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

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

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## 12 **ABSTRACT**

13

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

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

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

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

32 In order to evaluate whether the effect elicited by MSG could be due to glutamate uptake in boar  
33 spermatozoa, fertilization trials were performed in presence of either 1mM MSG or 1mM MSG +  
34  $100 \mu\text{M}$  DL-threo-beta-hydroxyaspartic acid (THA), a non selective inhibitor of glutamate uptake. A  
35 significant increase ( $P < 0.05$ ) in the penetration rate in both MSG and MSG + THA groups compared  
36 to control was recorded ( $39.8 \pm 15.7$ ,  $53.7 \pm 22.1$ ,  $52.2 \pm 23.7$ , Control, MSG and MSG + THA  
37 respectively) while no difference in penetration rate between MSG and MSG+THA treatment was  
38 observed suggesting that sperm glutamate transporters are not involved in the pathway mediating  
39 this effect.

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

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

## 44 **1. INTRODUCTION**

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

54 The two subunit of the umami receptor dimer (T1R1 and T1R3), which form the functional receptor  
55 for monosodium glutamate in tongue taste buds, are expressed in mature murine and human  
56 spermatozoa and in murine spermatids, while transcripts for sweet taste receptor (T1R2) were not  
57 detected [5,6]. Moreover we have recently demonstrated the expression  $\alpha$ -gustducin and  $\alpha$ -  
58 transducin, G proteins accepted as specific markers of chemosensitive cells, in boar spermatozoa  
59 [7]. The expression of  $\alpha$ -gustducin was also demonstrated in mouse, rat, bull and human male  
60 gametes [6,8]. Both  $\alpha$ -gustducin and  $\alpha$ -transducin are involved in the umami taste signaling [9] in  
61 response to aminoacids and, among them, glutamate is the most extensively studied ligand for  
62 umami receptor in taste buds.

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

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

70

## 71 **2. MATERIALS AND METHODS**

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

### 73 **2.1. In vitro maturation of porcine oocytes (IVM)**

74 Porcine cumulus–oocyte complexes (COCs) were aspirated using a 18 gauge needle attached to a  
75 10 mL disposable syringe from 4 to 6 mm follicles of ovaries collected at a local abattoir and  
76 transported to the laboratory within 1 h. Under a stereomicroscope, intact COCs were selected and  
77 transferred into a petri dish (35 mm, Nunclon,Denmark) prefilled with 2 mL of modified PBS  
78 supplemented with 0.4% BSA. After three washes in NCSU 37 [10] supplemented with 5.0  
79 mg/mLinsulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 50 μM β-  
80 mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were  
81 transferred to a Nunc 4-well multidish containing 500 μL of the same medium per well and cultured  
82 at 39 °C in a humidified atmosphere of 5% CO<sub>2</sub>/7% O<sub>2</sub> in air. For the first 22 h of in vitro maturation  
83 the medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL, eCG (Folligon, Intervet, Boxmeer,  
84 The Netherlands) and 10 IU/mL hCG (Corulon, Intervet). For the last 22 h COCs were transferred to  
85 fresh maturation medium [11]. At the end of the maturation period the oocytes were denuded by  
86 gentle repeated pipetting.

## 87 **2.2. Semen collection and preparation**

88 Sperm-rich fraction of ejaculates were collected by gloved-hand technique from three mature  
89 boars of proven fertility and extended in equal volume of Androhep TM (Minitub, Tiefenbach,  
90 Germany). Only ejaculates with sperm viability higher than 85% were used in the experiments. In  
91 order to minimize the boar effect, ejaculates were pooled.

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

## 95 **2.3. Effect of MSG sperm-zona pellucida binding and fertilization parameters**

### 96 **2.3.1. Effect of MSG sperm-zona pellucida binding**

97 For the sperm-zona pellucida binding assay, spermatozoa were diluted to a concentration of  
98  $500 \times 10^3$  spermatozoa /ml and 500 μL of the sperm suspensions were preincubated for 1 h in  
99 presence or absence of different concentrations of MSG dissolved in water (0, 0.1, 1, 10 mM).

100 At the end of the maturation period 30-35 denuded oocytes were added into each well and after 1  
101 h of gamete co-incubation at 38 °C in 95% humidity and 5% CO<sub>2</sub> in air the oocytes were washed four  
102 times in PBS 0.4% BSA with a wide bore glass pipette in order to remove the spermatozoa loosely  
103 attached to zona pellucida. The oocytes were then fixed in 4% paraformaldehyde for 15 min at room

104 temperature and then incubated with 8.9  $\mu$ M 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  $750 \times 10^3$  spermatozoa /ml and  
112 500  $\mu$ L 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  $50 \times 10^6$   
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  $\mu$ l of a 300  $\mu$ M propidium iodide (PI) stock  
132 solution and 2  $\mu$ l of a 10  $\mu$ M SYBR green-14 stock solution, both obtained from the live/dead sperm

133 viability kit (Molecular Probes, Inc., Eugene, OR, USA) for 5 min at 37°C in the darkness. At least 200  
134 spermatozoa per sample were scored with a Nikon Eclipse E 600 epifluorescence microscope (Nikon  
135 Europe BV, Badhoevedop, The Netherlands). Spermatozoa stained with SYBR green-14 and not  
136 stained with PI were considered as viable. Spermatozoa SYBR and PI positive and those SYBR  
137 negative / PI positive were considered as cells with non-intact membrane or dead.

138 Acrosome integrity was evaluated by using a FITC-conjugated lectin from *Pisum Sativum* (FITC-PSA)  
139 which label acrosomal matrix glycoproteins. Spermatozoa were washed twice in PBS, resuspended  
140 in ethanol 95% and fixed/permeabilized at 4°C for at least 30 min. Aliquots of sperm suspensions  
141 were dried on heated slides and incubated with FITC-PSA solution (5 µg PSA-FITC/1 mL H<sub>2</sub>O) for 20  
142 min in darkness. After staining, samples were washed in PBS and mounted with Vectashield  
143 mounting medium containing propidium iodide (PI) counterstain for DNA (Vector Laboratories). The  
144 slides were then observed with the above described epifluorescence microscope and at least  
145 200 spermatozoa per sample were evaluated.

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

149

#### 150 **2.4.2. Immunolocalization of tyrosine-phosphorylated proteins**

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

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

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

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



165 A: positivity in the Equatorial Subsegment (EqSS) and acrosome;  
166 B: positivity in the acrosome, EqSS and principal piece of the tail;  
167 C: positivity in the tail and (not constant) in the EqSS.

168

## 169 **2.5. Effect of MSG in presence of DL-threo-beta-hydroxyaspartic acid (THA)**

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

## 175 **2.6. Statistical analysis**

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

182

## 183 **3. RESULTS**

### 184 **3.1. Effect of MSG sperm-zona pellucida binding and fertilization parameters**

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

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

194

### 195 **3.2. Effect of MSG addition during in vitro induced sperm capacitation**

196 The addition of MSG during in vitro capacitation of boar spermatozoa at the concentrations of 0.1,  
197 1 and 10 mM did not induce any significant difference on the percentage of viable cells and  
198 spermatozoa with intact acrosome as compared with control (Tab. 1).

199 The results on immunolocalization of tyrosine-phosphorylated proteins of spermatozoa capacitated  
200 in presence of 0.1, 1 and 10 mM MSG are reported in Fig. 1. A not significant tendency to a decrease  
201 of the percentage of cells displaying A pattern (typical of non capacitated cells) and to a parallel  
202 increase of B pattern (typical of capacitated cells) compared with control group was recorded  
203 (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,  
204 64.2±13.7, MSG 0, 0.1, 1 and 10mM respectively).

205

### 206 **3.3. Effect of MSG in presence of DL-threo-beta-hydroxyaspartic acid (THA)**

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

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

215

216

## 217 **4. DISCUSSION**

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

223 Sperm viability and percentage of acrosome intact cells were not influenced by MSG during in vitro  
224 induced capacitation. Therefore, the positive effect on sperm oocyte-binding and fertilization does  
225 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-oocyte 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  $\alpha$ -gustducin and  $\alpha$ -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.

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

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

267

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272

## 273 **5. REFERENCES**

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320

	MSG (mM)			
	0	0.1	1	10
<b>Effect of MSG on in vitro capacitated spermatozoa</b>				
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
<b>Effect of MSG on sperm - zona pellucida binding</b>				
No. oocytes examined	166	153	177	167
Sperm bound to ZP (%)	12.3±9.0 <sup>a</sup>	12.6±9.1 <sup>a</sup>	17.8±11.3 <sup>b</sup>	17.6±10.8 <sup>b</sup>
<b>Effect of MSG on IVF parameters</b>				
No. oocytes examined	279	184	264	266
Penetrated oocytes (%) <sup>*</sup>	36.8±16.2 <sup>a</sup>	44.3±20.1 <sup>ab</sup>	53.7±20.4 <sup>b</sup>	47.1±19.3 <sup>ab</sup>
Monospermic oocytes (%) <sup>#</sup>	79.4±15.5	74.0±21.8	72.8±24.9	78.0±14.4
Efficiency of fertilization (%) <sup>§</sup>	27.7±8.5	30.6±13.0	35.3±9.9	35.0±9.5

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

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

328 # Percentage of monospermic oocytes/ number of penetrated oocytes.

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

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

332

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

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

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

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

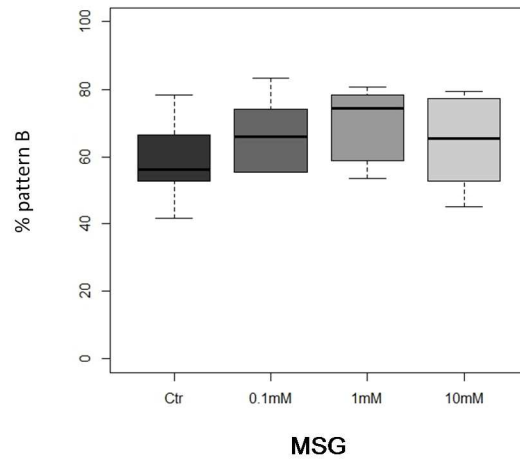
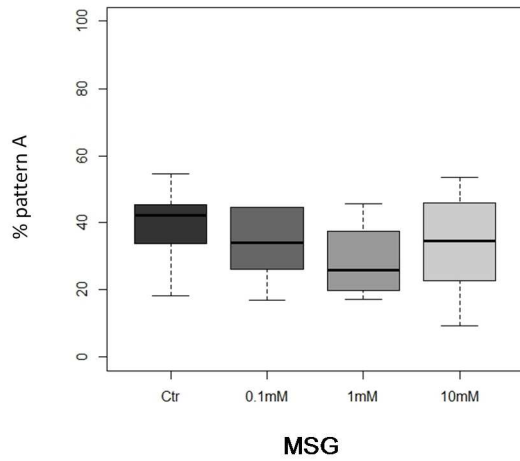
339 # Percentage of monospermic oocytes/ number of penetrated oocytes.

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

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

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