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

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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

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 trancrips for sweet taste receptor (T1R2) were not
57 detected [5,6]. Moreover we have recently demonstrated the expression α-gustducin and α-
58 transducin, G proteins accepted as specific markers of chemosensitive cells, in boar spermatozoa
59 [7]. The expression of α-gustducin was also demonstrated in mouse, rat, bull and human male
60 gametes [6,8]. Both α-gustducin and α-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 ß-
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_2/7% O_2 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
98 500×10^3 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% CO2 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 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 $50\text{x}10^6$
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 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

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, Badhoeverdop, 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 H2O) 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 ± 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 *t*yrosine-phosphorylated proteins of spermazotoa 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-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.

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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271
272

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319

320

	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

321

322

323

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 preicubation 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.

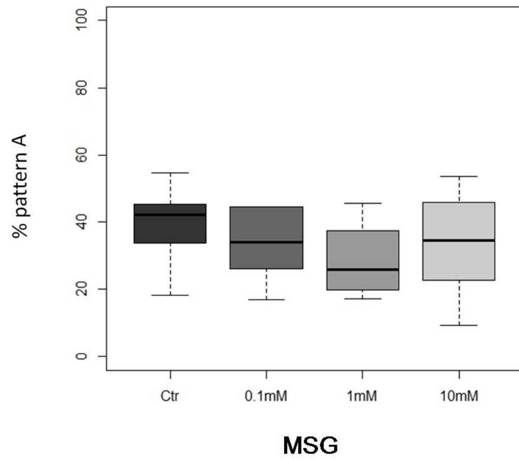
332

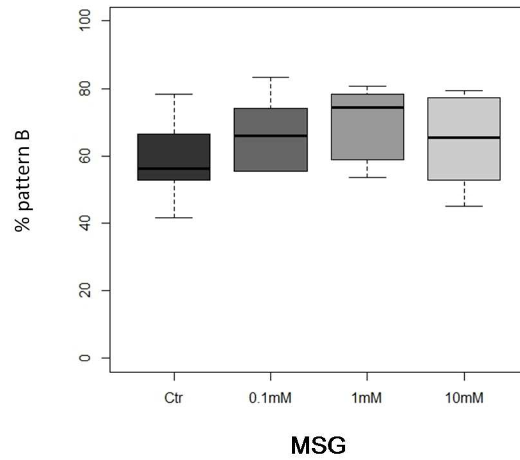
333

	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

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.
341

342





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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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