UNIVERSIDAD POLITECNICA DE VALENCIA

DEPARTAMENTO DE CIENCIA ANIMAL

# OOCYTE GLUTATHIONE CONCENTRATION IN A RABBIT LINE SELECTED FOR OVULATION RATE

*TÉSIS DE MÁSTER*

*Patricia Laborda Vidal*

*DIRECTORAS: Dra. Mª Antonia Santacreu Jerez*
*Dra. Lorena* Mocé *Cervera*

*Valencia, Diciembre de 2007*

# OOCYTE GLUTATHIONE CONCENTRATION IN A RABBIT LINE SELECTED FOR OVULATION RATE

**Laborda, P.[1], Santacreu, M.A. [1], García, E. [2], Mocé, E. [3], Mocé, M.L. [2]**

[1] Departamento Ciencia Animal, Universidad Politécnica Valencia, Valencia, Spain

[2] Departamento Producción Animal, Universidad Cardenal Herrera C.E.U., Valencia, Spain

[3] Centro Tecnología Animal (CTA-IVIA), Segorbe, Castellón, Spain

## ABSTRACT

The present study was designed to determine glutathione concentration in rabbit oocytes and to establish a relation between oocyte glutathione concentration and ovulation rate. Glutathione concentration is used to assess oocyte quality. A total of 59 does belonging to a line selected for ovulation rate during seven generations were mated with vasectomized males to induce ovulation. Females were slaughtered fifteen hours later and oocytes were collected by flushing the oviducts. Oocytes were processed and glutathione concentration was determined by the enzymatic recycling assay of the 5,5-dithio-bis (2-nitrobenzoic acid)-glutathione disulfide reductase. Mean glutathione concentrations were 8.6 ± 2.7 pmol/oocyte. Oocyte glutathione concentration was significantly lower in oocytes from females with high (7.6 ± 0.4 pmol/oocyte) and medium (8 ± 0.4 pmol/oocyte) ovulation rates than in oocytes from females with low ovulation rates (9.4 ± 0.4 pmol/oocyte). It seems that high ovulation rates could be associated with poorer oocyte quality than low ovulation rates.

Key words: Glutathione concentration, Ovulation Rate, Oocyte Quality, Rabbit

## INTRODUCTION

Selection for ovulation rate was proposed as an indirect way to improve litter size. In pigs, mice and rabbits, response in ovulation rate was high, but correlated response in litter size was small due to an increase of prenatal

mortality (Ibañez et al., 2006; Mocé et al., 2007 in rabbit; Bradford, 1969; Land and Falconer, 1969 in mice; Lamberson et al., 1991; Rosendo et al., 2007 in pig). High ovulation rate might cause ovulation of inmature oocytes (Koenig et al., 1986), and might increase prenatal mortality.

In order to be a competent oocyte, cytoplasmic and nuclear maturation has to be completed successfully. Glutathione (GSH) is considered as a relevant biochemical marker of the cytoplasmic maturation and consequently of the development and viability of oocytes (Zuelke et al., 2003). Mature oocytes have higher glutathione concentration than inmature ones (Kim et al., 2007; Luciano et al., 2006). Glutathione is a tripeptide thiol (γ-glutamylcysteinylglycine), the major non-enzymatic sulphydryl compound in cells (Luberda, 2005). It plays an important role in protecting cells against the effects of the reactive oxygen intermediates and free radicals, also called oxygen reactive species (ROS) (Meister, 1983 quoted by Luciano et al., 2006) and maintaining the intracellular redox status. Glutathione also regulates protein and DNA synthesis and participates in microtubule assembly (Luciano et al., 2006). After fertilization, it plays an active role in the formation of the male pronucleus, which depends upon the maturational state of the oocyte (Yoshida et al., 1993). Glutathione synthesis is a critical part of oocyte cytoplasmic maturation.

The aim of this work is to determine oocyte glutathione concentration in rabbits and to study its relation to ovulation rate in a line selected for ovulation rate.

## MATERIALS AND METHODS

### Reagents and Media

All chemicals and reagents were purchased from Sigma Chemical Company, Madrid, Spain: calcium-free Dulbecco´s Phosphate Buffered Saline (DPBS), Bovine Serum Albumine (BSA), hyaluronidase (Hyaluronidase), EDTA

(ethylenediaminetetraacetic acid), 5,5-dithio-bis (2-nitrobenzoic acid), glutathione (GSH), glutathione reductase from Bakers Yeast, NADPH, phosphoric acid, sodium dihydrogen phosphate monohydrate ($NaH_2PO_4H_2O$), di-sodium hydrogen phosphate ($Na_2HPO_4$).

The stock buffer solution pH 7.2 is prepared by mixing 2 solutions: solution A, pH 4 ($NaH_2PO_4H_2O$, 27.6 mg/mL and EDTA, 3.72 mg/mL) and solution B, pH 8 ($Na_2HPO_4$, 28.39 mg/mL and EDTA, 3.72 mg/mL).

Animals

All experimental procedures involving animals were approved by the Polytechnic University of Valencia Research Ethics Committee.

A total of 59 multiparous, lactating and non-lactating females that belonged to a line selected for ovulation rate during 7 generations were mated with vasectomized males to induce ovulation. They were slaughtered 15 hours after mating and the reproductive tracts were removed.

Does were housed individually at the experimental farm of the Polytechnic University of Valencia. Animals were fed with a commercial diet and kept under controlled 16 h light- 8 h darkness photoperiod.

Oocytes

Oocyte glutathione concentration ($GSH_{oocyte}$) was measured as described by Funahashi et al., (1994) with slight modifications. Oocytes were recovered by flushing each oviduct with 5 mL of DPBS supplemented with 2 mg/mL of BSA at room temperature. The number of corpora haemorragica in both ovaries was counted in order to estimate the ovulation rate (OR). Recovery rate (RR) was calculated as the ratio between recovered oocytes and ovulation rate.

Denuded oocytes were obtained by washing cumulus-oocyte complexes in 0.1% hyaluronidase, and then in DPBS-BSA, and by stripping off their

cumulus cells by careful aspiration in and out of a narrow-bore glass pipette 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 -20ºC. Oocytes from each female were frozen in separate microfuge tubes.

Glutathione determination:

Glutathione determination is based on an enzymatic recycling assay of the 5,5-dithio-bis (2-nitrobenzoic acid)-glutathione disulfide reductase (DTNB-GSSG reductase). The DTNB-GSSG reductase recycling assay is a specific and sensitive procedure (Anderson, 1985). As indicated in reaction (1) the reduced form of glutathione (GSH) is oxidized by 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) to give glutathione disulfide (GSSG) with formation of 5-thio-2-nitrobenzoic acid (TNB). GSSG is reduced to GSH by the action of the highly specific DTNB-GSSG reductase and NADPH. The rate of TNB formation is followed at 412 nm and is proportional to GSH (reviewed by Anderson, 1985).

$$2\ GSH + DTNB \longrightarrow GSSG + TNB \qquad (1)$$

$$GSSG + NADPH + H^{+} \xrightarrow[\text{REDUCTASE}]{\text{DTNB-GSSG}} 2\ GSH + NADP^{+} \qquad (2)$$

Assay

The assay was performed at room temperature and within two different sessions. At least 10 (15 ± 2.6) oocytes per microfuge tube were used for the glutathione assay. Five samples were prepared with the following glutathione concentrations: 0.00mM, 0.01mM, 0.02mM, 0.10mM and 0.20mM. After addition of 5 µL of phosphoric acid, 100 µL of 6 mM DTNB, 700 µL of NADPH (0.3 mg/mL) and 190 µL of distilled water, 10 µL of glutathione reductase (266 U/mL) was added to initiate the assay. The increasing absorbance was

monitored every 30 seconds during 3 minutes at 412 nm (UV) with the spectrophotometer (Thermo electron corporation, Heλlos α). Standard curves were estimated with the increasing concentrations of glutathione and the absorbance measured.

After that, the samples were thawed at room temperature and were assayed under the same conditions. The amount of glutathione in each sample was determined by comparison with the standard curve. This amount was divided by the number of oocytes in the sample to obtain glutathione concentration per oocyte ($GSH_{oocyte}$).

Traits

The following traits were recorded: ovulation rate (OR), recovery rate (RR) and oocyte glutathione concentration ($GSH_{oocyte}$). These traits were described before.

Statistical Analisys

Descriptive analyses were realized for OR, RR and $GSH_{oocyte}$ with Statgraphics Plus 4.0.

Oocyte glutathione concentration ($GSH_{oocyte}$) was analyzed fitting the model:

$$y_{ijkl} = \mu + S_i + OR_j + OOF_k + e_{ijkl}$$

where S is the effect of session, with 2 levels, OR is the effect of ovulation rate, with 3 levels (Level $_{OR}$ 1: low ovulation rate (10-14 corpora haemorragica); Level $_{OR}$ 2: medium ovulation rate (15-16 corpora haemorragica); Level $_{OR}$ 3: high ovulation rate (17-24 corpora haemorragica)) and OOF is the effect of number of oocytes frozen, with 3 levels (Level $_{OOF}$ 1: low number of oocytes frozen (10-13 oocytes frozen); Level $_{OOF}$ 2: medium number of oocytes frozen (14-16 oocytes frozen); Level $_{OOF}$ 3: high number of oocytes frozen (17-21 oocytes frozen)). The GLM procedure of SAS (SAS, 1998) was used.

## RESULTS AND DISCUSSION

In the present experiment, 3 of the 59 does did not ovulate after mating and were excluded from the analysis. Raw means, standard deviations and maximum and minimum values of ovulation rate, recovery rate and oocyte glutathione concentration are summarized in table 1.

**Table 1:** Raw means, standard deviation and maximum and minimum values of ovulation rate, recovery rate and oocyte glutathione concentration

| | Mean | Standard deviation | Minimum | Maximum |
|---|---|---|---|---|
| OR | 16.0 | 2.7 | 10.0 | 24.0 |
| RR (%) | 96.1 | 5.6 | 78.6 | 100.0 |
| $GSH_{oocyte}$ | 8.6 | 2.7 | 3.9 | 14.1 |

OR: ovulation rate, expressed as number of corpora haemorragica; RR (%): recovery rate expressed as the ratio between recovered oocytes and ovulation rate; $GSH_{oocyte}$: oocyte glutathione concentration, expressed in pmol/oocyte

Mean ovulation rate is high compared to other studies in rabbits (García and Baselga, 2002; Santacreu et al., 2005); in this study, females belonged to a line selected for ovulation rate during seven generations. Recovery rate is high and similar to results published by Santacreu et al. (1997) using vasectomized males.

In this experiment the mean glutathione concentration per oocyte was 8.6 pmol and the number of oocytes frozen range from 10 to 21 with a mean value of 15. To our knowledge, there is no study reporting the measurement of oocyte glutathione in rabbits. The results obtained in this experiment indicate that the method employed is able to determine glutathione content when at least 10 oocytes per microfuge tube are used.

A great variability among species in terms of glutathione concentration has been reported for *in vivo* matured oocytes. In pigs, Brad et al. (2003) studied a mean number of 10 oocytes per tube and obtained 36.26 ± 11.01 pmol/oocyte. Other studies found 19.2 pmol/oocyte in dogs (Kim et al., 2007)

and 5.89 ± 0.48 pmol/oocyte in equine (Luciano et al., 2006). In these studies, the mean number of oocytes per tube was 10 and 11 respectively.

Table 2 presents oocyte glutathione concentration at the different levels of ovulation rate. Oocytes from females with low ovulation rates have higher glutathione concentrations than oocytes from females with medium and high ovulation rates. These differences are significant and relevant. Glutathione concentration is a tool to determine *in vitro* matured oocytes´ quality. High glutathione concentrations indicate good oocyte quality. Moreover *in vivo* matured oocytes have higher glutathione concentration than inmatured oocytes. Thus, the data obtained in this experiment support that females with low ovulation rate might have better oocyte quality than females with high ovulation rate. Females with high ovulation rate could ovulate inmature oocytes that might give rise defective embryos. These might not be able to undergo normal development and might die. However, no studies reporting the association between glutathione concentration and ovulation rate have been found.

**Table 2:** Least square means and standard errors for oocyte glutathione concentration ($GSH_{oocyte}$)

| | $GSH_{oocyte}$ (pmol/oocyte) | | $GSH_{oocyte}$ (pmol/oocyte) |
|---|---|---|---|
| Level $_{OR}$ 1 | 9.4 ± 0.4 $^{a}$ | Level $_{OOF}$ 1 | 6.5 ± 0.5 $^{a}$ |
| Level $_{OR}$ 2 | 8.0 ± 0.4 $^{b}$ | Level $_{OOF}$ 2 | 8.7 ± 0.3 $^{b}$ |
| Level $_{OR}$ 3 | 7.6 ± 0.4 $^{b}$ | Level $_{OOF}$ 3 | 9.8 ± 0.6 $^{b}$ |

Level $_{OR}$ 1: low ovulation rate (10-14 corpora haemorragica); Level $_{OR}$ 2: medium ovulation rate (15-16 corpora haemorragica); Level $_{OR}$ 3: high ovulation rate (17-24 corpora haemorragica); Level $_{OOF}$ 1: low number of oocytes frozen (10-13 oocytes frozen); Level $_{OOF}$ 2: medium number of oocytes frozen (14-16 oocytes frozen); Level $_{OOF}$ 3: high number of oocytes frozen (17-21 oocytes frozen);
$^{a, b}$ different letters within column indicate significant differences, P<0.05

Oocyte glutathione concentration at different levels of oocytes frozen can be observed in table 2. Oocyte glutathione concentration was significantly lower for a small number of oocytes frozen than for a medium and a high number of oocytes frozen.

Considerable variation between sessions was observed for oocyte glutathione concentration (table 3). Variations in glutathione mean values between sessions could be explained by the laboratory temperature, which increased during the second session. The enzymatic reaction accelerates with the increasing temperature; therefore, oocyte glutathione concentration is higher in the second session.

**Table 3:** Least square means and standard errors of oocyte glutathione concentration ($GSH_{oocyte}$) in session 1 and 2

| | Session 1 | Session 2 |
|---|---|---|
| $GSH_{oocyte}$ (pmol/oocyte) | 6.1 ± 0.3 [a] | 10.6 ± 0.3 [b] |

[a, b] different letters within row indicate significant differences, P<0.05

As conclusions, we can say that oocytes from females with high ovulation rate have lower glutathione concentration than oocytes from females with low ovulation rate. Given that glutathione concentration is a measure of oocyte quality, females with high ovulation rate seem to have poor oocyte quality. That could be a possible reason for the increase of prenatal mortality found in the experiments of selection for ovulation rate. Besides, it is important to consider for further studies that the measurement of oocyte glutathione content varies with the number of oocytes frozen and with the laboratory temperature.

## ACKNOWLEDGMENTS

The authors would like to thank to Centro Tecnología Animal (CTA-IVIA, Segorbe, Castellón, Spain) and Dr. R. Romar for technical support in GSH assay.

This research was supported by Comisión Interministerial de Ciencia y Tecnología (CICYT) (AGL2005-07624-C03-01).

**REFERENCES:**


Anderson, M.E., 1985. Determination of glutathione and glutatathione disulfide in biological samples. Methods Enzymol. 113: 548-55.

Blasco, A., Bidanel, J.P., Bolet, G., Haley, C.S., Santacreu, M.A., 1993. The genetics of prenatal survival of pigs and rabbits: a review. Livest. Prod. Sci. 37: 1-21.

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

Bradford, G.E., 1969. Genetic control of ovulation rate and embryo survival in mice. I. Response to selection. Genetics 61: 905-921.

Funihashi, H., Cantley, T.C., Stumpf, T.T., Terlouw, S.L., Day, B.N., 1994. *In vitro* development of *in vitro* matured porcine oocytes following chemical activation or *in vitro* fertilization. Biol. Reprod. 50: 1072-1077.

García, M.L., Baselga, M., 2002. Estimation of genetic response to selection in litter size of rabbits using a cryopreserved control population. Livest. Prod. Sci. 74: 45-53.

Santacreu, M.A., Climent, A., Gallego, M., Blasco, A., 1997. Estudio de la tasa de fecundación y la calidad de los embriones en dos líneas seleccionadas de forma divergente por capacidad uterina en conejo. VII Jornadas sobre Producción Animal. ITEA Vol Extra 18 (I): 378-380.

Ibáñez, N., Santacreu, M.A., Martínez, M., Climent, A., Blasco, A., 2006. Selection for ovulation rate in rabbits. Livest. Sci. 101: 126-133.

Kim, M.K., Hossein, M.S., Oh H.J., Fibrianto, H.Y., Jang, G., Kim, H.J., Hong, S.G., Park, J.E., Kang, S.K., Lee, B.C., 2007. Glutathione content of in vivo and in vitro matured canine oocytes collected from different reproductive stages. J. Vet. Med. Sci. 69: 627-632.

Koenig, J.L.F., Zimmermann, D.R., Eldridge, F.E., Kopf, J.D., 1986. The effect of superovulation and selection for high ovulation rate on chromosomal abnormalities in swine ova. J. Anim. Sci. 63 (Suppl.1): 202.

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

Lamberson, W.R., Johnson R.K., Zimmerman, D.R., Long, T.E., 1991. Direct responses to selection for increased litter size, decreased age at puberty, or random selection following selection for ovulation rate in swine. J. Anim. Sci. 69: 3129-3143.

Land, R.B., Falconer, D.S., 1969. Genetic studies of ovulation rate in the mouse. Genet. Res. 13: 25-45.

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

Luciano, A.M., Goudet, G., Perazzoli, F., Lahuec, C., Gérard, N., 2006. Glutathione content and glutathione peroxidase expresión in *in vivo* and *in vitro* matured equine oocytes. Mol. Reprod. Dev. 73: 658-666.

Meister, A., 1983. Selective modification of glutathione metabolism. Science 220: 472-477.

Mocé M.L., Santacreu, M.A., Laborda, P., Climent, A., Blasco A., 2007. Selección por tasa de ovulación en conejo. Estimación de la respuesta en tasa de ovulación y tamaño de camada. XII Jornadas sobre Producción Animal. ITEA Vol Extra 28 (II): 390-392.

Rosendo, A., Druet, T., Gogué, J., Bidanel, J.P., 2007. Direct responses to six generations of selection for ovulation rate or prenatal survival in Large White pigs. J. Anim. Sci. 85: 356-364.

Santacreu, M.A., Mocé, M.L., Climent, A., Blasco, A., 2005. Divergent selection for uterine capacity in rabbits. II. Correlated response in litter size and components estimated with a cryopreserved control population. J. Anim. Sci. 83: 2303-2307.

Watson, A.J., 2007. Oocyte cytoplasmic maturation: a key mediator of oocyte and embryo developmental competence. J. Anim. Sci. 85 (E. Suppl.): E1-E3.

Yoshida, M., Ishigaki, K., Nagai, T., Chikyu, M., Pursel, V.G., 1993. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol. Reprod. 49: 89-94.

Zuelke, K.A., Jeffay, S.C., Zucker, R.M., Perreault, S.D., 2003. Glutathione (GSH) concentrations vary with the cell cycle in maturing hamster

oocytes, zygotes, and pre-implantation stage embryos. Mol. Reprod. Dev. 64: 106-112.