 acrosome reaction. Our results on the lack of effect of MSG on acrosome reaction agree with those
227 by Meyer et al. [5] who observed that neither MSG nor sweet tastants elicited an elevation in the
228 percentage of acrosome reaction in capacitated mouse sperm.
229 The presence of MSG at the concentrations of 1 mM during in vitro-induced capacitation seems to
230 represent a slight stimulus for tyrosine-phosphorylation of sperm tail proteins as a tendency to an
231 increase of cells displaying a immunopositivity in the acrosome, EqSS and principal piece of the tail
232 (69.1%)(pattern B, characteristic of capacitated cells) was recorded. It has to be taken into account
233 that the stimulating effect of MSG may have been partially masked by the stimulation of capacitating
234 medium itself that induced an increase of B pattern from around 1% in fresh semen (data not shown)
235 to 59.2% in capacitated sperm in absence of MSG.
236 This slight stimulation of one of the events underlying capacitation could explain, at least in part,
237 the positive influence of MSG on pig sperm-oocyte binding and fertilization. It can be hypothesized
238 that in vivo glutamate, one of the most abundant amino acids in pig follicular fluid [4], could be
239 entrapped in the cumulus oophorus after ovulation thus increasing its concentration in the
240 proximity of the oocyte enhancing events underlying sperm-oocye interactions.
241 Meyer et al. [5] observed that in uncapacitated mouse sperm, 10mM MSG induced a significant
242 increase in cAMP concentration compared to basal cAMP levels. Even if this effect was no more
243 significant in capacitated sperm, possibly due to a masking effect of the elevated cAMP level
244 induced by capacitation medium, these results suggest that MSG may be able to modulate the level
245 of this intracellular second messenger involved in sperm capacitation [5].
246 It could be hypothesized that MSG may act on boar spermatozoa through taste receptors as the
247 expression of the functional receptor for umami taste in tongue has been detected in murine and
248 human spermatozoa [5] and the expression of both G proteins involved in umami taste detection,
249 α-gustducin and α-transducin, have been documented in boar spermatozoa [7]. However Meyer et
250 al. [5] stated that it still remains debatable whether glutamate is an active ligand of the T1R1 in
251 mouse spermatozoa as MSG did not elicit an increase in intracellular calcium concentration and was
252 ineffective in inducing acrosome reaction.
253 The expression of Group III metabotropic glutamate receptors (mGlu 6 and 8) was detected in mice
254 spermatozoa [17] and ionotropic glutamate receptors (NR1, NR2B, GluR6 and KA2) and glutamate
255 transporters (GLT1 and EAAC1) have been identified in mouse and human sperm [15] suggesting a
256 possible role in sperm physiology.

To explore whether the effect elicited by MSG could be due to a glutamate uptake in boar spermatozoa, fertilization trials have been performed in presence of MSG (1 mM) and THA (a non selective inhibitor of glutamate uptake). THA was ineffective in influencing the MSG positive effect on fertilization rate; as a consequence, it is likely that glutamate transporters are not involved in the pathway mediating this effect.

In conclusion our study demonstrates for the first time that glutamate exerts a positive effect on sperm-oocyte binding and fertilization. Even if our results on tyrosine phosphorylation of sperm proteins may suggest that the beneficial effect could be mediated by a stimulation of capacitation process, further studies are needed to clarify the mechanism(s) by which glutamate exerts its effect and to verify whether glutamate may have any role during in vivo sperm- oocyte interaction in pig.

Acknowledgements

The work was supported by “Fondazione Sfameni” and “Fondazione del Monte di Bologna e Ravenna” grants.

5. REFERENCES

[1] Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. Theriogenology 2005;64(4):992-1006.

[2] Iritani A, Sato E. Secretion rates and chemical composition of oviduct and uterine fluids in sows. J Anim Sci 1974;39(3):582-8.

[3] Guérin P, Gallois E, Croteau S, Revol N, Maurin F, Guillaud J, Menezo Y. Techniques de récolte et aminogrammes des liquides tubaire et folliculaire chez les femelles domestiques. Rev Med Vet 1995;46:805-14.

[4] Hong J, Lee E. Intrafollicular amino acid concentration and the effect of amino acids in a defined maturation medium on porcine oocyte maturation, fertilization, and preimplantation development. Theriogenology 2007;68(5):728-35.

[5] Meyer D, Voigt A, Widmayer P, Borth H, Huebner S, Breit A, Marschall S, de Angelis MH, Boehm U, Meyerhof W, Gudermann T, Boekhoff I. Expression of Tas1 taste receptors in

mammalian spermatozoa: functional role of Tas1r1 in regulating basal Ca^{2+} and cAMP concentrations in spermatozoa. PLoS One 2012;7(2):e32354.

[6] Gong T, Wei Q, Mao D, Shi F. Expression patterns of taste receptor type 1 subunit 3 and α-gustducin in the mouse testis during development. Acta Histochem 2016;118(1):20-30.

[7] Spinaci M, Bucci D, Mazzoni M, Giaretta E, Bernardini C, Vallorani C, Tamanini C, Clavenzani P, Galeati G. Expression of α-gustducin and α-transducin, G proteins coupled with taste receptors, in boar sperm. Theriogenology 2014;82(1):144-51.e1

[8] Fehr J, Meyer D, Widmayer P, Borth HC, Ackermann F, Wilhelm B, Gudermann T, Boekhoff I. Expression of the G-protein alpha-subunit gustducin in mammalian spermatozoa. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 2007;193(1):21-34.

[9] He W, Yasumatsu K, Varadarajan V, Yamada A, Lem J, Ninomiya Y, Margolskee RF, Damak S. Umami taste responses are mediated by alpha-transducin and alpha-gustducin. J Neurosci 2004;24(35):7674-80.

[10] Petters RM, Wells KD (1993) Culture of pig embryos. J Reprod Fertil 48:61-73.

[11] Funahashi H, Cantley T, Day BN (1997) Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. Biol Reprod 57:49–53.

[12] Brackett BG, Oliphant G. Capacitation of rabbit spermatozoa in vitro. Biol Reprod 1975;12:260-274.

[13] Spinaci M, Volpe S, De Ambrogi M, Tamanini C, Galeati G. Effects of epigallocatechin-3-gallate (EGCG) on in vitro maturation and fertilization of porcine oocytes. Theriogenology 2008;9(7):877-85.

[14] Bucci D, Galeati G, Tamanini C, Vallorani C, Rodriguez-Gil JE, Spinaci M. Effect of sex sorting on CTC staining, actin cytoskeleton and tyrosine phosphorylation in bull and boar spermatozoa. Theriogenology 2012;77:1206–1216

[15] Hu JH, Yang N, Ma YH, Jiang J, Zhang JF, Fei J, Guo LH. Identification of glutamate receptors and transporters in mouse and human sperm. J Androl 2004;25(1):140-6.

[16] R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 2016.

316 [17] Marciniak M, Chruścicka B, Lech T, Burnat G, Pilc A. Expression
317 of group III metabotropic glutamate receptors in the reproductive system of male mice. Reprod
318 Fertil Dev 2016;28(3):369-74.
319

	MSG (mM)			
	0	**0.1**	**1**	**10**
Effect of MSG on in vitro capacitated spermatozoa				
Viable spermatozoa (%)	52.9±6.6	53.4±6.6	54.8±9.8	48.9±9.0
Acrosome-intact sperm (%)	90.7±2.6	89.9±3.5	91.3±3.6	89.8±3.3
Effect of MSG on sperm - zona pellucida binding				
No. oocytes examined	166	153	177	167
Sperm bound to ZP (%)	12.3±9.0[a]	12.6±9.1[a]	17.8±11.3[b]	17.6±10.8[b]
Effect of MSG on IVF parameters				
No. oocytes examined	279	184	264	266
Penetrated oocytes (%)*	36.8±16.2[a]	44.3±20.1[ab]	53.7±20.4[b]	47.1±19.3[ab]
Monospermic oocytes (%)#	79.4±15.5	74.0±21.8	72.8±24.9	78.0±14.4
Efficiency of fertilization (%)§	27.7±8.5	30.6±13.0	35.3±9.9	35.0±9.5

Table 1. Effect of MSG addition during 2h in vitro induced sperm capacitation (six replicates from six pooled ejaculates from three boars) and during sperm preicubation and gamete coincubation on sperm - zona pellucida binding (six replicates) and IVF parameters (seven replicates).

* Percentage of penetrated oocytes/ total number of inseminated oocytes .

Percentage of monospermic oocytes/ number of penetrated oocytes.

§ Percentage of monospermic oocytes/ total number of inseminated oocytes.

Values are mean ± SD. Different superscripts in the same row represent significant difference for $P < 0.05$ between treatments.

	Control	MSG	MSG + THA
No. oocytes examined	271	258	254
Penetrated oocytes (%)*	39.8±15.7[a]	53.7±22.1[b]	52.2±23.7[b]
Monospermic oocytes (%)#	68.6±13.4	63.1±21.0	60.6±31.9
Efficiency of fertilization (%)§	27.7±12.1	31.2±12.3	25.93±10.8

Table 2. Effect of of 1mM MSG and 100µM THA (non selective inhibitor of glutamate uptake) on IVF parameters.

Values are mean ± SD of six replicates. Different superscripts in the same row represent significant difference for $P < 0.05$ between treatments.

* Percentage of penetrated oocytes/ total number of inseminated oocytes .

Percentage of monospermic oocytes/ number of penetrated oocytes.

§ Percentage of monospermic oocytes/ total number of inseminated oocytes.

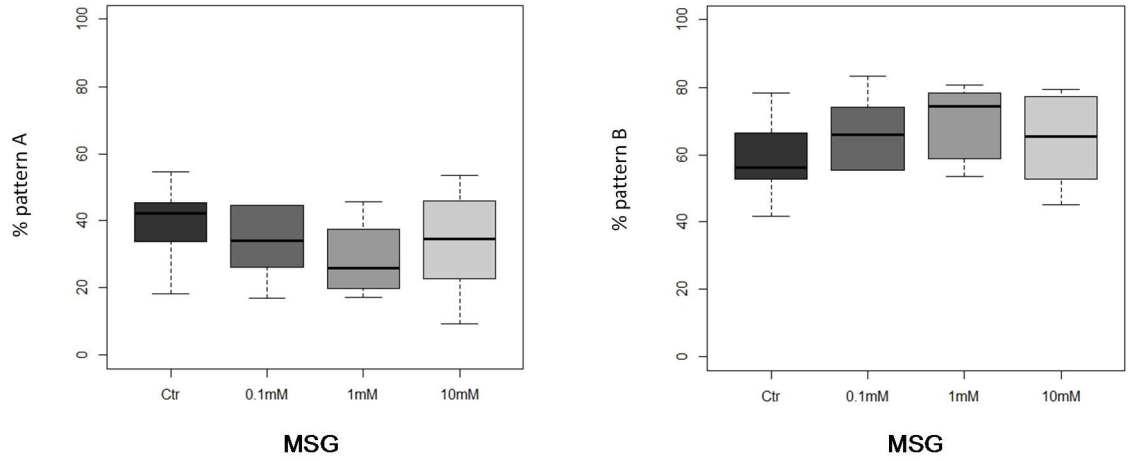

Fig. 1. Boxplots representing the percentage of sperm cells displaying tyrosine phosphorylation pattern A (positivity in the Equatorial Subsegment (EqSS) and acrosome) or pattern B (positivity in the acrosome, EqSS and principal piece of the tail) after 2h of incubation under capacitating conditions in presence of different concentrations of MSG. Each experiment was repeated seven times.