OOCYTE GLUTATHIONE CONCENTRATION IN A RABBIT LINE SELECTED FOR OVULATION RATE

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ABSTRACT

The present study was designed to determine glutathione concentration in rabbit oocytes and to establish its relation to 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. Glutathione concentration ranged from 4.7 to 10.3 pmol/oocyte. Ovulation rate was classified into three levels: low (10-14 corpora haemorragica), medium (15-16 corpora haemorragica) and high (17-24 corpora haemorragica). Oocyte glutathione concentration was significantly lower in oocytes from females with high (6.6±0.3 pmol/oocyte) and medium (7.3±0.4 pmol/oocyte) ovulation rates than in oocytes from females with low ovulation rates (8.4±0.3 pmol/oocyte). It seems that high ovulation rates could be associated with poorer oocyte quality in comparison with 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 and mice, response to selection for ovulation rate was high, but the correlated response in litter size was not significantly different from zero due to an increase of prenatal mortality (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 immature oocytes (Koenig *et al*., 1986), and might increase prenatal mortality.

In order to develop adequate oocytes, 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 immature ones (rewieved by Rausell and Tarín, 2005). Glutathione is 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 (Meister, 1983 quoted by Luciano *et al*., 2006) and maintaining the intracellular redox status. After fertilization, glutathione provides reducing power to initiate chromatin decondensation, prior to male pronucleus formation. Formation of the male pronucleus depends upon the maturational state of the oocyte (reviewed by Luberda, 2005; Rausell and Tarín, 2005). 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. Animals belonged to a line selected for ovulation rate (Ibañez *et al.*, 2006). A total of 59 multiparous, lactating and non-lactating females from seven 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 of this solution. Then, 5 µl of phosphoric acid 1.25 M was added, and it was immediately frozen at -20°C. Oocytes from each female were frozen in separate tubes.

Assay

Glutathione determination is based on an enzymatic recycling assay of the 5,5-dithio-bis (2-nitrobenzoic acid)-glutathione disulfide reductase (DTNB-GSSG reductase). This is a specific and sensitive procedure (Anderson, 1985).

The assay was performed at room temperature and within two different sessions. At least 10 oocytes per tube were used for the glutathione assay, mean was 15 ± 2.6 oocytes per tube. Five samples were prepared with the following glutathione concentrations: 0.00 mM, 0.01 mM, 0.02 mM, 0.10 mM and 0.20 mM. 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}).

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. Sixteen females were included in level $_{OR}$ 1, 15 females in Level $_{OR}$ 2 and 19 females in Level $_{OR}$ 3.

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	16.1	2.8	10.0	24.0
RR	96.1	5.6	78.6	100.0
GSH_{oocyte}	7,6	1.3	4.7	10.3

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 if 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 varied between 4.7 and 10.3 pmol. To our knowledge, there is no study reporting the measurement of oocyte glutathione in rabbits. 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. Zuelke *et al*. (2003) obtained 3.24 ± 0.49 pmol/oocyte in hamster (25-30 oocytes/tube). 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. In our experiment, the number of oocytes frozen ranged from 10 to 21 with a mean value of 15.

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* mature oocytes have higher glutathione concentration than immature 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 immature oocytes that might give rise defective embryos that 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}		GSH_{oocyte}
Level $_{OR}$ 1	8.4 ± 0.3 [a]	Level $_{OOF}$ 1	6.2 ± 0.4 [a]	Session 1	6.7±0.3 [a]
Level $_{OR}$ 2	7.3 ± 0.4 [b]	Level $_{OOF}$ 2	7.6 ± 0.2 [b]	Session 2	8.1±0.2 [b]
Level $_{OR}$ 3	6.6 ± 0.3 [b]	Level $_{OOF}$ 3	8.5 ± 0.5 [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); GSH_{oocyte}: oocyte glutathione concentration, expressed in pmol/oocyte
[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 2). Variations in glutathione mean values between sessions could be explained by the laboratory temperature, which increased more during the second session. The enzymatic reaction accelerates with the increasing temperature; therefore, oocyte glutathione concentration is higher in the second session.

CONCLUSIONS

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 in prenatal mortality found in the experiments of selection for ovulation rate.

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