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Revised

Improved embryo development using high cysteamine concentration during IVM and sperm co-culture with COCs previous to ICSI in bovine.

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Abstract

In contrast to other species, intracytoplasmic sperm injection (ICSI) in bovine remains inefficient, resulting in low embryo developmental rates. It is unclear whether such inefficiency is due to the poor response of bovine ooplasms to the injection stimulus, or to the inability of bull sperm to induce oocyte activation. In order to facilitate these events, two strategies were assessed: the use of high concentration of cysteamine [Cys] during IVM; and the selection of sperm attached to cumulus cells after incubation with COCs for

ICSI. First, COCs were IVM with increasing [Cys] and subjected to IVF. Zygotes from all groups were cultured under different O_2 tensions and development to blastocyst was evaluated. In a second experiment, sperm were co-cultured for 3 h with COCs and acrosome reaction was studied. Afterwards, the best IVM and IVC conditions determined on Experiment 1 were used for ICSI assay. COCs were matured for 21 h with 1 (Cys 1) or 0.1 mM Cys (Cys 0.1 groups, standard condition). In addition, COCs were incubated for ≥3 h with $16x10^6$ sperm/ml and only sperm attached to cumulus cells were selected for ICSI (ICSI + Co-cult groups). After chemical activation, embryos were cultured in SOF medium under low O_2 tension. Cleavage and blastocyst rates were evaluated at days 2 and 7 of IVC, respectively. Finally, the relative expression of eight genes indicators of embryo quality was compared between ICSI and IVF control blastocysts by qPCR. Cleavage rates were higher for Cys 0.1 ICSI + Co-cult and Cys 1 ICSI + Co-cult groups (n=117, 92% and n=116, 79%, respectively) compared to their controls (n=132, 60% for Cys 0.1 ICSI and n=108, 52% for Cys 1 ICSI) ($p\leq0.05$). Interestingly, the combined treatment (Cys 1 ICSI + Co-cult) showed higher blastocyst rates than all other ICSI groups (23 vs. 11, 18 and 14% for Cys 0.1 ICSI + Co-cult, Cys 1 ICSI, and Cys 0.1 ICSI, respectively) ($p\leq0.05$). Moreover, incubation with COCs increased the rates of live acrosome reacted sperm ($p\leq0.05$). The relative abundance of mRNAs coding for *INFτ, CAT, DNMT1, OCT4,* and *HDAC3* did not differ between treatments ($p\leq0.05$). *SOD2, HADC1* and *HADC2* expression was higher for Cys 0.1 ICSI than for IVF embryos ($p\leq0.05$). Group Cys 1 ICSI did not differ from IVF for those three genes, neither did Cys 1 ICSI + Co-cult, except for *HDAC1* ($p\leq0.05$). In conclusion, the use of 1 mM Cys during IVM and of sperm incubated with mature COCs might be a good strategy to improve ICSI outcomes in cattle.

Keywords: sperm injection, cumulus cells, pretreatment, *in vitro* maturation, oxygen tension, gene expression.

1. Introduction

Since the first intracytoplasmic sperm injection (ICSI) performed in bovine [1], many efforts have been made worldwide to improve *in vitro* embryo development and birth rates. Despite the great advances achieved in terms of *in vitro* embryo production, ICSI in cattle continues being inefficient [reviewed by 2]. In this species, low embryo development would be related to abnormal levels of sperm decondensation and pronuclei formation observed after ICSI [3-6]. It is still unclear whether such inconsistencies are due to the inability of bull sperm to induce complete oocyte activation, or to the poor response of bovine ooplasms to the injection stimulus, which provokes an incorrect sperm head decondensation. For this reason, several oocyte activation protocols and sperm pretreatments have been developed, though with varying results [7-13]. The aim of this work was to evaluate treatments that better resemble physiological processes occurring to oocytes and sperm during fertilization, in order to facilitate ICSI embryo development.

Upon regular fertilization, the hypercondensed sperm chromatin decondenses resulting into a male pronucleus. This complex process is mediated by the reduced glutathione (GSH), which accumulates in the ooplasm during oocyte maturation [reviewed by 14]. After fertilization, the endogenous GSH reduces the disulfide bonds of protamines from the sperm nucleus, leading to their replacement by histones and allowing sperm chromatin

decondensation [15, 16]. For *in vitro* embryo production, 0.1 mM cysteamine (Cys) is added to the *in vitro* maturation (IVM) medium as the main precursor of GSH [17]. For species with frequent fertilization failure after IVF, five fold Cys concentration has been used to increase endogenous GSH levels into the oocyte, resulting in higher rates of pronuclei formation [18, 19]. In bovine, while fertilization failure does not usually occur after IVF, it is highly frequent after ICSI [13, 20-22]. Thus, we propose the use of higher concentrations of Cys during IVM to improve fertilization rates after ICSI in cattle. To this aim, an IVF assay was initially performed to determine the highest concentration of Cys to be used for *in vitro* maturation of COCs. Since Cys has an anti-oxidant action [23], high and low O_2 tension were tested during *in vitro* culture of IVF embryos until the blastocyst stage and the best combination of IVM and IVC conditions (Cys concentration and O_2 tension, respectively) were used for ICSI assay.

Prior to *in vivo* fertilization, mammalian spermatozoa become capacitated in the female reproductive tract, turning competent to undergo the acrosome reaction. It consists on the release of the acrosome content, which allows the sperm to penetrate the zona pellucida for further fertilization. During acrosome reaction, the complex of membranes that surrounds the sperm nucleus is removed, facilitating the entry of the sperm nucleus into the oocyte [24]. The ICSI procedure bypasses these events and introduces additional membrane barriers between sperm nucleus and ooplasm [25-27] that might be responsible for the reduced developmental competence of the resulting embryos [26, 28, 29]. Recently, it was suggested that *in vitro* matured bovine oocytes might be unable to decondense sperm that have not suffered *in vivo* capacitation and acrosome reaction [22]. In addition, sperm interaction with cumulus cells was shown to increase acrosome reaction, sperm capacitation

and subsequent *in vitro* fertilization rates [30, 31]. For this reason, we intended to closely mimic physiological processes that usually precede fertilization by allowing the interaction of sperm with cumulus cells in a capacitating medium. After incubation, sperm that were attached to cumulus cells were separated from COCs by aspiration with the injection pipette and immediately used for ICSI.

In summary, this report intended to facilitate sperm decondensation after ICSI by means of specific treatment of oocytes and sperm, by the supplementation of the IVM medium with high Cys concentration and the incubation of sperm with COCs prior to ICSI. Results were evaluated in terms of blastocyst production and quality of the generated blastocysts, based on the relative expression of important developmental genes by qPCR.

2. Materials and Methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.1. Experimental design

In Experiment 1, the maximum Cys concentration that could be supplemented on IVM medium with no detrimental effects over blastocysts development was determined. COCs were *in vitro* matured under standard conditions (with 0.1 mM Cys) or with increasing concentrations of Cys (0.5, 1 and 10 mM) and IVF was performed. Embryos from all groups were cultured under low and high O_2 tension. In Experiment 2, the effect of sperm

115 co-culture with COCs on sperm acrosome membrane integrity was studied. In Experiment
116 3, the best IVM and IVC conditions determined on Experiment 1 were used for ICSI assay.
117 The effect of sperm-COCs co-culture prior to ICSI on embryo development was evaluated.
118 In experiment 4, the quality of ICSI blastocysts was analyzed based on relative expression
119 of *INFτ, CAT, SOD2, DNMT1, OCT4, HDAC1, HDAC2* and *HDAC3* genes.

120

121 *2.2 Cumulus–oocyte complexes (COCs) collection and in vitro maturation (IVM)*

122 COCs collection was performed as previously described by Canel et al. [32]. The
123 maturation medium was TCM-199 with Earle's salts (11150042; Gibco, NY, USA)
124 containing 10% v/v fetal bovine serum (FBS, Internegocios, Mercedes, Argentina), 1% v/v
125 penicillin/streptomycin/Fungizone® (ATB/ATM, 15240-096; Gibco, NY, USA), 0.3 mM
126 sodium pyruvate (P2256) and 2 μg/ml Follicle Stimulating Hormone (FSH, NIH-FSH-P1,
127 Folltropin®, Bioniche, Australia). For Experiment 1, IVM medium was supplemented with
128 0.1, 0.5, 1 or 10 mM cysteamine (Cys, M9768). For Experiment 3, IVM medium was
129 supplemented with 0.1 or 1 mM Cys, depending on the groups. In all cases, groups of 20-
130 22 COCs were randomly allocated in 100 μl droplets of IVM medium covered with mineral
131 oil (M8410) and incubated at 39ºC in a humidified atmosphere of 6% CO_2 and 20% O_2 in
132 air. After 20-22 h of IVM, cumulus cells were removed from COCs by vortexing for 3 min
133 in 1 mg/ml hyaluronidase solution (H4272) and washed three times in Hepes-TALP [33].
134 Oocytes with an extruded first polar body (PB) were selected for ICSI or chemical
135 activation.

136

137 *2.3 In vitro fertilization (IVF)*

138 For IVF groups, COCs were matured *in vitro* for 21 h, washed in Hepes-TALP and
139 immediately co-incubated with sperm. The IVF procedure was previously described by
140 Brackett and Oliphant [34]. Briefly, frozen semen from two bulls was thawed in a 37°C
141 water bath for 30 sec. Sperm were washed twice by centrifugation at 490 X g for 5 min
142 with 5 ml of Brackett's defined medium. Sperm concentration was adjusted to $15x10^6$/mL
143 in Brackett's fertilization (BO) medium and co-incubated for 5 h with groups of 20-22
144 COCs in 100 µl droplets covered with mineral oil. Afterwards, presumptive zygotes were
145 vortexed for 30 to 60 sec and washed several times in Hepes-TALP and cultured *in vitro* as
146 described below.

147

148 *2.4 Co-incubation of sperm with COCs (Co-cult) previous to ICSI*

149 This procedure was performed as was explained in IVF section, with slight differences.
150 Groups of 2-3 *in vitro* matured COCs were washed in TALP-H and placed in 20 µl droplets
151 containing $15x10^6$/mL sperm. The co-incubation of sperm and COCs (co-culture) was
152 carried out for a minimum of 3 h. Then, COCs were placed directly into a PVP droplet,
153 carrying the spermatozoa that were attached to the cumulus cells. These spermatozoa were
154 aspirated with the ICSI pipette, then immobilized and finally injected into the cytoplasm of
155 *in vitro* matured oocytes as explained in the following section (Figure 1).

156

157 *2.5 Intracytoplasmic sperm injection (ICSI)*

After 21 h of IVM, sperm injection was performed in 100 µl droplets of Hepes-TALP under mineral oil in 100 x 20 mm culture dishes (430,167; Corning, NY, USA), using hydraulic micromanipulators (Narishige, Medical Systems, Great Neck, NY, USA) mounted on a Nikon Eclipse TE-300 microscope (Nikon, Melville, NY, USA). According to the experimental group, spermatozoa or COCs previously incubated with sperm from two bulls were placed in a 2 µl droplet of 10% v/v polyvinylpyrrolidone (PVP, Irvine Scientific, Santa Ana, CA, USA) in Hepes-TALP. Each spermatozoon was immobilized by breaking its tail and aspirated tail-first into a 9 µM inner diameter injection pipette. The injection pipette was transferred to a droplet containing MII oocytes, which were held by negative pressure with a holding pipette, locating the polar body at the 6 or 12 o'clock position. Immediately, sperm injection was performed by aspiration (until the breakage of the oocyte membrane) and subsequent deposition of the spermatozoa and the previously aspirated ooplasm inside the oocyte. Sham controls were injected with the medium used for spermatozoa/COCs, but with no sperm, using a volume equivalent to that used for ICSI. A parthenogenetic group (PA) was also included as a control of *in vitro* development. After injection, all groups were subjected to chemical activation as described below.

2.6 Chemical activation

Sperm injected oocytes were treated with 5 µM ionomycin (I24222; Invitrogen, Carlsbad, CA, USA) in Hepes-TALP for 4 min, followed by incubation for 3 h in TCM-199 medium (to allow second polar body extrusion), and finally treated with 1.9 mM 6-DMAP (D2629) diluted in TCM-199 medium for 3 h. In the case of PA control group, metaphase II oocytes

were treated with 6-DMAP immediately after ionomycin incubation to inhibit second polar body extrusion. Afterwards, oocytes were thoroughly washed in Hepes-TALP and cultured as described below.

2.7 In vitro culture (IVC) and determination of blastocyst cell number

Activated oocytes and presumptive zygotes were cultured in 50 µl droplets of SOF medium [35, 36] supplemented with 2.5% FBS, covered with mineral oil. Incubation was performed at 39ºC in a humidified atmosphere of 6% CO_2 and 5% O_2 in air for 7 days. For Experiment 1, some groups were incubated under an atmosphere of 6% CO_2 and 20% O_2 in air (high oxygen tension condition). The number of cleaved embryos and blastocysts was evaluated at days 2 and 7 respectively, and 100% of the medium was replaced at days 2 and 5 of *in vitro* development. Total cell number of IVF blastocysts was determined at day 7 of *in vitro* development, by staining with 3 µg/ml of Hoechst Bisbenzimide 33342 (H33342: B-2261) for 10 min. Embryos were placed between a slide and a coverslip, and nuclei were visualized and counted under an inverted epifluorescence microscope, using an excitation/emission wavelength of 350/461 nm.

2.8 Evaluation of acrosome membrane integrity

Sperm previously incubated in BO medium for 3 h with COCs (Co-cult group), without COCs (BO group) or just thawed (Thawed group) were analyzed. In the case of co-cult group, sperm attached to the cumulus cells were separated from COCs using an ICSI

pipette, as was performed prior to sperm injection. All groups were resuspended in 200 µl of BO medium and incubated with 10 µg/ml of the fluorescence probe peanut agglutinin conjugated with fluorescein isothiocyanate (PNA/FITC, L7381) and 5 µg/ml propidium iodide (PI, P4170) for 15 min at 39ºC in the dark. Then, samples were washed by centrifugation at 490 X g for 5 min and the pellet was resuspended in 50 µl of BO medium for evaluation of acrosome membrane integrity. Sperm samples used as PI positive controls were treated with 0.8% (v/v) Triton X-100 (T-9284) in PBS for 15 min (Triton X group). Sperm samples used as PNA/FITC positive controls were treated with 10 µM ionomycin for 45 min in the dark (Ionomycin group). All samples were placed between a glass slide and a coverslip and observed under an epifluorescence microscope using excitation wavelengths of 488 and 544 nm.

2.9 RNA extraction and real time PCR

Pools of five expanded blastocysts from IVF, ICSI Control, Cys 1 ICSI + Co-cult and Cys 1 ICSI groups were kept in RNA Later® (AM 7020, Ambion, CA, USA) at -50°C until RNA extraction. Total RNA was extracted using the commercial Pico Pure Isolation kit (Arcturus, Carlsbad, CA, USA) and residual genomic DNA was removed by DNAsa I digestion with the RNase-Free DNase Set kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using the Revert Aid ™ H Minus First Strand kit (Thermo Scientific Inc., Pittsburgh, PA, USA) and quantitative real-time PCR (qPCR) using the Brilliant II SYBR Green QPCR masterbatch (Stratagene Agilent Technologies, Inc., Palo Alto, CA, USA) and the Q-PCR MX3000PTM kit (Stratagene Agilent Technologies) in a

MX3000P thermocycler (Stratagene Agilent Technologies). Genes analyzed were *IFNτ, CAT, SOD2, DNMT1, OCT4, HDAC1, HDAC2* and *HDAC3*. A detailed table including gene accession numbers, primers and amplification products is included (Table 1). Negative controls consisted on the same mix but substituting the cDNA with water, and run in parallel with the samples. IVF blastocysts were used as calibrators and the values are shown as n times of relative difference with respect to the calibrator. Dissociation curves were performed after each PCR run to ensure that a single PCR product had been amplified. Quantification of relative mRNA expression levels was performed using the MXPro-MX300P Version 4.10 program (Stratagene Agilent Technologies), based on the ΔΔCt method and using the amplification efficiency of each gene as a correction factor [37]. As a reference, the geometric average of *YWHAZ* and *GAPDH* were used, after being analyzed with the geNorm Visual Basic program (Microsoft Excel).

2.10 Statistical analysis

Each experiment was repeated at least three times. Fisher's nonparametric analysis was performed to compare *in vitro* development and sperm membrane integrity results, with a confidence interval of 95% of the data obtained using Graph Pad PRISM® software, 5.01 version. The mean cell number of blastocysts and standard deviation (SD) were compared using the Kruskal-Wallis non-parametric test, with Dunn's correction for multiple comparisons. For calculations of relative quantification of mRNAs, differences between treatments in each experiment were determined by one-way ANOVA, after transforming the proportional data to its arc sine. In cases where significant differences were observed,

245 the Least Significant Difference (LSD) test was used to determine the magnitude of the
246 differences. For Kruskal-Wallis non-parametric test and all other analyzes, a difference of p
247 ≤0.01 and 0.05 were considered significant, respectively.

248

249 **3. Results**

250 *3.1 Experiment 1: Effect of Cys concentration during IVM followed by IVC under low or*
251 *high O_2 on development of IVF embryos.*

252 The effect of increasing concentrations of Cys during IVM (0.5, 1 and 10 mM vs. 0.1 mM
253 control) on the development of IVF bovine embryos was evaluated (Figure 2). All these
254 treatments were combined with *in vitro* culture of embryos until the blastocyst stage under
255 high (20%) or low (5%) O_2 tension. Total cell number of blastocysts from all groups was
256 determined. Results are detailed in Table 2. No detrimental effect over cleavage/blastocysts
257 rates and mean cell number of blastocysts was observed for COCs matured with 0.5 and 1
258 mM Cys when embryos were cultured under 5% O_2, nor for COCs matured with 0.5 mM
259 Cys and embryos cultured under 20% O_2 ($p \leq 0.05$). However, a decline in cleavage and
260 blastocyst rates was observed after IVM with 10 mM Cys, using both high and low O_2
261 tension during IVC. The same effect was observed when maturation was performed with 1
262 mM Cys and embryo culture under 20% O_2 ($p \leq 0.05$). A decrease of mean cell number was
263 observed for blastocysts subjected to IVM with 1 and 10 mM Cys and IVC under 20% O_2
264 compared to 0.1, 0.5 and 1 mM Cys groups, cultured under 5% O_2 ($p \leq 0.01$). On the basis of
265 these results, 1 mM Cys and 5% O_2 were the conditions chosen for IVM and IVC of ICSI
266 assays.

267

268 *3.2 Experiment 2: Sperm acrosome membrane integrity after incubation with COCs*

269 The effect of sperm incubation with COCs on sperm acrosome membrane integrity was
270 tested (Table 3, Figure 3). All treatments showed similar rates of live non-reacted and dead
271 reacted acrosome sperm ($p \leq 0.05$). Nonetheless, incubation with COCs (Co-cult group)
272 resulted in more live reacted and less dead non-reacted acrosome sperm, in comparison
273 with BO and Thawed groups ($p \leq 0.05$). As expected, Triton X and Ionomycin positive
274 control groups showed the highest rates of dead non-reacted and live reacted acrosome
275 sperm, respectively ($p \leq 0.05$).

276

277 *3.3. Experiment 3: ICSI after high Cys IVM and sperm-COCs co-incubation*

278 Treatments on oocytes and sperm were evaluated for ICSI in bovine: IVM with 1 mM Cys
279 (Cys 1 ICSI group) and co-incubation of sperm with COCs for a minimum of 3 h (ICSI +
280 Co-cult group) before injection. In addition, both treatments were combined (Cys 1 ICSI +
281 Co-cult group). ICSI, Sham and PA control groups subjected to standard IVM conditions
282 were also included (ICSI, ICSI + Co-cult, Sham and PA Cys 0.1). All embryos were
283 cultured under low O_2 tension. Results are summarized in Table 4. Cleavage rates were
284 higher for ICSI groups subjected to co-culture treatment (Cys 0.1 ICSI + Co-cult and Cys 1
285 ICSI + Co-cult groups) compared to their controls (Cys 0.1 ICSI and Cys 1 ICSI groups,
286 respectively) ($p \leq 0.05$). Interestingly, the combined treatment (Cys 1 ICSI + Co-cult)
287 showed higher blastocyst rates than all other ICSI groups ($p \leq 0.05$). See Figure 4.

288

289 *3.4 Experiment 4: Gene expression analysis of ICSI blastocysts by qPCR*

290 Since the combination of IVM with high Cys and the co-incubation of sperm-COCs
291 previous to ICSI improved both cleavage and blastocyst rates, the quality of blastocysts
292 generated with this treatment (Cys 1 ICSI + Co-cult group) was analyzed in terms of
293 relative expression of eight genes. Groups Cys 1 ICSI, Cys 0.1 ICSI and IVF were used as
294 controls. Real time PCR results are sown in Figure 4. No differences were found for the
295 relative abundance of mRNAs coding for *INFτ, CAT, DNMT1, OCT4,* and *HDAC3* between
296 groups ($p \leq 0.05$). However, *SOD2, HADC1* and *HADC2* expression was higher for Cys 0.1
297 ICSI compared to IVF embryos ($p \leq 0.05$). On the other hand, *SOD2* and *HADC2* expression
298 did not differ between Cys 1 ICSI, Cys 1 Co-cult and IVF groups.

299

300 **4. Discussion**

301 Unlike other species, ICSI in cattle results in low blastocyst rates and poor quality embryos.
302 In this work, we increased the concentration of Cys during IVM, and sperm were incubated
303 with COCs prior to ICSI. This alternative protocol, designed to closer mimic physiological
304 conditions, avoids the use of harmful chemical agents that may damage the oocyte and
305 spermatozoa (ex. dithiothreitol, triton X-100, lysolecithin, methyl-β-cyclodextrin) [10, 12,
306 38, 39, 40], and improves *in vitro* development of ICSI bovine embryos.

307 In Experiment 1, the maximum Cys concentration that can be supplemented to the IVM
308 medium without showing a negative impact on blastocyst development was determined.
309 The addition of Cys to the maturation medium is known to increase GSH levels in mature
310 oocytes, and oocytes with higher GSH content show increased developmental competence

311 [17, 41, 42]. As well as reducing the disulfide bonds of protamines, GSH acts as a major
312 antioxidant within cells [43, 44]. For this reason we considered important to evaluate the
313 effect of increasing Cys concentration during IVM on embryo development, performing
314 IVC in more or less oxidative conditions (20% or 5% oxygen tension, respectively). For
315 this assay, embryos were produced by IVF and results indicate that Cys concentration
316 during IVM can be increased up to 1 mM, with no detrimental effects on embryo
317 development when IVC is performed under 5% O_2 tension. Oppositely, their counterparts
318 cultured with 20% O_2 showed lower blastocyst rates and total cells number. This is
319 expected, since the physiologic environment where oocyte maturation and embryo
320 development take place are highly hypoxic [45]. When embryos are exposed to more
321 oxidative conditions, the electron donors and metabolic intermediates required for ATP
322 production are diverted to biosynthetic pathways supply and antioxidant defense
323 regeneration. Given that mitochondria are the only source of ATP for early embryos,
324 developmental competence becomes affected by the lower energy supply [46]. Regarding
325 to the maximum Cys concentration (10 mM) used during IVM, developmental rates
326 substantially decreased for both 5 and 20% O_2 tension culture conditions. This toxic effect
327 might be explained, at least in part, by the availability of NADPH. The activity of
328 glutathione reductase, which maintains most of glutathione molecules in its reduced state,
329 requires NADPH as electron donor. If the concentration of glutathione is excessive, the
330 availability of NADPH may not be enough for allowing glutathione reductase activity,
331 leading to the accumulation of glutathione in its oxidized form, thus increasing the
332 oxidative stress of the embryo. Moreover, glutathione transferases, which covalently link
333 reactive chemicals with GSH, cooperate with other systems for the detoxification of cells.
334 The excess of the components of one of these systems (such as GSH) might also affect the

335 functioning of the others, altering the redox state of the embryos, and even the expression
336 of certain genes [reviewed by 44].

337 In Experiment 2, the effect of co-incubation of sperm with COCs for 3 h (Co-cult) on
338 sperm acrosome membrane integrity was analyzed. Cumulus cells have been proposed to
339 generate a microenvironment that is beneficial for the fertilizing sperm, to prevent the entry
340 of abnormal sperm into the oocyte, and to enrich the sperm population with capacitated and
341 reacted spermatozoa [47, 30, 31]. Indeed, clinical studies indicate the use of cumulus cells
342 to select human spermatozoa before ICSI. It was reported that sperm populations that
343 manage to get through the cumulus cells show higher percentages of capacitated and
344 acrosome reacted sperm, beyond certain parameters of normality [48, 49]. In agreement
345 with those reports, our results show that the rates of live and acrosome reacted spermatozoa
346 increase after Co-cult treatment. Some authors have evidenced that the use of acrosome
347 reacted sperm for ICSI improves *in vitro* embryo development and implantation rates in
348 cattle and human, respectively [12, 28]. For all the exposed above, we employed the Co-
349 cult treatment for ICSI assay.

350 In Experiment 3, IVM was performed with 1 vs. 0.1 mM Cys and all ICSI embryos were *in*
351 *vitro* cultured under 5% O_2. In addition, IVM and IVC conditions were combined or not
352 with the injection of sperm subjected to Co-cult. Our results show that the Co-cult increases
353 cleavage rates of ICSI embryos, independently of IVM conditions. This is expected since
354 certain structures of the intact sperm head were observed to obstruct sperm chromatin
355 decondensation after ICSI [50]. Additionally, the Co-cult method restricts the sperm
356 selection to those that are able to interact and stay attached to cumulus cells, and to
357 maintain their motility for at least 3 h of incubation in a capacitating medium. Hence,

results from Experiments 2 and 3 suggest that the Co-cult treatment is not only enriching the sperm population with acrosome reacted ones, but also with those sperm that would be selected by the cumulus cells on an IVF protocol, which ultimately might increase cleavage rates. Nonetheless, Co-cult treatment only improved blastocyst rates when combined with the previous maturation of COCs with 1 mM Cys. These results could be explained by the protamin content in the bovine sperm nucleus. While protamines P1 and P2 are found in other species, only P1 is present in cattle, which displays higher affinity for DNA than P2 [51-53]. It provokes a greater degree of chromatin compaction that might interfere with the development of ICSI embryos. However, by employing high [Cys] during IVM, competent oocytes could increase their ability to synthesize GHS, facilitating the replacement of sperm protamins by histones during the male pronucleus formation, resulting in better embryo development. Following the line of this hypothesis, we can infer that even though several changes on sperm physiology are needed to allow early development of bovine ICSI embryos, other changes into the ooplasm must also occur, that are not well achieved by current IVM systems.

Finally, the incidence of Cys 1 ICSI and Cys 1 ICSI + Co-cult treatments on relative expression of eight genes from ICSI blastocysts was analyzed by qPCR. Treated ICSI groups were compared with ICSI and IVF control groups, for which COCs were matured with 0.1 mM Cys and spermatozoa were not subjected to any pre-treatment (standard conditions). No differences were observed for *INFτ, OCT4* and *DNMT1* expression between groups, which might reflect capability of pregnancy maintenance and inner cell mass/trophoectoderm number of cells ratio closer to those of IVF blastocysts [54-57, 6]. Arias et al. [6] reported overexpression of *CAT* and *SOD2* in ICSI bovine embryos. In our

hands, while ICSI control group overexpressed *SOD2*, this effect was reversed by both Cys 1 ICSI and Cys 1 ICSI + Co-cult treatments, reflecting a better regulated oxidative state of embryos [58]. A similar observation was detected for *HDAC1* and *HDAC2* expression, whereas *HDAC3* did not differ between groups. The expression of *HDAC* genes in ICSI bovine embryos had not been studied so far. They code for enzymes implicated in histone acethylation, which can induce transcriptional repression [59, 60]. Since level of these transcripts is expected to decrease after embryo genome activation [61], our results suggest an epigenetic profile less altered by *in vitro* production system for both ICSI treatments using 1 mM Cys concentrations.

4.1 Conclusions

The addition of high concentrations of Cys during IVM and co-incubation of sperm with COCs previous to injection improves *in vitro* development of ICSI bovine embryos, when IVC is performed under low O_2 tension. This work evidences the need to study the changes that both male and female gametes suffer before fertilization under physiological conditions. This would allow the design of novel protocols, not only for sperm preparation, but also for improving IVM and IVC, in order to facilitate the development of ICSI embryos in cattle.

Authorship

N.G.C. performed oocyte collection and *in vitro* maturation, IVF, ICSI, the experimental design, data analysis, and manuscript writing. M.S. and R.B. took part in ICSI protocols, study design, manuscript drafting, critical discussion and data analysis. N.G.C. and M.S.

403 performed sperm membrane integrity analysis. M.E.A. performed RNA extraction and
404 qPCR. M.E.A. and R.F. were involved in qPCR experimental design and analyzed the data.
405 D.S. was in charge of research coordination, contributed to the experimental design, critical
406 discussion and manuscript editing and submission. All the authors revised and accepted the
407 draft version of the manuscript.

408

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412

413 **Conflict of interest**

414 None.

415

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627 **Table 1.** Analyzed genes, sequence of specific primers, product size and access source in
628 GenBank.

Gene	Abbreviation	Primer sequence (5'-3')	Product size (bp)	GeneBank
Interferon tau	***IFNτ***[a]	TGGCCCTGGTGCTGGTCAGC TCATTCGGGCCAGGAGCCTG	108	XM_001250591.1
Catalase	***CAT***[b]	ACCCTCGTGGCTTTGCAG ACTCAGGACGCAGGCTCC	192	NM_001035386.1
Mn-superoxide dismutase of the mitochondria	***SOD2***[c]	ACCTCAACGTCGCCGAGG CCAACCGGAGCCTTGGAC	260	L22092.1
Dimethyltransferase 1	***DNMT1***[d]	CGCATGGGCTACCAGTGCACCTT GGGCTCCCCGTTGTATGAAATCT	312	X63692
Octamer-binding transcription factor 4	***OCT4***[e]	GGTTCTCTTTGGAAAGGTGTTC ACACTCGGACCACGTCTTTC	314	AF022987
Histone deacetylase 1	***HDAC1***[f]	GGCTCTGACTCCTTGTCTGG GCATAGGCAGGTTGAAGCTC	103	AY504948
Histone deacetylase 2	***HDAC2***[g]	ACAGGGTCATCCCATGAAAC TTCTTCGGCAGTGGCTTTAT	115	AY504949
Histone deacetylase 3	***HDAC3***[h]	ATCTGGATGGAGCGTGAAGT GTGGCTACACTGTCCGGAAT	137	AY504950
Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activating zeta protein	***YWHAZ***[i]	GCATCCCACAGACTATTTCC GCAAAGACAATGACAGACCA	120	BM446307
Glyceraldehyde 3-phosphate dehydrogenase	***GAPDH***[j]	GGAGCCAAACGGGTCATCATCTC GAGGGGCCATCCACAGTCTTCT	223	XM_583628

629 [a]Protein secreted by the trophoectoderm involved in establishment and maintenance of pregnancy [57].
630 [b,c]Mitochondrial enzymes with important role as antioxidant defense of the cells [44].
631 [d]Dimethyltransferase involved in epigenetic regulation of pluripotency and cell differentiation [61].
632 [e]Transcription factor involved in embryonic stem cell pluripotency [62, 63].
633 [f,g,h]Enzymes involved in transcriptional repression through histone acetilation [64, 65].
634 [i,J]Housekeeping genes used as a reference [6,66].
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640 **Table 2.** Effect of high cysteamine (Cys) concentrations during *in vitro* maturation on
641 development of bovine IVF embryos, using low or high O_2 tension.

O_2 tension	[Cys]	N	Cleavage (%)	Blastocysts (%)	Mean cell n° ± SD
Low (5%)	0.1 mM	139	115 (82.73)^{ac}	52 (37.41)^{a}	131±41 (n=22)^{a}
	0.5 mM	135	107 (79.26)^{ac}	44 (32.59)^{ab}	123±53 (n=43)^{a}
	1 mM	153	119 (77.78)^{ac}	63 (41.18)^{a}	120±42 (n=28)^{a}
	10 mM	116	15 (12.93)^{b}	4 (3.45)^{c}	85±20 (n=4)^{ab}
High (20%)	0.1 mM	140	107 (76.43)^{ac}	55 (39.29)^{a}	98±42 (n=32)^{ab}
	0.5 mM	158	135 (85.44)^{a}	64 (40.51)^{a}	99±39 (n=34)^{ab}
	1 mM	107	80 (74.77)^{c}	26 (24.3)^{b}	72±36 (n=18)^{b}
	10 mM	112	19 (16.96)^{b}	10 (8.93)^{c}	64±14 (n=10)^{b}

642 ^{a,b,c}: different superscripts in the same column indicate significant difference (Fisher's exact test, $p \leq 0.05$ for
643 cleavage and blastocyst rates; Kruskal-Wallis non-parametric test with Dunn's correction for multiple
644 comparisons, $p \leq 0.01$ for mean cell n° ± SD).

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647 **Table 3.** Effect of incubation with COCs on sperm acrosome membrane integrity.

Treatments	N	% Live, non reacted acrosome (PI-/PNA-)	% Dead, non reacted acrosome (PI+/PNA-)	% Live, reacted acrosome (PI-/PNA+)	% Dead, reacted acrosome (PI+PNA+)
Co-cult	271	16.6^{a}	21.03^{a}	27.31^{c}	35.06
BO 3h	361	21.6^{a}	35.46^{b}	6.93^{a}	36.01
Thawed	503	19.48^{a}	29.22^{b}	11.33^{b}	39.96
Triton X	439	31.44^{b}	68.56^{c}	-	-
Ionomicyn	537	18.62^{a}	-	81.38^{d}	-

648 ^{a,b,c,d}: different superscripts in the same column indicate significant difference (Fisher's exact test, $p \leq 0.05$).
649 Sperm previously incubated in BO medium for 3 h with (Co-cult) or without COCs (BO) or just thawed
650 (Thawed) and exposed to PI and PNA/FITC. Triton X: PI positive controls. Ionomycin: PNA/FITC positive
651 controls.

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Table 4. Effect of 1 mM cysteamine (Cys) during IVM and sperm co-incubation with COCs previous to ICSI over *in vitro* embryo development.

[Cys] on IVM	Groups	N	Cleavage (%)	Blastocysts (%)
1 mM	**ICSI + Co-Cult**	116	92 (79.31)[a]	27 (23.28)[a]
	ICSI	108	56 (51.85)[b]	19 (17.59)[b]
0.1 mM	**ICSI + Co-Cult**	117	107 (91.45)[c]	13 (11.11)[b]
	ICSI	132	79 (59.85)[b]	18 (13.64)[b]
0.1 mM	**Sham**	106	94 (88.68)[c]	22 (20.75)[ab]
(controls)	**PA**	144	138 (95.83)[c]	88 (61.11)[c]

[a,b,c]: different superscripts in the same column indicate significant difference (Fisher's exact test, $p \leq 0.05$).
1 mM and 0.1 mM groups: COCs were IVM in the presence of 1 mM or 0.1 mM Cys (standard condition) before ICSI. ICSI + Co-cult groups: sperm were co-incubated with COCs for ≥ 3 h before intracytoplasmic injection. Sham: oocytes injected with an equivalent volume of the medium used for spermatozoa and activated with Io+3h+DMAP. PA: oocytes activated with Io+DMAP (parthenogenetic control).

Figure captions

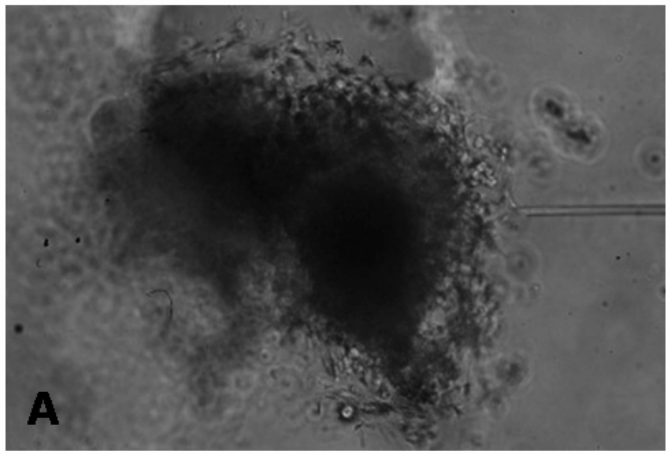
Figure 1. Micromanipulation technique for the production of ICSI embryos with sperm previously co-incubated with COCs (Co-cult). A) Selection of sperm attached to cumulus cells (100X magnification). B) Immobilization of sperm with the injection pipette (200X). C) Mature oocyte prior to be injected with the selected sperm (200X).

Figure 2. COCs after 21 h of *in vitro* maturation under increasing Cys concentrations.

Figure 3. Sperm acrosome membrane integrity assay. Sperm previously incubated in BO medium for 3 h with (Co-cult) or without COCs (BO) or just thawed (Thawed) and exposed to PI (red) and PNA/FITC (green). A) Sperm observed under bright field. The same sperm observed by fluorescence microscopy, positive for PI staining (B) and PNA/FITC probe (C). 200X magnification.

Figure 4. ICSI blastocysts at day 7 of *in vitro* culture. Cys 1 ICSI: oocytes were IVM with 1 mM Cys before sperm injection. Cys 0.1 ICSI + co-cult: oocytes were IVM with 0.1 mM Cys and sperm were co-incubated with COCs for ≥ 3 h before sperm injection. Cys 0.1 ICSI: oocytes were IVM with 0.1 mM Cys before sperm injection (control). Cys 1 ICSI + co-cult: oocytes were IVM with 1 mM Cys and sperm were co-incubated with COCs for ≥ 3 h before sperm injection. 40X magnification.

Figure 5. Relative quantification of mRNA by qPCR from ICSI blastocysts. [a,b,c]: different superscripts indicate significant difference (one-way ANOVA and LSD test, $p \leq 0.05$). IFV: *in vitro* fertilized oocytes. White bars: fertilized/injected oocytes previously subjected to IVM in the presence of 0.1 mM Cys (controls). Black bars: injected oocytes previously subjected to IVM in the presence of 1 mM Cys. Co-cult: sperm co-incubated with COCs for ≥3 h before ICSI.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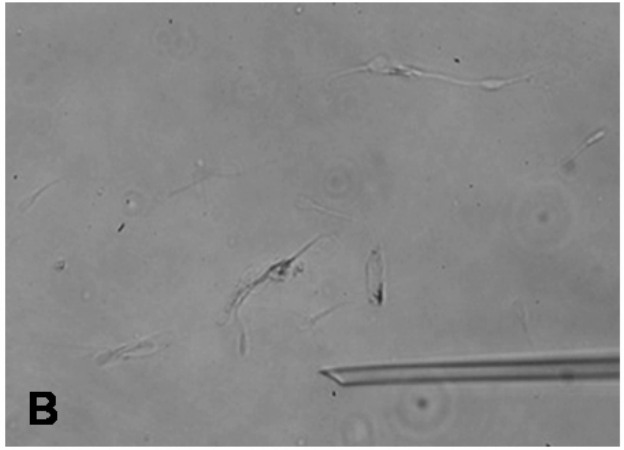
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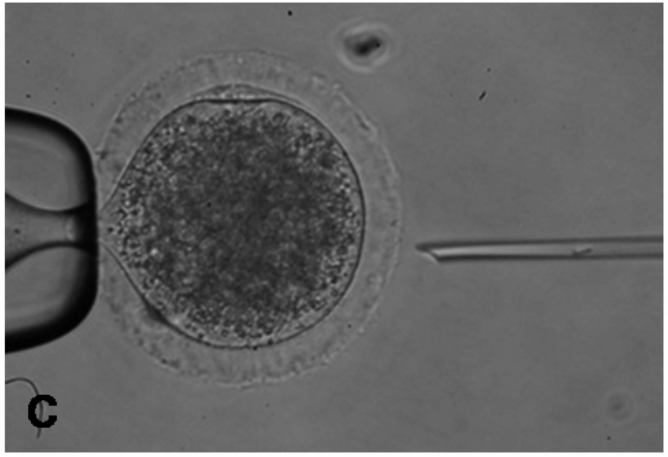
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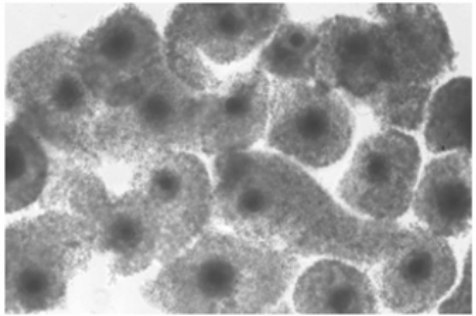
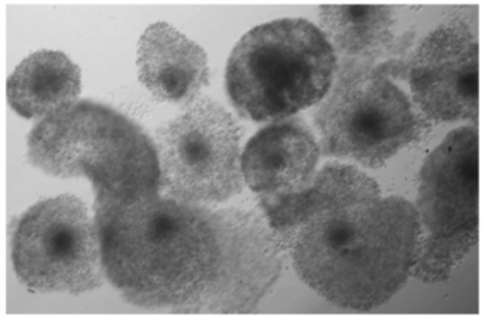
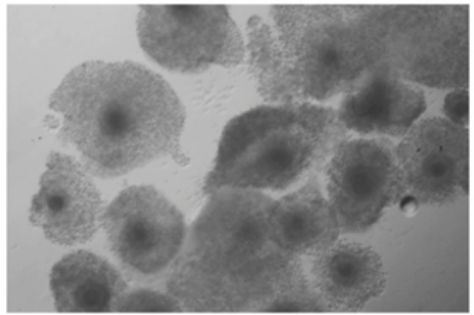


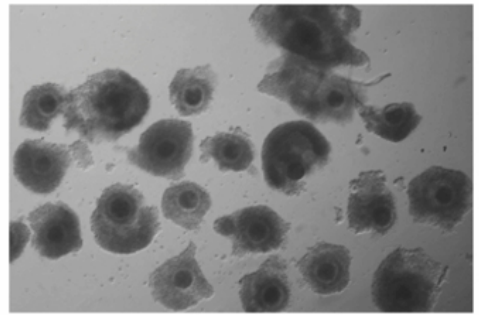
Cys 0.1 mM



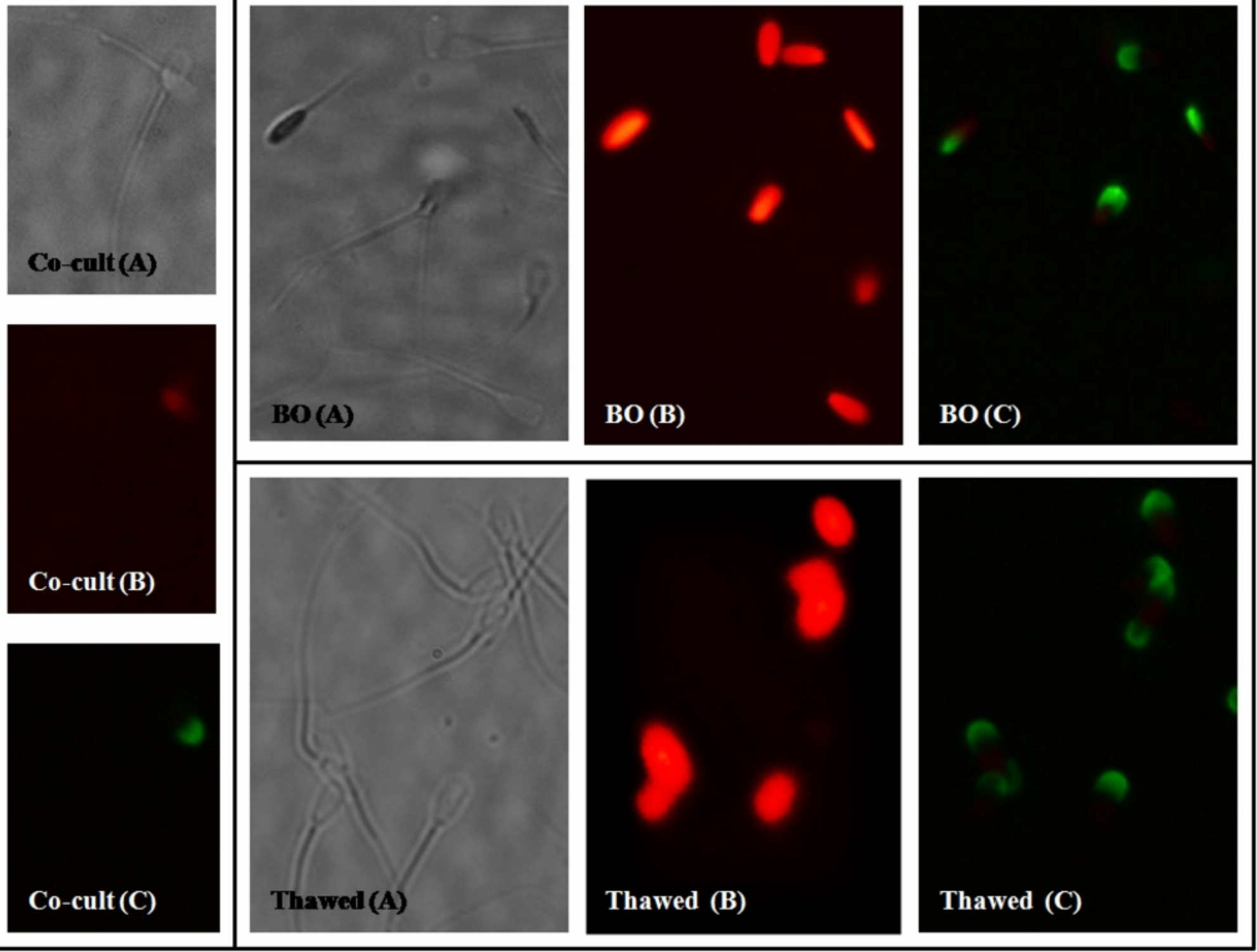
Cys 0.5 mM

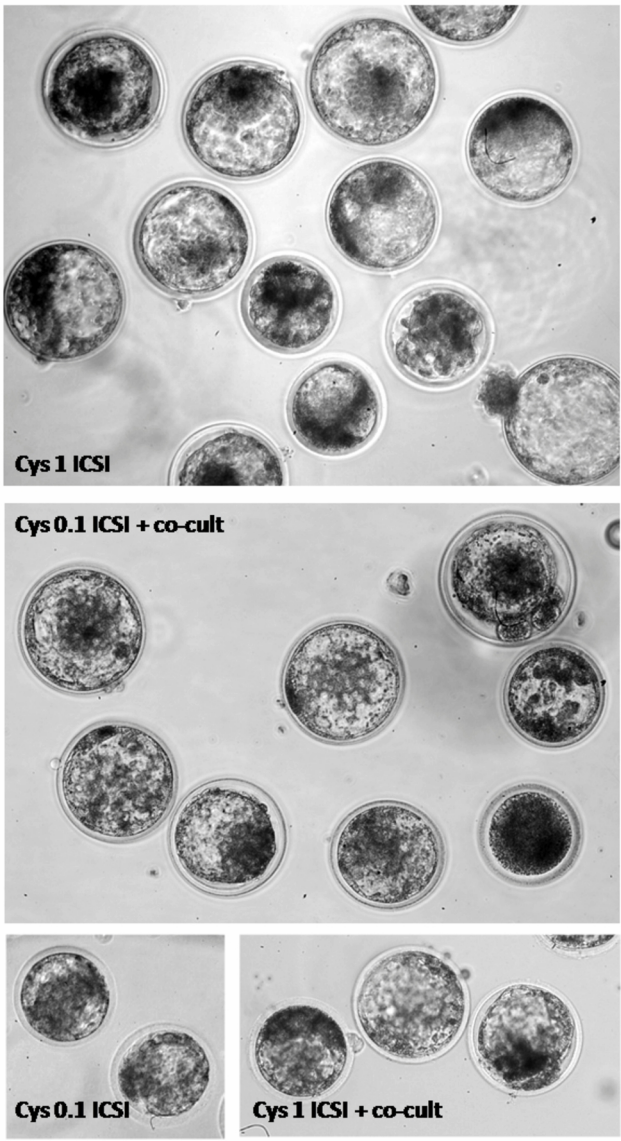


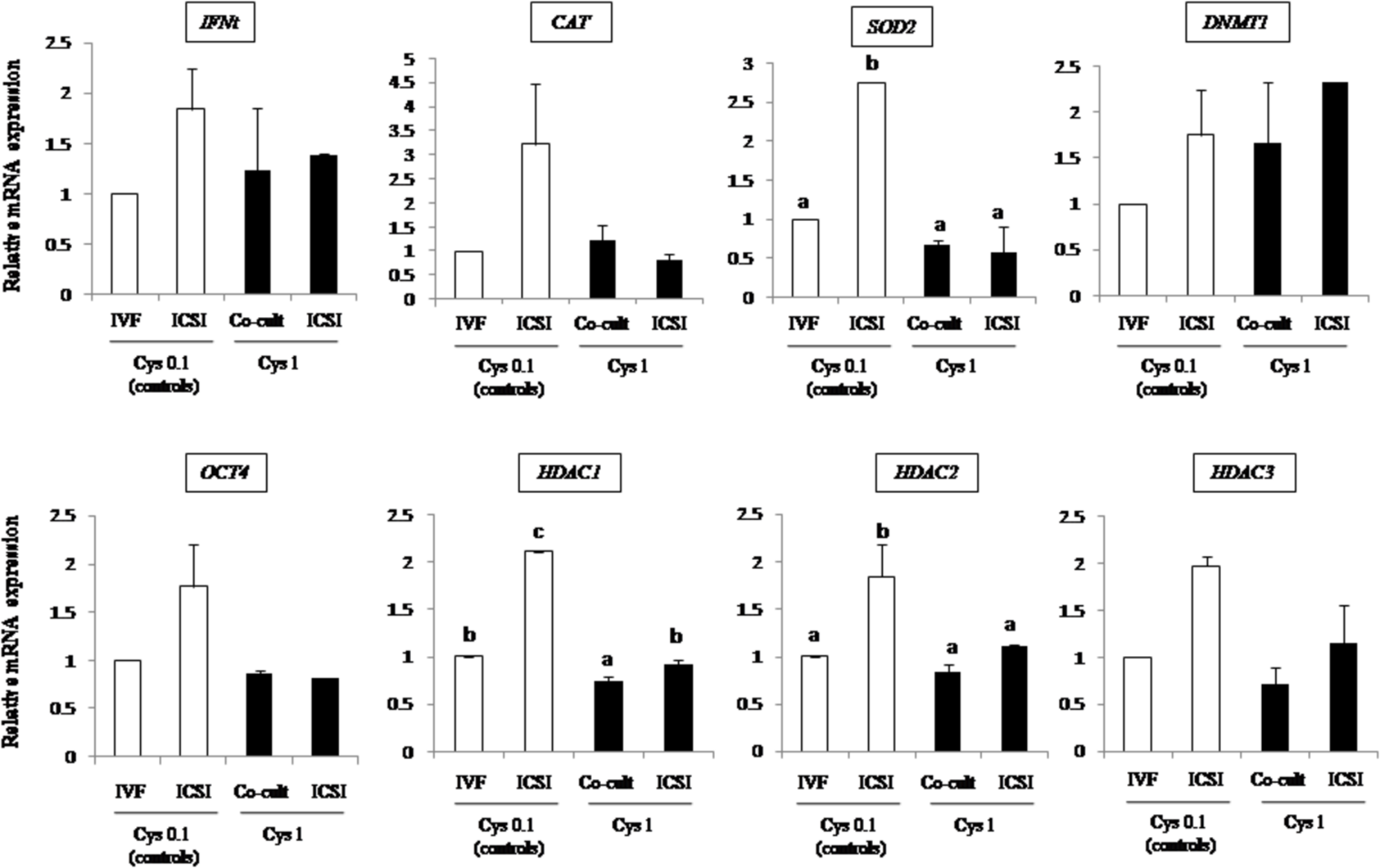
Cys 1 mM



Cys 10 mM


Co-cult (A)
Co-cult (B)
Co-cult (C)
BO (A)
BO (B)
BO (C)
Thawed (A)
Thawed (B)
Thawed (C)


Cys 1 ICSI
Cys 0.1 ICSI + co-cult
Cys 0.1 ICSI
Cys 1 ICSI + co-cult


IFNt
CAT
SOD2
DNMT1
OCT4
HDAC1
HDAC2
HDAC3
Relative mRNA expression
IVF
ICSI
Co-cult
ICSI
Cys 0.1 (controls)
Cys 1
a
b
c