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CONCENTRATION OF GLUTATHIONE AND ATP AS A MEASURE OF OOCYTE MATURATION IN RABBIT FEMALES

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ABSTRACT

The present study was designed to determine the concentrations of ATP and glutathione (GSH) in ovulated and not-ovulated rabbit oocytes in a line of rabbits selected for ovulation rate. ATP and GSH concentrations were used to assess oocyte quality. A total of 58 does belonging to the 9th generation of a line selected for ovulation rate were used to assess ATP concentrations. A total of 10 does belonging to the 10th generation of the same line were used to assess GSH concentrations. Ovulation was induced by an intramuscular injection of 1 mg of buserelin acetate and animals were slaughtered 16 hours thereafter. Ovulated oocytes were collected by flushing the oviducts. Not-ovulated oocytes were aspirated from preovulatory follicles with a 1ml syringe and a 25G needle. Oocytes were processed. ATP concentration was determined by bioluminiscency. ATP concentration ranged from 0.6 to 2.4 pmol/oocyte in not-ovulated oocytes and from 0.8 to 5.0 pmol/oocyte in ovulated oocytes. GSH concentration was determined by HPLC. Glutathione concentration ranged from 0.5 to 2.2 pmol/oocyte in not-ovulated oocytes and from 5.2 to 10.2 pmol/oocyte in ovulated oocytes. These differences in ATP and GSH concentrations between not-ovulated and ovulated rabbit oocytes might indicate that ATP and GSH are synthesized during oocyte maturation.

Key words: ATP, glutathione, ovulated oocyte, not-ovulated oocyte

INTRODUCTION

The nuclear and cytoplasmic maturation of mammalian oocytes has to be completed successfully in order to lead to a competent oocyte. Several authors have proposed the measurement of glutathione (GSH) and ATP inside the cell as a method to assess oocyte cytoplasmic maturation (reviewed by Krisher, 2004). GSH is the major non-enzymatic sulphydryl compound in cells. It plays an important role in protecting cells against the effects of the reactive oxygen intermediates and free radicals and maintaining the intracellular redox status (reviewed by Luberda, 2005). ATP is an important energy source for maintaining protein synthesis and other cellular functions (reviewed by Krisher, 2004). GSH and ATP are stored during the follicular maturation of the oocyte, and are necessary for its development. Higher levels of GSH and ATP have been related to more developed oocytes, which have a lower probability to die (reviewed by Krisher, 2004).

The aim of this work was to estimate the oocyte concentration of GSH and ATP in ovulated and not-ovulated rabbit oocytes.

MATERIAL AND METHODS

Animals

All experimental procedures involving animals were approved by the Research Ethics Committee of the Universitat Politècnica de València. Animals came from an experiment of selection for ovulation rate (line OR) described by Laborda *et al.* (2011). A total of 58 does belonging to the 9th generation

were used to assess ATP concentrations in ovulated and not-ovulated oocytes. A total of 10 does belonging to the 10th generation were used to assess GSH concentrations in ovulated and not-ovulated oocytes. Ovulation was induced by an intramuscular injection of 1 mg of buserelin acetate (Hoechst, Marion Roussel, Madrid, Spain). Animals were slaughtered 16 hours thereafter and the reproductive tracts were removed. Animals were housed at the experimental farm of the Universitat Politècnica de València in individual cages. They were kept under controlled 16-h light: 8-h dark photoperiods.

Oocytes

Ovulated cumulus-oocyte complexes (COCs) were recovered by flushing each oviduct with 5 mL of DPBS supplemented with 2 mg/mL of BSA at room temperature. Not-ovulated COCs were aspirated with a 1ml syringe and a 25G needle from preovulatory follicles measuring 1-2 mm diameter.

Pools containing 10-12 oocytes were used to measure GSH in ovulated and not-ovulated oocytes. Ovulated and not-ovulated oocytes were denuded separately by washing them first in 0.1% hyaluronidase and then in DPBS-BSA, and by stripping off their cumulus cells, using a binocular stereoscopic microscope, Leica MZ75-200x. After that, oocytes were washed three times in stock buffer solution and were transferred to a microfuge tube in 5 µL. Then, 5 µL of phosphoric acid 1.25 M was added, and it was immediately frozen at -80ºC and stored until used.

COCs used to measure ATP were similarly denuded, without removing the corona radiate in order to limit excessive oocytes manipulation which can provoke stress-induced ATP release by the cells. After washing ovulated and not-ovulated oocytes separately 3 times in DPBS, 2-5 oocytes from each female were transferred to a straw in 50 µL of DPBS. Straws were stored in liquid nitrogen until they were used.

GSH Assay

Equipment and chromatographic conditions

Concentrations of GSH in 16h oocytes were determined according to the method described by Nolin et al. (2007) with some modifications. Samples were analysed in a Waters HPLC system (Milford, MA, USA) equipped with two 515 pumps, a 717 autosampler and a 745 fluorescence detector. The injected volume into the HPLC system was 50 µL. The separation was performed on a Waters (Milford, MA, USA) Symmetry C-18 column (100 mm x 4.6 mm x 3.5 µm) coupled with a Symmetry C-18 guard column (20 mm x 4.6 mm x 5 µm) at 32 ºC. Elution from the stationary phase was carried out at a flow rate of 1 ml min^{-1} using two mobile phases consisted of A: 0.1 M sodium acetate buffer (pH 4.5)/methanol [97:3 (v/v)] and B: methanol. The gradient changed linearly from 100% A to 97%:3% A:B in 0.5 min, from this point to 80%:20% in 8 min and in 1 min the mobile phase reached to 75%:25% A:B, then was maintained for 3.5 min and modified to initial 100% A in 0.5 min, conditions maintained until the run finished at 20 min. Excitation and emission wavelengths for the fluorescence detector were 385 and 515 nm, respectively. An eight-point calibration curve (7.81-125 pmol L^{-1} GSH/tube) containing 125 pmol L^{-1} of 2-mercaptopropionylglycine (MPG, internal standard) dissolved in ultrapure grade water was used daily. Retention times for GSH and MPG were 3.15 and 9.18 min respectively. The ratio GSH/MPG peak areas against the ratio of quantity injected for GSH and MPG were employed for plotting the calibration curve.

Standard preparation

Standards were prepared at the beginning of the validation experiment by weighting GSH and appropriate diluting it with ultrapure grade water at the concentrations previously given. The MPG internal standard was dissolved in ultrapure grade water to prepare a 125 mM solution. Standards were stored at −20ºC.

Sample preparation

MPG internal standard was added to the samples (50 µL) and briefly vortex-mixed. Following addition of TCEP (100 g/L; 10 µL) tubes were capped, briefly vortex-mixed, and incubated at room

temperature for 30 min. Trichloroacetic acid 100 g/L with 1mM EDTA (90 µL) was then added to each sample, briefly vortex-mixed and then centrifuged at 13,000×g for 10 min. Supernatant (50 µL) was added to autosampler vials containing 10 µL of 1.55 M NaOH, 125 µL of 0.125 M borate buffer, pH 9.5, with 4 mM EDTA, and 50 µL of 1 g/L SBD-F in borate buffer (0.125 M with 4 mM EDTA). Samples were capped, briefly vortex-mixed, incubated at 60ºC for 1 h, placed in the autosampler, and then a 10 µL aliquot was injected onto the HPLC system for analysis.

ATP Assay

Concentrations of ATP were determined using the bioluminescent somatic cell assay kit FL-ASC as previously described by Stojkovic *et al.* (2001). The oocytes contained in straws with 50 µL of DPBS were transferred into 5 ml plastic tubes equilibrated on ice (0°C) and 100 µL of ice-cold somatic cell reagent (FL-SAR) were added to all tubes. The mix was incubated for another 5 min on ice cold water. Subsequently, 100 µL of ice-cold assay mix (dilution 1:25 with ATP assay mix dilution buffer, FL-AAB) was added, and the tubes were kept for 5 min at room temperature in the darkness to avoid the flash reaction. Then, ATP content of the samples was measured using a luminometer (LB 9509 Junior; Berthold Technologies) with high sensitivity (0.01 pmol). In order to have simultaneous measurements of sample luminescence, tubes with oocytes were all measured once and then a second measure was performed in the reverse order. The mean of these two values expressed in Relative Light Unit was recorded to establish our final value. An eight-point standard curve (0-100 pmol/tube) was routinely included in each assay. The ATP content was determined by the formula derived from the linear regression of the standard curve and the luminescence values.

Traits

The following traits were recorded: the oocyte ATP concentration and the oocyte GSH concentration expressed in pmol/oocyte were calculated dividing the concentration of ATP or GSH in each sample by the number of oocytes in the sample; ovulation rate (OR), counted as the number of corpora haemorragica in both ovaries; recovery rate (RR), calculated as the ratio between recovered oocytes and ovulation rate.

Statistical analyses

The mean oocyte GSH concentration (GSH_{oocyte}) in ovulated and not-ovulated oocytes was analyzed fitting the model:

$$y_{ij} = \mu + M_i + e_{ij}$$

where M_i is the effect of maturational state of the oocytes and has 2 levels (mature or ovulated oocytes and immature or not-ovulated oocytes); and e_{ij} is the residual of the model.

The model used to analyse the mean oocyte ATP concentration in ovulated and not-ovulated oocytes was:

$$y_{ijkl} = \mu + M_i + S_j + p_k + e_{ijkl}$$

where S_j is the Session effect, with 2 levels; p_k is the permanent environmental effect of the female; and M_i and e_{ijkl} were explained before. The GLM procedure of SAS (SAS, 1998) was used for both analyses.

RESULTS AND DISCUSION

Raw means, standard deviations and maximum and minimum values of ovulation rate, recovery rate, oocyte GSH concentration and oocyte ATP concentration are summarized in Table 1.

As shown in Tables 1 and 2, GSH and ATP concentrations were higher in ovulated than in not-ovulated oocytes in agreement with other studies found in the literature (reviewed by Krisher, 2004).

The concentration of GSH determined by HPLC was 8.3 ± 0.5 pmol/oocyte in ovulated oocytes and 1.5 ± 0.5 pmol/oocyte in not-ovulated oocytes (Table 2). The concentration of ATP was 2.8 ± 0.1

Table 1: Raw means, standard deviations (SD) and maximum and minimum values of the oocyte GSH concentration and oocyte ATP concentration in ovulated and not-ovulated oocytes

		Mean	SD	Minimum	Maximum
GSH_{oocyte} (pmol/oocyte)	Not-ovulated	1.5	0.6	0.5	2.2
	Ovulated	8.3	2.0	5.2	10.2
ATP_{oocyte} (pmol/oocyte)	Not-ovulated	1.2	0.5	0.6	2.4
	Ovulated	2.8	0.9	0.8	5.0

pmol/oocyte in ovulated oocytes and 1.1 ± 0.2 pmol/oocyte in not-ovulated oocytes. To our knowledge, there is no other study reporting the GSH concentration measured by HPLC in rabbit oocytes. An enzymatic method has been used in another study in rabbits to determine the GSH concentration in ovulated oocytes, showing similar results (Laborda et al., 2008). In other species, the mean GSH concentration per oocyte determined by HPLC was approximately 8 pmol/oocyte for in vivo mature oocytes and 1.5 pmol/oocyte for immature oocytes (Funahashi *et al*., 1999 in rat; Gasparrini *et al*., 2003 in buffalo; Zuelke *et al*., 1997 in hamster). The oocyte concentrations of ATP in this study were similar to the concentrations measured in other studies in rabbits (Salvetti *et al*., 2010), pigs (Brad *et al*., 2003; Herrick *et al*., 2003), bovine (Stojkovic *et al*., 2001) and human (Slotte *et al*., 1990) with the same methodology (1.6-2.5 pmol/oocyte for *in vivo* mature oocytes and 0.9-1.8 pmol/oocyte for immature oocytes).

Table 2: Least square means and standard errors for oocyte GSH concentration and oocyte ATP concentration in ovulated and not-ovulated oocytes

	GSH_{oocyte} (pmol/oocyte)	ATP_{oocyte} (pmol/oocyte)
Not-ovulated	1.5 ± 0.5 [a]	1.1 ± 0.2 [a]
Ovulated	8.3 ± 0.5 [b]	2.8 ± 0.1 [b]

[a, b] different letters within column indicate significant differences, P<0.01.

The different GSH and ATP concentrations between ovulated and not-ovulated rabbit oocytes indicate that GSH and ATP are synthesized during oocyte maturation. High GSH and ATP concentrations have been related with oocyte quality (reviewed by Krisher 2004), although the relationship between oocyte quality and high levels of ATP has not been always positive (Brad *et al*., 2003; Herrik *et al*., 2003).

CONCLUSION

There are relevant differences in GSH and ATP concentrations between ovulated and not-ovulated rabbit oocytes, indicating a higher developmental competence in ovulated oocytes.

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REFERENCES

Brad A.M., Bormann C.L., Swain J.E., Durkin R.E., Johnson A.E., Clifford A.L., Krisher R.L. 2003. Glutathione and adenosine triphosphate content of *in vivo* and *in vitro* matured porcine oocytes. *Mol. Reprod. Dev., 64, 492-498.*

Funahashi H., Bandoh N., Nakahira S., Oh S., Tsuboi S. 1999. Changes in intracellular content of glutathione and thiols associated with γ-glutamyl cycle during sperm penetration and pronuclear formation in rat oocytes. *Zygote, 7, 301–305.*

Gasparrini B., Sayoud H., Neglia G., de Matos D.G., Donnay I., Zicarelli L. 2003.Glutathione synthesis during in vitro maturation of buffalo (Bubalus bubalis) oocytes: effects of cysteamine on embryo development. *Theriogenology, 60, 943–952.*

Herrick J.R., Brad A.M., Krisher R.L., Pope W.F. 2003. Intracellular ATP and glutathione concentrations in oocytes from first estrous, multi-estrous, and testosterone-treated gilts. *Anim.Reprod. Sci., 78, 123-131.*

Krisher R.L. 2004. The effect of oocyte quality on development. *J. Anim. Sci., 82 (E. Suppl.), E14-E23.*

Laborda P., Santacreu M.A., García E., Mocé E., Blasco A., Mocé M.L. 2008. Oocyte glutathione concentration in a rabbit line selected for ovulation rate. *9th World Rabbit Congress*. Verona, Italy. 375-380.

Laborda P., Mocé M.L., Santacreu M.A., Blasco A. 2011. Selection for ovulation rate in rabbits: genetic parameters, direct response and correlated response on litter size. *J. Anim. Sci*., 89, 2981-2987.

Luberda Z. 2005. The role of glutathione in mammalian gametes. *Biol. Reprod., 5, 5-17.*

Nolin T.D., McMenamin M.E., Himmelfarb J. 2007. Simultaneous determination of total homocysteine, cysteine, cysteinylglycine, and glutathione in human plasma by high-performance liquid chromatography: Application to studies of oxidative stress*. J. Chromatogr. B, 852, 554-561.*

Salvetti P., Buff S., Afanassieff M., Daniel N., Guérin P., Joly T. 2010. Structural, metabolic and developmental evaluation of ovulated rabbit oocytes before and after cryopreservation by vitrification and slow freezing. *Theriogenology, 74,847-855.*

Slotte H., Gustafson O., Nylund L., Pousette A. 1990. ATP and ADP in human pre-embryos. *Human Reprod., 5,319-322.*

Stojkovic M., Machado S.A., Stojkovic P., Zakhartchenko V., Hutzler P., Gonçalves P.B., Wolf E. 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation; correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol. Reprod., 64, 904-909*.

Zuelke, K.A., Jones D.P., Perreaults S.D. 1997. Glutathione oxidation is associated with altered microtubule function and disrupted fertilization in mature hamster oocytes. *Biol. Reprod., 57, 1413-1419.*