Theriogenology Insight: 8(1): 21-32, April 2018
DOI: 10.30954/2277-3371.01.2018.4

The Possible Role of Certain Glycosidase on Cow Reproduction

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ABSTRACT

Glycosidase are associated with cumulus cells expansion, sperm capacitation, sperm oviductal epithelial cells interaction, sperm zona pellucida binding and polyspermy block. The aim of this review is to summarize the role of certain glycosidase (α-mannosidase - α-MAN, β-N-acetyloglucosaminidase – β-NAGASE and β-galactosidase - β-GAL) on cow reproduction. The activity of certain glycosidase in: i) the cervical mucus after spontaneous or induced estrous and its relation to cow fertility, ii) the uterine luminal fluid after superovulation, iii) the follicular fluid and the maturation, fertilization or culture medium during IVM-IVF is presented in this review. Furthermore, it is mentioned if: a) the endometrium or oocytes or embryos release or use glycosidase during their development, b) the addition of certain glycosidase in culture medium affects embryo development, and c) glycosidase could be used as markers of embryo quality or superovulatory response (SR). Glycosidase activity was significantly lower in the cervical mucus of spontaneous estrous compared to induced estrous cows. A high superovulatory response is related to low β-NAGASE, probably because of the poor quality of embryos. The β-NAGASE affects negatively embryonic development when added to culture medium. COCs release β-NAGASE and use β-GAL during maturation. Embryos release β-NAGASE and α-MAN during their development, but they use only α-MAN. Degenerated embryos release less β-NAGASE and α-MAN compared to good embryos, whereas β-NAGASE seems to be related to retarded morulae. Glycosidase affects the developmental competence of oocytes collected from different sized follicles during IVF.

Keywords: glycosidase activity, cervical mucus, uterine luminal fluid, oocyte, embryo culture

Glycosidase are considered intracellular enzymes that degrade oligosaccharides on glycoconjugates destined for the endocytic/ lysosomal pathway (Miller *et al.,* 1993b). However, glycosidase can also be secreted extracellularly. Furthermore, it has been shown that in certain cells undergoing rapid division (e.g., tumor cells) the structure of glycosidase is altered (Hakamori and Murakami, 1968; Glick and Murakami, 1968; Dabelsteen and Pindborg, 1973) and they may facilitate tumor cell metastasis (rapid division) in collaboration with protease activities (Liotta *et al.,* 1986). It is not known whether the modified structure of glycosidase in tumors is a consequence of rapid growth or whether this is a primary cause of the rapid growth, possibly by altering membrane permeability, thus allowing entry of important metabolites (Holley, 1972). On a cellular level, early embryonic development is a rapid process that might be influenced by the presence of glycosidase in the luminal fluid of the uterus or in the maturation, fertilization and culture media.

Glycosidase play an important role in the synthesis of glycosides; they catalyse the hydrolysis of glycosidic bonds to liberate monosaccharides and oligosaccharides of lower molecular weight (MW) than the native simple as well as complex carbohydrate substrates (Divakar, 2013). Beta-*N*-acetyloglucosaminidase (β-NAGASE) is reported to liberate terminal β-linked *N*-acetylglucosamine and

N-acetylgalactosamine from a variety of substrates; α-mannosidase (α-MAN) liberates mannose from a variety of synthetic and natural α-mannosides; whereas β-galactosidase releases galactose from the nonreducing end of complex oligosaccharides. The mechanism by which these enzymes influence embryonic metabolism is unclear.

According to previous studies in several species, glycosidase are associated with cumulus cells expansion (Takada *et al.,* 1994), sperm capacitation (Taitzoglou *et al.,* 2007), sperm-oviductal epithelial cells interaction (Lefebvre *et al.,* 1997), sperm-zona pellucida binding (Miller *et al.,* 1993a,b; Miranda *et al.,* 2000; Zitta *et al.,* 2006) and polyspermy block (Miller *et al.,* 1993a,b). However, there is limited data available regarding the presence of glycosidase in bovine follicular fluid or the secretion of glycosidase by the cumulus-oocyte-complex (COC) during oocyte maturation, fertilization and culture. Furthermore, cervical mucus plays an important role during fertilization. On the other hand, uterine luminal fluid is important during very early gestation. In previous studies, we investigated the activity of certain glycosidase [β-N-acetyloglucosaminidase (NAGASE), α-mannosidase (α-MAN) and β-galactosidase (β-GAL)] in the: a) cervical mucus at spontaneous or induced estrous, b) uterine luminal fluid after superovulation and the relationship between these glycosidase and the superovulatory response (number of corpora lutea and embryos recovered), c) follicular fluid collected from small or large bovine follicles and the quality of oocytes collected from those follicles, d) maturation, fertilization and culture medium and their relationship to the maturation rate of oocytes or cleavage rate of resulting zygotes. Our main objectives were a) to define if these glycosidase may be used as markers of embryo quality, b) to designate if embryos use these enzymes during their development and c) to determine the effects of addition of these glycosidase in culture medium on early embryo development.

***In vivo* studies**

Cervical mucus

Several attempts have been made to improve sperm transport in many species by adding compounds such as glycosidase (β-glucuronidase) to bull semen diluents and fertility improved significantly (Hafs *et al.,* 1971; O'Hagan *et al.,* 1974). Therefore, the aim of our first research (Tsiligianni *et al.,* 2003a) was to determine for the first time the activity of α-MAN, β-NAGASE, β-glucuronidase and β-GAL in cervical mucus of cows at either spontaneous estrus or induced estrus at the time of first artificial insemination (AI). Estrous was induced by using progesterone intravaginal device (PRID) for 12 d plus eCG at PRID removal, or double $PGF_{2\alpha}$ injection 11 days apart, or PRID for 7 days plus $PGF_{2\alpha}$ 1 day before PRID removal.

Cervical mucus collected from spontaneous estrus was abundant, clear and transparent, while that from most induced estrus cows was decreased in quantity and thick (Tsiligianni *et al.,* 2003). The activity of all glycosidase studied were significantly lower in cervical mucus collected from cows with spontaneous estrus than that collected from cows with induced estrus (Table 1).

Despite differences in the glycosidase activity in cervical mucus among spontaneous and induced-estrus groups, conception rates did not differ significantly, suggesting that enzyme activity of cervical mucus does not directly affect fertility in cows. In induced-estrus groups a second AI was performed 24 h after the first one. It is possible that these enzymes' activity in cervical mucus at the time of the second AI was similar to that for spontaneous estrus, but this suggestion needs further investigation. It is well known that in synchronization programs there are substantial differences in the interval from luteolysis to estrus and to ovulation (Garcia-Winder and Gallegos-Sanchez, 1991; Roche *et al.,* 1981). On the basis of this finding,

Print ISSN: 2249-6610

Table 1: Glycosidase activity (α-mannosidase - α-MAN, β-N-acetyloglucosaminidase – β-NAGASE and β-galactosidase - β-GAL) in cervical mucus collected just before AI from spontaneous or inducted (by PRIDx12d, double $PGF_{2\alpha}$ 11 days apart or PRIDx7d plus $PGF_{2\alpha}$ 1d before PRID withdrawal) estrous cows

Enzyme activity	Spontaneous estrous	PRINDx12 induced estrus	$PGF_{2\alpha}$ induced estrous	(PRIDx7) + $PGF_{2\alpha}$ induced estrus
α-MAN (μg/mL)	18.1 ± 2.0^a	83.6 ± 14.1^b	63.7 ± 15.5^b	80.7 ± 29.9^b
β-NAGASE (μg/mL)	534.5 ± 45.6^a	1407.5 ± 311.6^b	1955.7 ± 470.6^b	2077.9 ± 729.5^b
β-GAL (μg/mL)	196.4 ± 8.5^a	401.8 ± 78.6^b	356.2 ± 58.5^b	388.3 ± 121.8^b

^{a,b} lines with different superscripts are significantly different ($P<0.001$) (Tsiligianni *et al.,* 2003a)

glycosidase activity in cervical mucus could be used as an indicator for the time of estrus manifestation, in order to identify the optimal time for AI.

Uterine luminal fluid

It was not known whether glycosidase activity is acting as inhibitor or promoter during early embryonic development. The aims of our second project (Tsiligianni, *et al.,* 2007a) were: a) to evaluate the activity of β-NAGASE, α-MAN and β-GAL in the uterine luminal fluid of cows after superovulation; and b) to investigate the relationship between these glycosidase and the superovulatory response (number of corpora lutea and embryos recovered). Activity of β-NAGASE, α-MAN, and β-GAL was observed in the uterine luminal fluid of cows on the day of embryo collection after superovulation treatment (Table 2). The presence of these enzymes in the cows' uterine luminal fluid on day 7 after artificial insemination may be due to the embryos' rapid development. Increased activity of certain glycosidase during early pregnancy may be required so that sufficient enzyme is released to uncover enough sites for firm adhesion of the embryo (Roberts and Parker, 1974).

Furthermore, glycosidase activity was detected in 4 samples collected on day 7 after superovulation treatment from 2 cows that they did not respond to ovarian stimulation (Tsiligianni *et al.,* 2007a). This finding is in agreement with the observation of Roberts and Parker (1974) for glycosidase activity in uterine fluid of unmated cows 12 to 18 d after estrus, at levels similar to those of cows at 13 and 15 d of pregnancy. Thus, the source of the glycosidase detected in the uterine luminal fluid of cows after superovulation treatment could be either the endometrium alone (when there is no embryo) or the cortical granule envelope and the endometrium (in all the other cases).

The β-NAGASE activity in the uterine luminal fluid after superovulation was increased by the presence of embryos in the uterus; a particular number of embryos was a limiting factor (Tsiligianni *et al.,* 2007a). When more than 4 corpora lutea developed per ovary and more than 4 embryos were collected per horn, the activity of all glycosidase declined, although the differences were significant only for β-NAGASE. The positive relationship between β-NAGASE activity and the total number of embryos and between β-NAGASE activity and the number of transferable embryos when 1 to 4 corpora lutea developed per horn indicates a superovulatory response threshold (in this study 4 corpora lutea per ovary), up to which β-NAGASE activity and superovulatory response are positively related (Tsiligianni *et al.,* 2007a). Furthermore, a relationship between glycosidase activity and hormonal levels exists.

No α-MAN or β-GAL activity was detected and β-NAGASE activity was lowest when more than 4 transferable embryos were collected per horn; however, there was no significant difference among the groups studied (Tsiligianni *et al.,*

Table 2: The activity (mean ± SEM) of α-mannosidase (α-MAN), β-N-acetyloglucosaminidase (β-NAGASE) and β-galactosidase (β-GAL) in the uterine luminal fluid of cows after superovulation in relation to total embryos collected per uterine horn (group T1: 0, group T2: 1-2, group T3: 3-4 and group T4: >4 embryos) and in relation to transferable embryos collected per uterine horn (group TR1: 0, group TR2: 1-2, group TR3: 3-4 and group TR4: >4 embryos)

	α-MAN (IU/L)	β-NAGASE (IU/L)	β-GAL (IU/L)
Total embryos			
T1:0	1.1 ± 0.8	129.3 ± 34.6[a]	2.7 ± 2.1
T2:1-2	3.9 ± 2.5	222.27 ± 62.6[a,b]	1.5 ± 1.5
T3:3-4	5.3 ± 4.2	290.3 ± 93.8[a]	1.7 ± 1.7
T4: >4	1.8 ± 1.8	50.1 ± 8.2[c]	0.5 ± 0.5
Transferable embryos			
TR1: 0	1.1 ± 0.8	129.3 ± 34.6	2.7 ± 2.1
TR2: 1-2	3.5 ± 2.3	215.1 ± 58.2	1.6 ± 1.4
TR3: 3-4	7.7 ± 4.7	270.0 ± 108.0	1.9 ± 1.9
TR4: >4	0.0 ± 0.0	48.0 ± 10.9	0.0 ± 0.0

[a,b,c] values with different letter in the same column differ significantly (P<0.01) (Tsiligianni *et al.,* 2007a)

2007a). On day 7 after insemination, embryos that fail to develop to blastocysts are generally considered as having delayed developmental competence (Van Soom *et al.,* 1997b). Thus, increased β-NAGASE activity could be deemed a possible indicator of retarded embryo development (Tsiligianni *et al.,* 2006). Increased cell number has been found at days 6 and 7 of in vitro produced blastocysts (Van Soom, *et al.,* 1997b; Enright et al.; 2000); however, the increase was mainly due to growth of trophectoderm cells and not inner cell mass (ICM) (Van Soom *et al.,* 1996). A slower transition from morula to blastocyst, as seen in embryos produced in vivo, allows sufficient time for allocation of inner cells to the ICM (Van Soom *et al.,* 1997b) and may explain the decreased β-NAGASE activity in our study when more than 4 corpora lutea developed and more than 4 embryos were collected. In any case, the exact mechanism leading to the reduced β-NAGASE activity needs further investigation.

In conclusion, a good reaction to superovulation treatment (up to 4 corpora lutea per ovary and 3 to 4 embryos per horn) is related to high β-NAGASE activity. However, a high superovulatory response (more than 4 corpora lutea per ovary and more than 4 embryos per horn) is related to low β-NAGASE activity, probably because of the poor quality of the embryos.

In vitro studies

The association between certain glycosidase activity (α-MAN, β-NAGASE and β-GAL) and oocyte quality and developmental competence was also evaluated (Cordova *et al.,* 2016; Dovolou *et al.,* 2016). The size of the follicle is considered an important indicator of oocyte developmental competence, so it is used as a criterion for oocyte selection (Lonergan *et al.,* 1994; Blondin and Sirard 1995; Lequarre et al.; 2005). The glycosidase activity was studied in the follicular fluid collected from small (2-5 mm) and large (>5-8 mm) follicles and in the maturation (Dovolou *et al.,* 2016) and fertilization (Cordova *et al.,* 2016) medium where these oocytes matured and fertilized either individually or in groups. The maturation rate of oocytes was assessed either based on nuclear maturation or by cleavage rate of

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zygotes. The aims of another project were: a) to evaluate the presence or absence of β-NAGASE and α-mannosidase in culture medium during in vitro production of bovine embryos, b) to investigate if these glycosidase may be used as markers of embryo quality and c) to determine if embryos use these enzymes in order to develop (Tsiligianni *et al.,* 2006; 2007b).

Follicular fluid (FF)

Several studies report on various differences in the ability of FF to assist oocyte maturation (Carolan *et al.,* 1996; Ali *et al.,* 2004). All three glycosidase presented (Table 3) significantly lower activity in the FF collected from large (>5-8 mm) compared to small (2-5 mm) follicles (Cordova *et al.,* 2016; Dovolou *et al.,* 2016), while in the FF of control (2-8 mm) group the activity was intermediate (Cordova *et al.,* 2016); the significance of the difference between control group and either small or large follicle groups probably depended on the small/large ratio within the control follicle group. Considering that in ruminant steroid hormones concentration in the FF 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 glycosidase activity in FF collected from large follicles 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. Given that more grade A oocytes were collected from small follicles, while more grade BC oocytes were collected from large ones, the hypothesis that the presence of grade A oocytes may be associated with increased glycosidase activity in the FF was supported (Dovolou *et al.,* 2016). Furthermore, regression analysis showed a positive correlation between β-GAL activity in FF of small follicles and the number of grade D COCs, and a negative correlation between β-GAL in FF and blastocyst formation rate (Cordova *et al.,* 2016) or maturation rate (Dovolou *et al.,* 2016), when IVF was performed in groups. Thus, increased β-GAL in FF seems to be associated with degenerating oocytes and, consequently, low maturation and blastocyst formation rate.

The α-MAN activity was higher in the FF collected from small follicles (in comparison to large follicles) and more grade A oocytes were collected from these follicles (Dovolou *et al.,* 2016). However, α-MAN activity in FF was negatively correlated to maturation rate. More grade A oocytes were collected and higher β-NAGASE activity was detected in the FF from small follicles; thus high β-NAGASE activity in the FF seems to be associated with high quality COCs (Dovolou *et al.,* 2016). The β-NAGASE activity in FF aspirated from small follicles was positively correlated to grade A oocytes and negatively to grade BC oocytes collected from these follicles. It has been reported that β-NAGASE activity increased at the late follicular phase to decrease after ovulation, whereas β-GAL, α-MAN and β-NAGASE in oviductal fluid showed higher activity at the early follicular phase, which decreased after ovulation (Takada *et al.,* 1994). Given that glycosidase can modify glycoproteins and the oviductal protein secretion is importantly regulated by ovarian steroids, mainly estrogen (Buhi *et al.,* 2000), it could be inferred that the changes in glycosidase activity during the estrous cycle might be hormonally regulated. In in vitro studies, COCs were aspirated from abattoir ovaries; the stage of the estrous cycle was not determined and steroid hormone concentrations were not assessed. It is possible that glycosidase activity in FF is associated to the functional maturity of the oocyte and not to the follicle size directly. Thus, the differences could be related to the hormonal status and the estrous cycle phase at slaughter.

Maturation medium (MM)

All three glycosidases presented (Table 3) higher activity in the MM of oocytes collected from large follicles (>5-8 mm) compared to that

Table 3: Role of certain Glycosidases in follicular fluid, maturation, fertilization and culture medium

Medium	β-N-acetyloglucosaminidase (β-NAGASE)	α-mannosidase (α-MAN)	β-galactosidase (β-GAL)
Follicular fluid	Higher activity in small compared to large (>5-8 mm) follicles.	Higher activity in small compared to large follicles.	Higher activity in small compared to large follicles.
	Higher activity is associated to high quality oocytes.	It is associated negatively to maturation rates.	High activity is associated with degenerated oocytes, low maturation and blastocyst rate.
Maturation medium	Higher activity in large compared to small follicles.	Higher activity in large compared to small follicles.	Higher activity in large compared to small follicles.
	Low activity is associated to high cleavage rate.	High activity is associated with high cleavage rate (in groups) and increased cell number (individual).	COCs use it.
	COCs release it.		
Fertilization medium	Higher activity in large compared to small follicles.	Higher activity in large compared to small follicles.	Activity doesn't differ between small and large follicles.
		It is associated negatively to cleavage rate.	It is associated negatively to blastocyst rate.
			COCs use it.
Culture medium	High activity is associated with retarded morulae.	Embryos release and use it.	
	Embryos release it. Degenerated embryos release less.	Degenerated embryos release less.	

Small follicles: 2-5 mm, Large follicles: >5-8 mm, Control follicles: 2-8 mm

collected from small (2-5 mm) follicles (Dovolou *et al.,* 2016). Regression analysis revealed that α-MAN activity in the MM of oocytes collected from large follicles and matured in groups was positively correlated (approaching significance) to the cleavage rate of resulting embryos. Furthermore, in the case of oocytes collected from small follicles and matured individually, α-MAN in MM was positively correlated to the cell number of embryos after fertilization. The α-MAN in MM could be released by COCs during maturation and/or may have affected maturation of oocytes and as a result embryo cleavage rate, when oocytes matured either individually or in groups. Taking all these into account, high α-MAN activity in MM could be an indicator of proper oocyte maturation resulting in increased embryo cleavage rate and increased cell number of embryos. Further research is needed to confirm this hypothesis.

In the case of large follicles, increased β-NAGASE activity in MM of oocytes was related to lower cleavage rate (Dovolou *et al.,* 2016). It has been reported that embryos derived from small follicles (2-3 mm, higher proportion of poor quality COCs) stopped cleaving more frequently during the third and fourth embryonic cell cycles leading to unevenly sized blastomeres (Lequarre *et al.,* 2005). It seems that oocyte quality and not follicular size affects the maturation of oocytes and probably also enzyme activity.

It was obvious that the activity of all three enzymes in the MM, when serum was added in the medium, comes from serum addition. Furthermore, the differences in glycosidase activity could be related to the source of serum (estrous cow or fetal calf). When oocytes collected from small and large follicles and matured in groups appear to consume α-MAN and β-GAL

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and release β-NAGASE in MM (Dovolou *et al.,* 2016). When oocytes collected from large follicles seemed to release α-MAN, β-NAGASE and β-GAL during maturation in groups, while they release α-MAN and β-NAGASE and consume β-GAL during individual maturation. Regarding α-MAN activity in the MM the findings were puzzling; it seems that oocytes collected from small follicles consumed α-MAN when maturing in groups, while they released it when maturing individually (Dovolou *et al.,* 2016). Grade A and BC COCs were put for maturation in groups or good quality COCs were matured separately. α-MAN activity was higher in the MM of oocytes collected from small follicles that after fertilization individually developed to ≥ 6 cell embryos (Dovolou *et al.,* 2016). Oocyte developmental competence is correlated with COC morphology (Blondin and Sirard, 1995; Hendriksen *et al.,* 2000), the morphology of the corona radiata (Laurincik *et al.,* 1996) and the degree of atresia (Mayes and Sirard, 2001; Feng *et al.,* 2007). Maybe the variations of the three enzymes activity during maturation are related mostly to the quality of the COCs and less to the size of the follicles.

Fertilization medium (FM)

The relationship between three glycosidase activities in the FM and the developmental competence of the resulting zygotes after oocytes being culture and fertilized in groups or individually was evaluated (Cordova *et al.,* 2016). Cleavage rate was found similar in control (2-8 mm), small (2-5 mm) and large (>5-8 mm) follicle groups. Blastocyst formation rate was significantly higher in control follicle group compared to large follicle group when oocytes were fertilized in groups. It could be suggested that oocytes collected from control follicle group (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 small follicles to develop into blastocysts in lower rate compared to those collected from large or control follicles when fertilized separately, but this tendency was observed in large follicle group, when they were fertilized in groups (Cordova *et al.,* 2016).

In the case of individually cultured oocytes, β-GAL activity in FM was significantly higher in oocytes collected from small follicles compared to control (Table 3). A tendency of the same pattern was observed when oocytes were fertilized in groups, however differences were not significant. Thus, β-GAL may be used during fertilization, which is obvious when oocytes were fertilized in groups. 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 glycosidase action remains to be clarified.

In the case of oocytes fertilized in groups, α-MAN and β-NAGASE were significantly lower in the FM of oocytes collected from small and control follicle group compared to large follicle 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 control follicle and fertilized in groups (Cordova *et al.,* 2016). This finding in relation to the higher blastocyst rate of control follicle 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. Taking all these into account, it could be suggested, that α-MAN and β-NAGASE facilitate the contact of the spermatozoa with the oocyte and prevent polyspermy at the same time.

The low α-MAN and β-NAGASE activity in FM of oocytes collected from small follicles might be related to the increased utilization of these enzymes during fertilization. This assumption is supported by the finding that α-MAN activity in the FM of oocytes collected from small follicles was significantly lower than that in FM with

no oocytes when sperm was added (Cordova *et al.,* 2016). 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 large follicles and fertilized in groups appear to release β-NAGASE and consume β-GAL, while those fertilized separately appear to release all three glycosidase. Furthermore, it seems that oocytes collected from small or control follicles utilize α-MAN and β-GAL during fertilization in groups and present a tendency to release β-NAGASE when fertilized either separately or in groups (Cordova *et al.,* 2016). It is clear that oocytes fertilized in groups utilize β-GAL, no matter if they originate from small, large or control follicles. 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 glycosidase in those samples was minimum (Cordova *et al.,* 2016). Furthermore, it seems that when oocytes are fertilized individually they release all three glycosidase.

Oocytes collected from small or control follicles presented a tendency to release less β-NAGASE compared to those collected from large follicles when fertilized in groups, while oocytes from control follicles release more β-NAGASE when fertilized individually. In the first case, blastocyst rate of controls was the highest. Increased β-NAGASE may also affect negatively fertilization when oocytes from large follicles are fertilized in groups, because its activity in FM of oocytes originating from large follicles was increased and the cleavage rate of these oocytes was the lowest (Cordova *et al.,* 2016).

Culture medium (CM)

It was not known: a) if the embryos produce/release glycosidase during in vitro embryonic development, b) if in in vitro embryonic development β-NAGASE and α-MAN are acting as putative inhibitors or as promoters or c) if embryos utilize β-NAGASE and α-MAN during their development. Thus, the aims of two other studies were: (a) to investigate the effects of these glycosidase on in vitro embryonic development (Tsiligianni *et al.,* 2006) and (b) to determine if embryos utilize these enzymes during development (Tsiligianni *et al.,* 2007b).

Both glycosidase (α-MAN and β-NAGASE) detected in spent CM on day 2 (Table 3), before serum addition in the CM (Tsiligianni *et al.,* 2006). This finding implies that even the spent medium contains glycosidase activity, which is probably originating from the embryos. It is possible that the presence of β-NAGASE in CM 24 h after fertilization is a consequence of the cortical reaction, but since the zygotes are washed after fertilization, another source of this enzyme activity is more likely. However, since spermatozoa and cumulus cells associated with the zygotes were removed by vortexing, it is very unlikely that the measured α-MAN is resulting from spermatozoa. Thus the source of these glycosidase might be the cortical granule envelope. Further research is needed in order to support this hypothesis.

Bovine embryos release glycosidase on day 6 and 7 of their development (Tsiligianni *et al.,* 2006). The activity of α-MAN and β-NAGASE was increased when degenerate embryos were absent from the culture droplets, implying that only good quality embryos release both glycosidase. Furthermore, the activity of β-NAGASE was increased in droplets containing embryos at morula stage, while this difference was not significant for droplets with blastocysts. Embryos, which are not at the blastocyst stage yet at 7 days post insemination are probably behind in development (Van Soom *et al.,* 1997a), making the increased β-NAGASE

Print ISSN: 2249-6610

activity a possible indicator for retarded embryo development.

In another study (Tsiligianni *et al.,* 2007b), glycosidase were added to the CM to study the effect of the presence of these enzymes on embryo development. When CM was supplemented with α-MAN fewer good embryos developed compared to controls (Tsiligianni *et al.,* 2007b), which is in contrast to earlier findings with rabbit embryos (Kane, 1986). The presence of α–MAN in CM could mediate archenteron development and have a negative effect on embryo development in invertebrates. The addition of β-NAGASE and a combination of both glycosidase to CM inhibited the development of all embryos (Tsiligianni *et al.,* 2007b). To ascertain the reason, the osmolarity of the CM was measured immediately after addition of the glycosidase. The addition of β-NAGASE or a combination of the two enzymes increased substantially osmolarity of the CM, while the addition of α-MAN did not. It is confident that the negative effect on embryonic development is due to high osmolarity caused by the addition of β-NAGASE. However, the reason for the increase in osmolarity is unknown (it was not due to an error in dilution) and remains to be clarified. Increased osmolarity can negatively affect embryonic development as described by McKiernan and Bavister (1990). The change in osmolarity observed after addition of β-NAGASE to SOF medium containing FCS is not due directly to the osmolarity-producing effects of enzymes. It is known that osmolarity depends on the number of particles and high osmolarities are produced by low molecular weight (MW) compounds, such as Na, Cl, glucose and amino acids. Thus, large weight concentrations of proteins would only produce a rise in osmolarity of about 1mOsm. However, the observed increase in osmolarity may arise from enzyme break-down of large MW compounds in the FCS to low MW compounds.

Another interesting finding was that the activity of α–MAN decreased in droplets containing embryos (Tsiligianni *et al.,* 2007b). Therefore, embryos might utilize or metabolize α-MAN during their development. About 2g of α-MAN and 100g of β-NAGASE were added to CM and enzyme activity measured (IU/l) in droplets with and without embryos; it was not possible to measure the exact enzyme concentration. The decrease in α–MAN activity in droplets with embryos, compared to droplets without embryos, may be due to inactivation of the enzyme by the embryos during development.

The addition of β-NAGASE to CM increases osmolarity and inhibits the development of embryos. However, α-MAN inhibits embryonic development without increase osmolarity. Embryos may metabolize α-MAN during development. However, the exact pathway of the action of the glycosidases it is still unknown.

DISCUSSION AND CONCLUSION

The activity of all glycosidase was lower in cervical mucus of spontaneous estrous compared to induced estrous. Furthermore, β-NAGASE activity in the uterine luminal fluid of cows was significantly higher when blastocysts were collected compared to when morulae or no embryos were collected. The activity of NAGASE, α-MAN and β-GAL was higher in the FF and lower in the MM of oocytes collected from small follicles compared to those collected from large follicles; whereas α-MAN and NAGASE were lower in the FM of oocytes collected from small follicles compared to those collected from large.

As concern β-NAGASE, taking in mind that the increased activity: a) in FF is related to high oocyte quality, b) in MM negatively affects cleavage rate and c) in CM is associated to retarded morulae, it could be assumed that the role of this enzyme is different in FF, MM and CM. Increased β-NAGASE activity in MM or CM affects negatively oocyte developmental competence, whereas increased activity in FF is associated to high quality oocytes.

As concern α-MAN, taking in mind that the increase activity: a) in FF is related to decreased maturation rate, b) in MM is associated to

high cleavage rate (in groups) or increased cell number (individual), c) in FM leads to low cleavage rate and d) degenerated embryos release less α-MAN, it could be suggested that the role of this enzyme is also different during maturation, fertilization and culture. The high activity in FF and FM has negative influence on maturation and cleavage rate. However, the high activity in MM and CM affects positively cleavage rate, embryo cell number and oocyte quality.

As concern β-GAL activity, having 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 control follicles to develop into 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.

The in vitro produced bovine embryos release β-NAGASE and α-MAN at the beginning of their development, and a negative correlation between both glycosidase and degenerate embryos exists. Furthermore, degenerate embryos release less β-NAGASE and α-MAN compared to good embryos, but β-NAGASE secretion is increased in retarded morulae. The presence of these enzymes in CM seems to be the consequence of rapid growth during embryo development. However, the exact pathway of these glycosidase' action remains to be clarified.

Glycosidase might be affecting the developmental competence of oocytes collected from different sized follicles; the role of these glycosidase in FF is different from that in FM. Factors - other than follicle size - resulting in variations of the oocyte quality may cause these differences. The results presented here could be used to properly direct the investigation of: a) the use of substances that reduce / antagonize the action of certain glycosidase, as well as b) the addition of specific concentrations of certain glycosidase into in vitro production media.

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 Print ISSN: 2249-6610