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Association between three glycosidases activity [α-mannosidase (α-MAN), β-N-acetyloglucosaminidase (NAGASE) and β-galactosidase (β-GAL)] and *in vitro* fertilization of bovine oocytes collected from different-sized follicles

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Abstract

We studied the role of three glycosidases (α-mannosidase - α-MAN, β-N-acetyloglucosaminidase - NAGASE and β-galactosidase - β-GAL) in follicular fluid (FF) and in fertilization medium (FM) of bovine oocytes. Oocytes were allocated into 3 groups according to the follicular size (controls - CF: 2-8 mm, small follicle group - SF: 2-5 mm, large follicle group - LF: >5-8 mm). Bovine embryos were produced *in vitro* either in groups (experiment 1, n = 2099 oocytes) or individually (experiment 2, n = 79 oocytes). In both experiments, the activity of all glycosidases in the FF of large follicles was significantly lower than in the FF of small follicles group. In the FM of LF-group oocytes, α-MAN and NAGASE were significantly higher compared to SF- and CF-group oocytes (experiment 1) and β-GAL was significantly higher in SF- compared to CF-group oocytes (experiment 2). Cleavage rate was similar among all groups in both experiments; however significantly higher blastocyst formation was noted in CF-group compared to LF- (days 7, 8, 9) and SF- (days 8, 9) groups (experiment 1). In follicular fluid of small follicle group, β-GAL was associated positively with degenerating oocytes' number and negatively with blastocyst rate at days 7, 8 (P = 0.065) and 9 (experiment 1). In fertilization medium of control group, α-MAN related negatively to cleavage rate (P < 0.05) and β-GAL to blastocyst rate at day 8 (P = 0.089) or day 9 (P = 0.072) (experiment 1). During fertilization, in experiment 1 all oocytes consumed β-GAL and only control or small follicle oocytes consumed α-MAN; in experiment 1, only large follicle oocytes released NAGASE, whereas all oocytes released all three glycosidases in experiment 2. In conclusion, glycosidases affect the developmental competence of oocytes collected from different sized follicles during *in vitro* fertilization, performed either in groups or individually; their role in follicular fluid is different from that in fertilization medium.

Keywords: fertilization medium, follicular fluid, glycosidases, *in vitro* embryo production.

Introduction

In vivo, oocytes and early embryos are in a suitable microenvironment, where surrounding fluids and tissues provide them with the required substrates for their proper development. Glycosidases are enzymes that might modify glycoprotein or glycolipid structure, affecting the zona pellucida and most cell membranes (Jeanloz, 1971; Winzler, 1972). One of them, NAGASE has been identified as participating in the sperm - oocyte interaction in several species (mainly hamster, mouse, human); although the specific stage is still unclear specifically at what stage (Zitta *et al*., 2006). Oocyte cortical granule NAGASE is released at fertilization and could participate in the block to polyspermy, since it inactivates the sperm 1,4-galactosyltransferase binding sites accounting for the block in sperm binding to the zona pellucida (Miller *et al*., 1993a). It has been suggested that it can also inactivate sperm receptors on oocytes (Lambert, 1989). Acrosomal NAGASE is released after initial sperm - oocyte binding and facilitates sperm penetration through the zona pellucida matrix in mice, presumably by removing the binding site for a sperm adhesion molecule on the oocyte coat (Miller *et al*., 1993b). It was found that alpha-D-mannose, N-acetylglucosamine and beta-D-galactose residues are all present in different amounts and distributions in the zona pellucida of pig oocytes, and beta-D-galactose and alpha-D-mannose residues appear to play important roles in initial sperm binding and subsequent penetration through the zona pellucida (Song *et al*., 2000). Galactose residues have been described previously in the bovine zona pellucida (Katsumata *et al*., 1996; Parillo *et al*., 2000; Ikeda *et al*., 2002). The oviductal beta-D-galactosidase and mannosidase may modify galactose and mannose residues contained in zona pellucida glycoproteins, affecting interaction with spermatozoa (Carrasco *et al*., 2008). Furthermore, in previous studies, we have found that embryos release both NAGASE and α-MAN during their development *in vitro*; whereas degenerated embryos release less NAGASE and α-MAN compared to good embryos (Tsiligianni *et al*., 2006). Therefore, it is reasonable to assume that glycosidases specific to these sugar residues may play a role in the permission or inhibition of processes that involve movement through zona pellucida such as sperm penetration and thus fertilization and developmental competence.

There is limited available data on the presence of glycosidases in bovine follicular fluid and their possible role during *in vitro* fertilization of bovine

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oocytes. Moreover, bovine oocytes recovered from small follicles develop to blastocyst stage *in vitro* at a significantly lower rate than their counterparts from larger follicles (Lonergan *et al*., 1994; Blondin and Sirad, 1995; Hagemann *et al*., 1999; Racedo *et al*., 2008). Therefore, oocytes isolated from follicles of different sizes may serve as a model to study factors involved in the acquisition of developmental competence. Having in mind all the above, this study investigates the activity of three glycosidases (NAGASE, α-MAN and β-GAL) in the follicular fluid (FF) collected from bovine follicles of different sizes. It also evaluates the relationship between these glycosidases' activity in the FF or in the fertilization media (FM) and the developmental competence of the resulting zygotes after oocytes being cultured and fertilized in groups or individually.

Materials and Methods

Unless differently stated, all chemicals were purchased from Sigma Chemical Company (Poole, UK).

Three laboratories have cultured and two experiments were designed in order to study the activity of glycosidases in the follicular fluid of different sized (small or large or controls) follicles and in the fertilization medium. In all experiments performed, cumulus oocyte complexes (COCs) obtained were allocated into three groups according to their follicular size estimated by visual examination [controls (CF group): 2-8 mm, small follicle group (SF group): 2-5 mm and large follicle group (LF group): >5-8 mm] as described by Racedo *et al.* (2008) and Dovolou *et al.* (2016). Semen from one bull of proven fertility was used throughout each experiment.

In vitro *maturation and fertilization of oocytes aspirated from follicles of different sizes and experiment set-up*

Experiment 1

In groups: cattle ovaries were collected from commercial slaughterhouse and transported in saline solution (0.9% NaCL) at 34°C. Cumulus oocyte complexes - (COCs; n = 2099) were aspirated using an 18-g needle connected to a sterile tube and aspiration pump. Follicles of each size group were aspirated separately; small (n = 1305), large (n = 198) or control (n = 596). *In vitro* maturation, fertilization and culture were performed as previously described (Dovolou *et al*., 2014). Briefly, COCs were matured in groups of 50 in TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor (EGF), at 39°C, under an atmosphere of 5% CO_2 in air, with maximum humidity. After 24 h of maturation, COCs were inseminated with frozen-thawed swim-up separated bull sperm at a final ratio of 1 x 10^6 spermatozoa/ml and gametes were co-incubated at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. At approximately 20 h post insemination (hpi), presumptive zygotes were denuded by 3 min gentle vortexing and cultured in groups of 25 in 25 µl droplets under mineral oil at 39°C, in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Synthetic oviduct fluid (SOF) supplemented with 5% FCS was used for embryo culture.

Experiment 2

Individual: cattle ovaries were collected from commercial slaughterhouse as previously described and COCs (n = 79) were aspirated from follicles of different sizes; small (n = 29), large (n = 21) or control (n = 29), separately. *In vitro* maturation, fertilization and culture were performed as previously described (Carolan *et al*., 1996; Holm *et al*., 1999; Donnay *et al*., 2004; Al-Darwich *et al*., 2010). Briefly, after aspiration, COCs were washed in 25 mM Hepes buffered TCM-199, containing BSA (0.4 g/L,) and gentamicin (40 µg/ml) and cultured individually into 10 µl droplets of maturation medium as previously described (Donnay *et al*., 2004), in humidified atmosphere 5% CO_2 in air at 38.8˚C for 24 h. After maturation, COCs were washed in fertilization medium (Tyrode medium supplemented with 25 mM bicarbonate, 10 mM lactate, 1 mM pyruvate, 6 mg/ml fatty acid free BSA, 100 µg/ml heparin and 40 µg/ml gentamicin) and transferred individually into 25 µl droplets of fertilization medium in 35 mm Petri dishes covered by 3.5 ml of mineral oil. Motile spermatozoa were obtained by centrifugation of frozen/thawed semen on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient (45/90%) and diluted in fertilization medium to obtain a final concentration of 1×10^6 spermatozoa per ml. A 25 µl aliquot of this suspension was added to each well containing a single COC. Gametes were co-incubated for 18 h at 38.8ºC in a humidified atmosphere with 5% CO_2 in air. Presumptive zygotes were denuded of surrounding cumulus cells and attached spermatozoa, washed in *in vitro* culture medium (SOF medium supplemented with 5% FCS; MP Biomedicals) and transferred into culture droplets (10 µl, in 35 mm Petri dishes) overlaid with mineral oil and cultured at 38.8°C in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2.

Embryo development assessment

In both experiments, cleavage rate was determined 48 hpi; according to the cell number, zygotes/embryos were classified as: 1 cell-not developed, 2-4 cells and >6 cells groups (experiment 2). Blastocyst rate was determined at days 7, 8 and 9 pi in experiment 1, and at days 6, 7 and 8 pi in experiment 2.

Sample collection

Nine (9) replicates have been performed in experiment 1 (in groups) and 3 replicates in experiment 2 (individual). In each replicate, all follicles in each category (CF, SF, LF) were aspirated separately. After the removal of COCs, 2 ml samples of the pooled FF were retrieved. FM samples were collected at the end of fertilization period. All samples were centrifuged (1100 g; 10 min) and stored at -20°C until analyzed for glycosidases activity. FM samples in which no ova were

added (no ova group) were also collected and assayed. An aliquot of sperm was added (experiment 1), or not (experiment 2) in FM of no ova group. In experiment 2, aliquots of 5 µl of FM were diluted in 495 µl MiliQ water just before measurement and used for glycosidases measurement.

Glycosidases activity measurement

The α-mannosidase (α-MAN), β-N-acetyloglucosaminidase (NAGASE) and β-galactosidase (β-GAL) activities either in FF or FM samples were determined colorimetrically by the method outlined by Levvy and Conchie (1966) as previously described (Tsiligianni *et al*., 2006). For this purpose p-nitrophenol, o-nitrophenol, 4-nitrophenyl-N-acetyl-b-D-glucosaminide 99%, p-nitrophenyl-a-D-mannopyranoside, and o-nitrophenyl-b-galactoside (all Fluka reagents) and glycine, citric acid, sodium chloride, sodium hydroxide, acetic acid, and disodium hydrogen phosphate were used. Fluorescence was read at 430 nm using a spectrophotometer (UV mini 1240, Shimadzu). Results were expressed in IU/L.

Statistical analysis

As mentioned, follicles were classified according to their size into 3 groups (CF, SF and LF). Moreover, after fertilization zygotes/embryos were classified into 3 categories according to the cell number: 1 cell-not developed, 2-4 cells and >6 cells (experiment 2). Q-Q test was used for distribution of normality. Analysis of variance was used to compare mean values of glycosidases activity in FF and FM. Comparisons between means were performed by Duncan's new multiple-range test. Pearson's chi-square test was used to compare cleavage or blastocyst formation rate (%) among groups. Linear regression analysis was performed to determine the relationship between the glycosidases activity and oocyte quality or cleavage rate or blastocyst rate after fertilization. The results were expressed as mean ± SEM. All analyses were performed using SPSS software.

Results

Developmental competence

Cleavage rate and blastocyst formation rate are presented in Table 1. Cleavage rate was similar in all groups for both experiments. In experiment 1, blastocyst formation rate was significantly higher ($P < 0.05$) in CF compared to SF and LF group at days 8 and 9 or LF group at day 7.

Glycosidases activity in the follicular fluid (FF)

Glycosidases activity in FF collected from CF, SF and LF groups is presented in Table 2. In both experiments, the activity of α-MAN, NAGASE and β-GAL was significantly lower in FF collected from LF ($P < 0.05$) compared to the one collected from SF. All three glycosidases activities in the FF of CF were found to be between those in LF and SF (experiment 1). In experiment 2, glycosidases activities in CF were significantly higher than those in LF ($P < 0.05$).

Table 1. Cleavage rate and blastocyst formation rate of oocytes collected from control (CF), small (SF) or large (LF) follicles.

	Cleavage rate (%)	Blastocyst rate (%)	Blastocyst rate (%)	Blastocyst rate (%)
Experiment 1		D7	D8	D9
CF (n = 596)	73.3[a]	29.3[a]	34.4[a]	36.9[a]
SF (n = 1305)	73.9[a]	21.6[a,b]	23.6[b]	23.9[b]
LF (n = 198)	67.6[a]	14.6[b]	14.6[b]	14.6[b]
Experiment 2		D6	D7	D8
CF (n = 29)	82.8[a]	20.8[a]	41.7[a]	45.8[a]
SF (n = 29)	87.5[a]	20.0[a]	40.0[a]	44.0[a]
LF (n = 21)	83.3[a]	29.4[a]	58.8[a]	58.8[a]

[a,b]Values with different superscripts in the same column differ significantly ($P < 0.05$; blastocysts = % of cleaved oocytes).

Table 2. Glycosidases activity in follicular fluid collected from control (CF), small (SF) and large (LF) follicles.

	α-MAN (U/L)	NAGASE (U/L)	β-GAL (U/l)
Experiment 1			
CF	87.9 ± 2.6[a,b]	47.3 ± 2.3[a,b]	15.7 ± 0.7[a,b]
SF	93.9 ± 2.1[a]	53.5 ± 2.4[a]	17.2 ± 0.5[a]
LF	73.4 ± 3.1[b]	40.5 ± 2.5[b]	15.1 ± 0.6[b]
Experiment 2			
CF	79.2 ± 1.3[a]	27.2 ± 1.9[a]	14.7 ± 1.4[a]
SF	81.6 ± 1.3[a]	31.2 ± 2.7[a]	15.2 ± 1.8[a]
LF	68.7 ± 1.5[b]	18.4 ± 1.7[b]	11.1 ± 1.5[b]

*$P < 0.05$, [a,b]In each experiment, values with different superscripts in the same column differ significantly ($P < 0.05$). α-MAN: α-mannosidase, NAGASE: β-N-acetyloglucosaminidase, β-GAL: β-galactosidase.

Moreover, in experiment 1 β-GAL activity in FF from SF presented: i) a positive relation with the number of degenerating oocytes collected (D) [β-GAL= (0.124 ± 0.05)D+(15.26 ± 0.78), sigF= 0.030] and ii) a negative relation with the blastocyst rate (BR) at day 7 [β-GAL = (-0.123 ± 0.035)BR + (18.68 ± 0.54), sig F = 0.017), at day 8 [β-GAL = (-0.100 ± 0.049) BR + (18.67 ± 0.87), sigF= 0.065] and at day 9 [β-GAL= (-0.129 ± 0.036) BR + (18.97 ± 0.60), sigF= 0.017).

Glycosidases activity determination in the fertilization media (FM)

Glycosidases activity in the FM of CF, SF and LF groups is presented in Table 3. The activities of α-MAN and NAGASE were significantly higher (P < 0.05) in FM of LF compared to SF and CF (experiment 1), while β-GAL activity was significantly higher (P < 0.05) in FM of SF compared to CF (experiment 2). In the experiment 1, all collected oocytes seem to consume β-GAL during IVF, while only oocytes collected from CF or SF seem to consume α-MAN. It seems that oocytes collected from LF release NAGASE during IVF in groups, while all three glycosidases are released during individual fertilization of oocytes collected from CF, SF or LF. On the other hand, oocytes collected from SF release more β-GAL compared to CF (experiment 2).

Glycosidases activity in FM of individually cultured oocytes was not associated to the cell number of resulting embryos in any group.

In the case of group culture and fertilization (experiment 1), regression analysis revealed a negative relation between: i) α-MAN (IU/l) in FM and cleavage rate (CR) of oocytes collected from CF [α-MAN= (-0.39 ± 0.15)CR+(55.97 ± 9.18), sigF= 0.037), ii) β-GAL (IU/l) in FM of oocytes collected from CF and blastocyst rate (BR) at day 8 [β-GAL= (-0.15 ± 0.08)BR+(22.88 ± 5.30), sigF= 0.089] or at day 9 [β-GAL= (-0.14 ± 0.07)BR + (22.26 ± 4.77), sigF= 0.072).

Table 3. Glycosidases activity in fertilization medium of oocytes collected from control (CF), small (SF) and large (LF) follicles.

Group (n)*	α-MAN (U/L) (P < 0.05)	NAGASE (U/L) (P < 0.001)	β-GAL (U/L) (P < 0.05)
Experiment 1			
CF (11)	26.6 ± 5.6[b]	23.4 ± 3.5[b]	42.2 ± 16.6[b]
SF (10)	27.1 ± 3.0[b]	22.1 ± 2.6[b]	48.8 ± 6.3[b]
LF (9)	44.5 ± 2.9[a]	106.2 ± 28.8[a]	47.5 ± 18.9[b]
No ova* (6)	43.4 ± 5.9[a]	17.4 ± 2.2[b]	71.9 ± 20.6[a]
Experiment 2			
CF (29)	119.8 ± 11.3[a]	28.9 ± 3.7[a]	28.3 ± 2.6[b]
SF (29)	107.7 ± 10.4[a]	12.1 ± 1.1[a]	42.5 ± 5.3[a]
LF (21)	100.6 ± 8.9[a]	16.2 ± 1.5[a]	36.6 ± 3.5[a,b]
No ova** (5)	3.1 ± 0.1[b]	1.0 ± 0.2[b]	0.6 ± 0.1[c]

[a,b,c]In each experiment, values with different superscripts in the same column differ significantly (P < 0.05). *(n) number of samples. **No ova group stands for fertilization medium without ova. α-MAN: α-mannosidase, NAGASE: β-N-acetyloglucosaminidase, β-GAL: β-galactosidase

Discussion

Mannose and N-acetyl-glucosamine found in the oocyte envelope (Miller *at al*., 1993b; Dupre *et al*., 2012) and galactose residues in the bovine zona pellucida (Katsumata *et al*., 1996; Parillo *et al*., 2000; Ikeda *et al*., 2002) indicate that these sugars could be the first receptors during gamete interaction. Moreover, specific glycosidases to these sugar residues could play a role during oocyte development in the follicle or during fertilization. Thus, this study has two targets. Firstly, to evaluate the activity of three glycosidases (NAGASE, α-MAN and β-GAL) in the follicular fluid (FF) collected from different size bovine follicles as well as in the fertilization medium (FM) of oocytes retrieved from those follicles. Secondly, to examine/study the relationship between the glycosidases activity found in FF or in FM and the developmental competence of the IVP zygotes cultured either in groups (experiment 1) or individually (experiment 2).

According to our results, all three glycosidases presented significantly lower activity in the FF collected from LF compared to SF, while in the FF of CF the activity was intermediate; the significance of the difference between CF and either SF or LF groups probably depended on the small/large ratio within the CF group. Considering that, in ruminants, steroid hormones concentrations in the follicular fluid are associated with follicular size, these differences could be related to the hormonal status of FF (Henderson *et al*., 1982; Aller *et al*., 2013; Tungal *et al*., 2014). The decreased glycosidases activity in FF collected from LF could be related to low synthesis or low release of these enzymes by COCs, or the increased utilization by the follicular oocytes during their maturation process within the follicle, before aspiration. Regression analysis showed a positive correlation between β-GAL activity in FF of SF and the number of grade D COCs, and a negative correlation between β-GAL in FF and blastocyst formation rate, when IVF was performed in groups. It seems that increased β-GAL activity in FF is related to impaired oocyte quality and, consequently, to a decreased blastocyst formation rate. Cleavage rate was found similar in all groups in any of the two experiments. Blastocyst formation rate was significantly higher in CF compared to LF group when oocytes were

fertilized in groups. It could be suggested that oocytes collected from CF (2-8 mm) and fertilized in groups help each other or act supplementary to each other. In general, there was a tendency for oocytes collected from SF to develop into blastocysts in lower rate compared to those collected from LF or CF when fertilized separately, but this tendency was observed in LF group, when they were fertilized in groups.

In the case of individually cultured oocytes, β-GAL activity in FM was significantly higher in oocytes collected from SF compared to CF. A tendency of the same pattern was observed when oocytes were fertilized in groups, however differences were not significant. It has been proposed that beta-D-galactosidase may remodel the zona pellucida oligosaccharides that are involved in porcine sperm-oocyte binding (Yonezawa *et al*., 2005) and finally reduce polyspermy (Carrasco *et al*., 2008). *In vivo*, beta-D-galactosidase could regulate the sperm binding sites present in the zona pellucida, reducing the sperm that could fertilize the oocyte and consequently reducing polyspermy (Carrasco *et al*., 2008). Thus, β-GAL is possibly used during fertilization, which is obvious when oocytes were fertilized in groups, and decreased β-GAL in FM could be related to better blastocyst formation rate, because β-GAL associated negatively to blastocyst rate; however the exact pathway of glycosidases action remains to be clarified.

In the case of oocytes fertilized in groups, α-MAN and NAGASE activities were significantly lower in the FM of oocytes collected from SF and CF compared to LF group, while this difference did not occur when oocytes were fertilized and cultured individually. Regression analysis revealed a negative relation between α-MAN activity in FM and cleavage rate of oocytes collected from CF and fertilized in groups. This finding in relation to the higher blastocyst rate of CF oocytes could lead to the reasonable assumption that increased α-MAN activity in the FM is associated with decreased fertilization capacity. However, it is unknown whether the increased α-MAN activity is responsible for the impaired fertilization, or the contrary. According to Carrasco *et al*. (2008), oviductal alpha-D-mannosidase activity decreases after ovulation and this enzyme may act on eliminating some mannose residues from the zona pellucida, thereby helping to control polyspermy by remodeling the sugar components and affecting interactions with spermatozoa, whereas β-NAGASE may facilitate contact of the spermatozoa with the oocyte by dispersing cumulus cells. Furthermore, a possible role of α-MAN in spermatozoa capacitation within the oviduct has been suggested, although the mechanism remains still unknown (Carrasco *et al*., 2008). Taking all these into account, it could be suggested, that during fertilization α-MAN and NAGASE facilitate the contact of the spermatozoa with the oocyte while preventing polyspermy at the same time.

The low α-MAN and NAGASE activity in FM of oocytes collected from SF might be related to the increased utilization of these enzymes during fertilization. This assumption is supported by another finding of the present study; α-MAN activity in the FM of oocytes collected from SF was significantly lower than that in FM with no oocytes when sperm was added. It has been proposed that mannose residues contained in bovine zona pellucida glycoproteins are involved in fertilization (Amari *et al*., 2001) and alpha-D-mannose affects the *in vitro* fusion between bovine gametes, because spermatozoa have receptors for mannose, which is necessary for fusion with the oolema (Tanghe *et al*., 2004). The α-MAN activity is related to alpha–D-mannose because α-MAN has been shown to cleave mannose from the glycans and other glycoproteins. The α-MAN in FM could be increased, because it was not used (to cleave mannose from glycoproteins of zona pellucida) or, reversely, it may have affected gamete fusion, thus impairing fertilization of the oocyte.

During fertilization, oocytes collected from LF and fertilized in groups appear to release NAGASE and consume β-GAL, while those fertilized separately appear to release all three glycosidases. Furthermore, it seems that oocytes collected from SF or CF utilize α-MAN and β-GAL during fertilization in groups and present a tendency to release NAGASE when fertilized either separately or in groups. It is clear that oocytes fertilized in groups utilize β-GAL, no matter if they originate from SF, LF or CF. It was reported that Beta-D-glucosaminidase and Beta-D-galactosaminidase activity detected in intact sperm is decreased after the induction of acrosome reaction in boars (De Ondiz *et al*., 2008). Given the increased β-GAL activity when sperm was present in the "empty" (no ova) FM, the decreased activity of β-GAL after fertilization in groups may be related to the sperm's acrosome reaction. During individual fertilization, where sperm was not added in no-ova FM, the activity of all glycosidases in those samples was minimum. Furthermore, it seems that when oocytes are fertilized individually they release all three glycosidases; however, in this case increased glycosidases activity could be due to sperm addition in FM.

In the present study, oocytes collected from SF or CF presented a tendency to release less NAGASE compared to those collected from LF when fertilized in groups, while oocytes from CF release more NAGASE when fertilized individually. In the first case, blastocyst rate of controls was the highest. The results concerning individual fertilization could be related to the observation of Coy *et al*. (2009) after incubation of pig oocytes with NAGASE, where the number of sperm attached to the ZP and the mean number of sperm per penetrated oocyte were decreased. Increased NAGASE may also affect negatively fertilization when oocytes from LF are fertilized in groups, because its activity in FM of oocytes originating from LF was increased and the cleavage rate of these oocytes was the lowest.

Taking in mind: a) the increased number of grade D oocytes when β-GAL activity was increased in FF, b) the decreased blastocyst rate when this enzyme activity was increased in FM and c) the tendency of oocytes collected from CF to develop in blastocyst in a lower rate when β-GAL activity in FM is increased, it could be suggested that increased β-GAL might impair

oocyte fertilization and embryo development when oocytes are fertilized and cultured either in groups or individually.

In conclusion, all three glycosidases activity was lower in the FF collected from LF compared to SF, while α-MAN and NAGASE were higher in the FM of oocytes collected from LF compared to those collected from SF. Glycosidases might be affecting the developmental competence of oocytes collected from different sized follicles; the role of these glycosidases in FF is different from that in FM. Further research is needed to clarify the exact role of these glycosidases on oocyte developmental competence.

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