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Male pronucleus formation after ICSI: effect of oocyte cysteine or sperm Triton X-100 treatments

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ABSTRACT: In pigs, intracytoplasmic sperm injection (ICSI) efficiency is still poor. The inadequate decondensation of the sperm chromatin, its transformation into the male pronucleus (MPN) together with the subsequent inability to activate the oocyte, seem to be the main causes of the low ICSI efficiency. In order to improve the MPN formation we took two different approaches. On the one hand, the *in vitro* culture (IVC) medium post-ICSI was supplemented with 1.71mM cysteine (CYS). Alternatively, the sperm membrane was digested with Triton X-100 (TX) before ICSI, to improve the exposure of the sperm chromatin to the oocyte cytoplasm. After 6 h post-ICSI, the activation rate was significantly higher in TX group (70.0%) compared with CYS and control groups (42.2% and 48.9%, respectively; $P < 0.05$). However, no significant differences between the three groups were observed in terms of the number of pronuclei, 2PN (oocytes with 2 pronuclei and no visible sperm), and 1PN + sperm (oocytes with 1 pronucleus and one sperm head). At 22 h post-ICSI, the activation rates were similar in TX, CYS, and control groups (73.1, 78.9, and 75.7%, respectively). In addition, we did not observe significant differences between TX, CYS, and control groups for the number of pronuclei, 2PN (52.6, 56.7, and 50%, respectively) or 1PN + sperm (21.1, 33.3, and 32.1%, respectively). While no cleavage was observed in the CYS group, no significant differences in the cleavage rate were observed between control (21.3%) and TX (10.5%) groups. In summary, and under our conditions, neither CYS supplement, nor sperm TX pre-treatment were able to improve MPN formation at 6 and 22 h post-ICSI. However, the sperm TX pre-treatment improved oocyte activation at 6 h post-ICSI, although 22 h post-ICSI such a beneficial effect did not persist.

Keywords: porcine; assisted reproductive technology; pronuclear formation

List of abbreviations: ICSI = intracytoplasmic sperm injection, PN = pronucleus, MPN = male pronucleus, IVC = *in vitro* culture, CYS = cysteine, TX = Triton X-100, IVP = *in vitro* production, IVM = *in vitro* maturation, IVF = *in vitro* fertilization, EGF = epidermal growth factor, GSH = glutathione, COC = cumulus oocyte complex, PZM-3 = porcine zygote medium-3, MM199 = maturation medium, HM199 = handling medium

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INTRODUCTION

In porcine species, *in vitro* production (IVP) technologies have been widely applied and improved over the last four decades (Grupen 2014). The ability of porcine oocytes to adequately develop after *in vitro* maturation (IVM) and fertilization (IVF), leading to live piglets, has been largely demonstrated by several laboratories (Kikuchi et al. 2002; Yoshioka et al. 2002, 2003; Suzuki et al. 2004, 2006; Coy et al. 2005; Garcia-Rosello et al. 2006a; Yong et al. 2006; Maehara et al. 2012; Kaneko et al. 2013). Despite this fact, the efficiency of IVP of blastocysts after intracytoplasmic sperm injection (ICSI) still remains low (Garcia-Rosello et al. 2009). The frequently inadequate sperm chromatin decondensation and its transformation into the male pronucleus (MPN) together with a failure to activate the oocyte seem to be the major causes behind the poor ICSI efficiency (Kren et al. 2003; Lee et al. 2003; Lee and Yang 2004).

There are several oocyte and sperm factors as well as conditions in which ICSI is performed, that could have a detrimental effect on the developmental ability of the resulting embryos (Garcia-Rosello et al. 2006b; Alfonso et al. 2009; Garcia-Mengual et al. 2011; Cheng et al. 2012). Related to the oocyte factors, the cytoplasmic environment is crucial to ensure the decondensation of the sperm head and then the MPN formation (Nagai 2001; Niemann and Rath 2001). Therefore, having a proper IVM for pig oocytes may guarantee, not only nuclear, but also the cytoplasmic competence required for full-term development.

In this regard, several IVM approaches have been applied, such as oocyte co-culture with follicular cells (Zheng and Sirard 1992; Ding and Foxcroft 1994; Agung et al. 2010), or supplement addition to the IVM defined media with: porcine follicular fluid (Naito et al. 1988; Yoshida et al. 1992; Ka et al. 1997), follicular fluid meiosis-activating sterol (Faerge et al. 2006), bovine serum albumin (Zheng and Sirard 1992), fetal bovine serum (Kishida et al. 2004), fetal calf serum (Naito et al. 1988), epidermal growth factor (EGF) (Ding and Foxcroft 1994; Uhm et al. 2010), β-mercaptoethanol (Abeydeera et al. 1998), cysteine (CYS) (Yamauchi and Nagai 1999; Yoshioka et al. 2003; Kobayashi et al. 2007; Choe et al. 2010), ascorbic acid (Tatemoto et al. 2001), leptin (Craig et al. 2005; Jin et al. 2009) or lycopene (Watanabe et al. 2010), among others.

Particularly interesting is the fact that the addition of CYS to IVM medium in pig oocytes results in increased glutathione (GSH, a derivative product of CYS) synthesis by the pig oocytes (Yoshida et al. 1993), enhancing *in vitro* development after IVF (Grupen et al. 1995; Kishida et al. 2004). In addition, GSH synthesis during oocyte maturation is an important factor for promoting their ability to form a MPN after IVF (Yoshida et al. 1993; Sawai et al. 1997), and GSH is known to be a prerequisite to ensure sperm chromatin decondensation (Perreault et al. 1984). Accordingly, porcine IVM media have routinely been supplemented with CYS. However, and contrarily to what happens after IVF, MPN formation of porcine oocytes *in vitro* matured with CYS is not improved after ICSI. The persistence of an intact sperm acrosome, plasma membrane and perinuclear theca (Katayama et al. 2002a, 2005), which are normally removed during sperm penetration in IVF and natural fertilization (Yanagimachi 1994), may be some of the factors causing an incorrect MPN formation in ICSI.

Regarding to embryo culture conditions, Katayama et al. (2007) observed improved fertilization rates after ICSI by increasing the CYS concentration in the *in vitro* culture (IVC) medium (up to 1.71mM, beyond the levels of the most commonly used concentration of 0.57mM CYS (Yoshioka et al. 2002; Petters and Wells 1993)), for the first 3–12 h post-ICSI. Therefore, our first efforts were focused on increasing the CYS levels in the IVC medium during the first 6 h post-ICSI.

Regarding to the strategies applied on the sperm to improve fertilization and embryo development rates after ICSI, several sperm pre-treatments have been tested to disrupt or even remove the plasma membrane and allow a more direct interaction with the oocyte cytoplasm. Among the mechanical pre-treatments, sonication (Kim et al. 1999) and repetitive freezing/thawing without cryoprotectants (Katayama et al. 2002b) have been reported to improve MPN formation after ICSI in porcine. With the same goal, chemical pre-treatments have also been tested, such as exposure to progesterone (Katayama et al. 2002b), incubation with Triton X-100 (TX) to induce membrane damage and dissolve nuclear proteins (Lee and Yang 2004; Tian et al. 2006), or dithiothreitol treatment to reduce disulfide bonds (Yong et al. 2005; Cheng et al. 2009). Although in a previous work we observed an improvement in MPN formation after ICSI with

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TX pre-treated sperm (Garcia-Mengual et al. 2011), the results on embryo *in vitro* development are controversial (Nakai et al. 2011). In particular, we observed an improvement in the MPN formation 18 h post-ICSI, however this effect was not observed on cleavage nor embryo development rates. Therefore in the present study we aim at deciphering the effects of sperm TX pre-treatment before ICSI on MPN formation, but this time assessing it at 6 and 22 h post-ICSI. We based our time to PN formation assessing selection in a previous report (Jin et al. 2009), which assessed PN formation at 6 h post-ICSI and observed an acceleration in PN formation after leptin treatment. We then selected 22 h in order to ensure that all zygotes have had enough time to reach PN timing.

In summary, the present study was conducted to investigate the effect of CYS supplementation in the IVC medium post-ICSI on MPN formation. Alternatively, we also studied the effect of exposing the sperm chromatin to the oocyte cytoplasm by means of sperm membrane elimination with TX before ICSI.

MATERIAL AND METHODS

In vitro maturation and embryo culture. Oocytes obtained by antral follicle puncture of ovaries collected from pre-pubertal gilts at local abattoir were IVM as described by Silvestre et al. (2007). Briefly, 50–60 cumulus oocyte complex (COCs) were cultured for 44 h in 500 µl maturation medium (MM199) consisting of medium 199 (M4530) supplemented with 0.1% (w/v) polyvinyl alcohol (P8136), 0.57mM CYS, and 10 ng/ml EGF and antibiotics (all Sigma-Aldrich Quimica, Madrid, Spain) (Abeydeera et al. 2000), in a Nunc 4-well multidish (Nunc, Roskilde, Denmark). During the first 22 h, COCs were cultured in MM199 supplemented with hormones (0.1 IU/ml recombinant human FSH and LH (Naito et al. 1988) (Gonal-F and Luveris, respectively; Serono, Madrid, Spain). After that, COCs were washed twice and cultured in hormone-free MM199 for additional 22 h. After IVM and ICSI, embryos were cultured in porcine zygote medium-3 (PZM-3) (Yoshioka et al. 2002). Both oocyte IVM and zygote culturing took place in humidified atmosphere with 5% O_2 and 5% CO_2 at 38.5°C.

Sperm pre-treatment. Sperm doses were supplied by a porcine artificial insemination centre and stored at 17°C. Semen sample was washed with handling medium (HM199), consisting of medium 199 supplemented with 25mM Hepes (M7528; Sigma-Aldrich Quimica), supplemented with 7.4% (v/v) heat-inactivated foetal bovine serum (10108-157, GIBCO®, Life Technologies, Madrid, Spain) and antibiotics, and submitted to light centrifugation (50 *g* for 3 min). For TX sperm pre-treatment, the upper fraction of the semen was put aside and 0.1% TX (T8787, Sigma-Aldrich Quimica) was added (Tian et al. 2006; Garcia-Mengual et al. 2011). Subsequently, the semen with TX was vortexed and washed with HM199. Then, semen was centrifuged for 5 min and the resulting pellet was resuspended in HM199.

Intracytoplasmic sperm injection. ICSI was performed in an inverted microscope with attached micromanipulators as described by Alfonso et al. (2009). Briefly, metaphase II (MII) cumulus-denuded oocytes were distributed over 6 HM199 microdrops placed surrounding central drops containing spermatozoa in 10% polyvinylpyrrolidone solution, all covered with mineral oil (both Sigma-Aldrich Quimica). One single sperm was immobilized by rubbing the midpiece with the injection pipette and aspirated by the tail. Oocytes were fixed by the holding pipette so that the first polar body was set in 6 or 12 o'clock position. Prior to sperm injection, a small amount of ooplasm was aspirated into the injection pipette. Temperature was maintained at 38.5°C throughout the whole micromanipulation process using a heated microscopic stage.

Cysteine treatment. The presumptive zygotes were cultured in PZM-3 supplemented with 1.71mM CYS (L-cysteine, Sigma-Aldrich Quimica) for the first 6 h of culture after sperm injection (Katayama et al. 2007).

Experimental design. After IVM, MII oocytes were randomly distributed into three experimental groups: CYS (oocytes were injected with control sperm (non pre-treated) and cultured in PZM-3 supplemented with CYS 1.71mM for 6 h. Presumptive zygotes were then washed twice and cultured in PZM-3 until 22 h of culture); TX (oocytes were injected with TX pre-treated sperm, and cultured in PZM-3 until 22 h of culture); Control (C) (oocytes were injected with control sperm and cultured in PZM-3 until 22 h of culture).

Assessment of nuclear maturation and pronuclear formation. After IVM, COCs were briefly incubated in HM199 supplemented with 1 mg/ml

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bovine testes hyaluronidase (H4272; Sigma-Aldrich Quimica) and then gently pipetted. Oocytes were evaluated under a stereomicroscope, and those with the presence of the first polar body were classified as nuclearly matured oocytes. In order to assess the pronuclear formation, half of the cultured presumptive zygotes were fixed in absolute ethanol and stained with Hoechst 33342 (10 mg/ml in DPBS) (B2261, D8662; Sigma-Aldrich Quimica) at 6 h post-ICSI, then mounted with glycerol on microscope slides. The other half of the embryos remained in the culture and was evaluated at 22 h post-ICSI. Pronuclei were scored under an epifluorescence microscope at 63× magnification.

Activation of injected oocytes was evaluated by the ability of the oocytes to resume meiosis, with no metaphase plate visible and with at least one pronucleus. The activated oocytes were classified into 4 groups based on our previous score (Garcia-Mengual et al. 2011): 2PN – oocytes with 2 pronuclei and no visible sperm; Total 1PN + sperm – oocytes with 1 pronucleus and one sperm head (condensed or decondensed); Cleaved – 2-cell embryo stage; Others – oocytes with other nuclear structures as more than 2 pronuclei or non-analyzable oocytes.

Statistical analysis. Four replicates were performed per treatment. Activation and pronuclear formation rate were compared between the three groups using a Chi-square test analysis. Differences were considered statistically significant at $P < 0.05$. When a single degree of freedom was involved, the Yates' correction for continuity was carried out.

RESULTS

The zygotes in TX group showed significantly higher activation rate after 6 h post-ICSI (70.0%) than those treated with CYS and the control group (42.2 and 48.9%, respectively; $P < 0.05$) (Table 1). However, no significant differences were observed in 2PN, and Total 1PN + sperm, nor in 1PN + decondensed sperm rates between the three groups. Rates of 2PN from activated oocytes ranged from 13.6 to 21.4%, whereas Total 1PN + sperm rates varied from 60.7 to 81.8% and 1PN + decondensed sperm from 38.9 to 57.1% (Table 1).

When the activation rates were assessed at 22 h post-ICSI, the results were similar between all three groups (73.1, 78.9, and 75.7%, respectively) (Table 2). In addition, we did not observe significant differences between TX, CYS, and control groups for 2PN (52.6, 56.7, and 50%, respectively) nor for Total 1PN + sperm (21.1, 33.3, and 32.1%, respectively) and 1PN + decondensed sperm (50.0, 30.0, and 44.4%, respectively) (Table 2). The cleavage rate was not significantly different between control (21.3%) and TX (10.5%) groups, nor between TX and CYS groups. No cleaved embryos were observed in the CYS group. The cleavage rate in the control was higher than in the CYS group ($P < 0.05$) (Table 2).

DISCUSSION

The developmental competence of zygotes generated by the ICSI technique in pigs is still low

Table 1. Effect of cysteine oocyte treatment (CYS) and sperm Triton X-100 pre-treatment (TX) on pronuclear formation of sperm injected oocytes, assessed at 6 h post-intracytoplasmic sperm injection

Treatment	No. of injected oocytes	No. of activated oocytes[1] (%)	Groups of activated oocytes (%)[1]				
			2PN	Total 1PN + sperm	Decondensed sperm[2]	Cleaved	Others[3]
TX	40	28 (70.0)[a]	6 (21.4)	17 (60.7)	8 (47.1)	0	5
CYS	45	19 (42.2)[b]	4 (21.1)	14 (73.7)	8 (57.1)	0	1
Control	45	22 (48.9)[b]	3 (13.6)	18 (81.8)	7 (38.9)	0	1

2PN = oocytes with 2 pronuclei and no visible sperm, Total 1PN + sperm = oocytes with 1 pronucleus and 1 condensed or decondensed sperm head, Cleaved = 2-cell embryo stage, Others = oocytes with other nuclear structures as 2 pronuclei and one sperm, 3 pronuclei or no analyzable oocytes

[1]activated indicates oocytes resuming meiosis, with no metaphase plate visible and at least with 1 pronucleus

[2]from Total 1PN + sperm (condensed + decondensed)

[3]data were not statistically analyzed

[a,b]values with different superscript in the same column are significantly different (Chi-square $P < 0.05$)

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Table 2. Effect of cysteine oocyte treatment (CYS) and sperm Triton X-100 pre-treatment (TX) on pronuclear formation of sperm injected oocytes, assessed at 22 h post- intracytoplasmic sperm injection

Treatment	No. of injected oocytes	No. of activated oocytes[1] (%)	Groups of activated oocytes (%)[1]				
			2PN	Total 1PN + sperm	Decondensed sperm[2]	Cleaved	Others[3]
TX	26	19 (73.1)	10 (52.6)	4 (21.1)	2 (50.0)	2 (10.5)[ab]	3
CYS	38	30 (78.9)	17 (56.7)	10 (33.3)	3 (30.0)	0 (0)[a]	3
Control	37	28 (75.7)	14 (50.0)	9 (32.1)	4 (44.4)	6 (21.3)[b]	2

2PN = oocytes with 2 pronuclei and no visible sperm, Total 1PN + sperm = oocytes with 1 pronucleus and 1 condensed or decondensed sperm head, Cleaved = 2-cell embryo stage, Others = oocytes with other nuclear structures as 2 pronuclei and one sperm, 3 pronuclei or no analyzable oocytes

[1]activated indicates oocytes resuming meiosis, with no metaphase plate visible and at least with 1 pronucleus

[2]from Total 1PN + sperm (condensed + decondensed)

[3]data were not statistically analyzed

[a,b]values with different superscript in the same column are significantly different (Chi-square $P < 0.05$)

(Garcia-Rosello et al. 2009). Specifically, the failure in MPN formation has been proposed as the main reason behind the low ICSI efficiency (Lee et al. 2003). Related to this, it has been suggested that the GSH may play an important role in fertilization but also in embryonic development (Maedomari et al. 2007). It is speculated that the GSH may exert a promoting effect on MPN formation by acting synergistically in the following ways: GSH promotes the disruption of the disulfide bond of the protamine in the sperm chromatin by shifting the oocyte cytoplasm into a redox state; and/or the GSH acts as a substrate of glutathione peroxidase and as a scavenger of free radicals in oocytes, enhancing their competence as a whole (Nagai 1996). Also CYS is a critical amino acid of GSH, the availability of which is crucial for ensuring GSH synthesis (Meister and Tate 1976).

Unfortunately, under our culture conditions, we observed that the addition of CYS did not significantly increase MPN formation as assessed neither at 6 h nor at 22 h post-ICSI. In a previous similar experiment, Katayama et al. (2007) hypothesized that the disassembly of the sperm plasma membrane in the ooplasm may be promoted by maintaining high levels of GSH in ooplasm, and this could be achieved by increasing the CYS supplement in the IVC medium. Despite they reported that the supplement of CYS in the culture medium did not affect the intracellular levels of GSH in cumulus-free matured oocytes after the ICSI, they observed improved fertilization rates after *in vitro* culture of ICSI derived zygotes in 1.71 and 3.71mM CYS for 3 to 12 h. In this sense, Li et al. (2014) observed significantly higher rates of cleavage (80.7%) and blastocyst formation (22.5%) in the culture medium supplemented with 1.71mM CYS than in other CYS-supplemented groups. Katayama et al. (2007) suggested that these improvements may be the result of better utilization of GSH by CYS-treated ICSI zygotes, which was consistent with improved MPN development as they could consume much of the ooplasmic GSH after fertilization. The discrepancy between our study and that of Katayama et al. (2007) in 2PN rate could lie in some technical details: while IVM medium and conditions were the same in both studies, and the IVC medium was supplemented with the same amount of CYS (1.71mM), we used PZM-3 (which also includes antioxidants (e.g. taurine and hypotaurine; Petters and Wells 1993)). Also, the benefits induced by CYS reported by Katayama et al. (2007) may have been conditioned by combining CYS with the use of piezo pulses for sperm-immobilization to disrupt the sperm plasma membrane, which it is known to improve the fertilization rates and the decondensation of the sperm chromatin after ICSI (Katayama et al. 2005). It should be highlighted that in our study the CYS supplement was tested alone, without any other sperm membrane disruptor than the sperm mid-piece rubbing step during ICSI (Garcia-Mengual et al. 2011).

Triton X-100 is an anionic detergent widely applied to disrupt and remove the sperm mem-

doi: 10.17221/8237-CJAS

brane prior to ICSI with the purpose to expose the naked sperm chromatin to the oocyte cytoplasm and promote the MPN formation (Lee and Yang 2004; Tian et al. 2006; Garcia-Mengual et al. 2011). However our attempt to assist the sperm chromatin decondensation, by disrupting or even removing the sperm membrane using TX pre-treatment, did not result in a significant improvement in MPN formation. The significantly higher activation rate observed at 6 h post-ICSI in TX group suggests a "faster" oocyte activation and MPN formation. However this result did not translate in significantly higher 2PN rate, nor Total 1PN + sperm rate, nor 1PN + decondensed sperm rate. When we assessed the activation rates at 22 h post-ICSI, TX and control groups showed similar results. On the other hand, the control group showed the highest cleavage rate, and this was similar to that in TX group and higher than in CYS group. In bovine, the timing of the first cleavage is crucial to ensure further *in vitro* embryo development, giving the embryos that cleaved earlier more chance to reach blastocyst stage (Lonergan et al. 1999). In our previous experience (Garcia-Mengual et al., 2011), when MPN formation was assessed at 18 h post-ICSI, oocytes injected with TX pre-treated sperm showed a significantly higher rate of MPN formation than the controls while no differences in *in vitro* embryo development, cleavage or blastocyst rates were observed. However, several reports showed that TX treatment did not improve 2PN formation, nor embryo development (Szczygiel 2002; Tian et al. 2006; Nakai et al. 2011). In this sense Parrilla et al. (2012) showed that ejaculate spermatozoa from individual boars can respond in a boar-dependent manner to different semen-processing techniques. It could be then hypothesized that different qualitative characteristics, such as DNA fragmentation index (Lopez-Fernandez et al. 2008) of sperm samples, could be compromising the TX pre-treatment reproducibility within different boar samples and could be the basis of the different fertilization rates obtained from different males, since DNA fragmentation in boar sperm has been correlated with poor fertility outcomes (Boe-Hansen et al. 2008; Didion et al. 2009).

Moreover, in further experiments it could be interesting to study the effect of the TX treatment in later embryo development stages.

In summary, in our conditions, neither the CYS supplementation on IVC medium, nor the sperm TX pre-treatment before ICSI improved MPN formation when assessed at 6 and 22 h post-ICSI. However, the sperm TX pre-treatment improved oocyte activation when assessed at 6 h post-ICSI, which could suggest different activation timing.

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