EFFECT OF TISSUE INHIBITOR OF METALLOPROTEINASE 1 (*TIMP1*) GENE FOR EMBRYO SURVIVAL AND DEVELOPMENT IN A F2 RABBIT CROSS

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ABSTRACT

The effect on early embryo survival and development of a diallelic 1423A>G SNP found in the promoter region of the tissue inhibitor of metalloproteinase 1 gene (*TIMP1*) was studied in an F_2 rabbit population. The F_2 population comes from High (H) and Low (L) lines selected divergently for uterine capacity during 10 generations. A total of 171 and 159 intact does were slaughtered at 48 h and 72 h of gestation respectively to determine whether *TIMP1* gene was associated with ovulation rate, fertilization rate, early embryo survival and embryonic stage of development. We did not find differences between homozygote genotypes in ovulation rate and fertilization rate at 48 and 72 h of gestation. At 48 h of gestation, AA genotype showed a similar early embryo survival and embryonic stage of development to GG genotype. However AA genotype, the genotype most frequent in the line selected to increase uterine capacity, had 0.88 embryos (P(D>0)=90%) more at 72 h of gestation than GG genotype and also had a higher embryonic stage of development, showing a lower percentage of early morulae (D_m=-16.33%, P(D<0)=92%), a higher percentage of compacted morulae (D_m=19.85%, P(D>0)=92%), and similar percentage of blastocysts (D=3.52%) at this stage of gestation. A more advanced embryonic stage of development seems to be associated with a higher embryo survival at 72 h of gestation.

Key words: Rabbits, *TIMP1* gene, Embryo survival and development, F_2 population.

INTRODUCTION

Selection on uterine capacity has been proposed as an alternative method to improve litter size (Blasco *et al.*, 1994, in rabbits, Bennett and Leymaster, 1989, in pigs; Clutter *et al.*, 1990, in mice). In the Polytechnic University of Valencia (UPV), a divergent selection experiment on uterine capacity in rabbits was carried out (Argente *et al.*, 1997). After 10 generations of divergent selection, Santacreu *et al.* (2005) found a difference between the High line (H) and the Low (L) line of 1.79 embryos at implantation and 2.35 kits at birth in intact females. Besides, the H line showed a more advanced embryonic stage of development at 48 h (Peiró *et al.*, 2007a) and at 72 h of gestation (Mocé *et al.*, 2004), which could explain a lower mortality in the H line at 72 h of gestation (Mocé *et al.*, 2004) and at implantation (Santacreu *et al.*, 2005) than in the L line. The results of a segregation analyses performed by Argente *et al.* (2003) suggested the existence of major genes with a moderate effect on uterine capacity and a large effect on number of implanted embryos in this population.

The matrix metalloproteinases (MMPs) are involved in endometrial tissue remodelling in association with embryogenesis and angiogenesis processes that take place during the normal progress of gestation. The enzymatic activity of the MMPs is specifically regulated by tissue inhibitors of metalloproteinases (TIMPs). One of these tissue inhibitors of metalloproteinases (TIMP1) seems to play an important role in embryonic development (Hwang *et al.*, 2000). Estellé *et al.* (2006) reported

higher expression of *TIMP1* gene in the oviduct at 62 h of gestation in the H line than in the L line of selection experiment on uterine capacity of UPV.

A diallelic 1423A>G SNP was found in the promoter region of *TIMP1* gene and an association was detected between the frequency of this SNP and the lines of the selection experiment on uterine capacity, being the allele A more frequent in the H line (Merchán, 2007). The objective of this study was to analyze the effect of 1423A>G SNP of *TIMP1* gene on embryo survival and development at 48 and 72 h of gestation in an F2 rabbit population. The F2 population was generated from reciprocal cross of the H and L lines of divergent selection experiment on uterine capacity by UPV.

MATERIALS AND METHODS

Animals

An F_2 rabbit population was used in this study. This population came from reciprocal cross of the High (H) and Low (L) lines of a divergent selection experiment on uterine capacity (more details in Peiró *et al.*, 2007b). Does were mated with F_2 males. All animals were individually housed in cages from 18 weeks of age in the experimental farm of Universidad Miguel Hernández de Elche. They were fed a commercial diet. The photoperiod used was 16 h light: 8 h dark. A total of 171 and 159 females were slaughtered respectively at 48 or 72 h post coitus in their fifth gestation by intravenous injection of sodium thiopental at a dose of 50 mg/kg body weight (Tiobarbital®, B. Braun Medical S.A., Barcelona, Spain). All data came from non-lactating females, the range in the interval between the last weaning and the slaughter of female was from 3 to 140 days. The reproductive tract was collected after slaughtering. The number of corpora lutea was recorded on both ovaries. Oviducts and uterine horns were separated and flushed once with 5 and 10 ml of 150 mM ammonium bicarbonate solution at room temperature in order to recover and count the number of cleaved and uncleaved ova. The total number of embryos (TE) and oocytes (OO) were estimated as the number of cleaved and uncleaved ova, respectively. Embryos were classified as normal (NE) or abnormal (AE) according to morphological criteria (Hafez, 2000). At 48 h of gestation, all embryos and oocytes were recovered from oviducts (96% of the total ova shed). The normal embryos were classified as early morulae (EM) or compact morulae (CM). At 72 h of gestation, embryos and oocytes were recovered from oviducts and uterine horns (86% of the total ova shed). The normal embryos were classified as EM, CM and blastocysts (B).

Traits

The measured variable were ovulation rate (OR), estimated as the number of corpora lutea, fertilization rate (% FR = 100 x (TE/TE + OO)), percentage of early morulae (% EM = 100 x EM/NE), percentage of compacted morulae (% CM = 100 x CM/NE) and percentage of blastocysts (% B = 100 x B/NE). Early embryo survival (EES) was analyzed as the number of normal embryo recovered fitting ovulation rate as a covariate.

Genotyping of the rabbit *TIMP1* gene in an F_2 population

Genomic DNA was isolated from 80 µl venous blood collected in EDTA following the protocol of the ABI PRISM™ 6100 Nucleic Acid PrepSation (Applied Biosystem). Genotyping of the 1423A>G SNP in the promoter region of *TIMP1* gene was performed by Pyrosequencing (Pyrosequencing AB) in a PSQ HS 96 instrument (Merchán, 2007). The animals genotyped as A/A_{1423}, A/G_{1423} and G/G_{1423} are named AA, AG and GG, respectively.

Statistical Analysis

Ovulation rate at 48 and 72 h of gestation was analyzed with the following model:

$$y_{ijklmno} = \mu + YS_i + FH_j + I_k + O_l + G_m + S_n + e_{ijklmno}$$

where YS_i was the effect of year-season (with 3 levels), FH_j was the effect of hemorrhagic follicles (with 3 levels: zero, between one to five follicles and six or more), I_k was the effect of interval between weaning of the last litter and the slaughter of doe (with 2 levels: until one month or more), O_l is the effect of operator (with 3 levels) and G_m is the effect of TIMP1 gene genotype (with 3 levels: AA, AG and GG) and S_n was the effect of the time of gestation (with 2 levels: 48 h and 72 h after mating).

Fertilization rate and embryonic stage of development at 48 h of gestation of 11 AA, 87 AG and 73 GG were analyzed using the following model:

$$y_{ijklmn} = \mu + YS_i + FH_j + I_k + O_l + G_m + e_{ijklmn}$$

where the effects are described before. The EES was analyzed with the same model, including OR as a covariate.

Fertilization rate and embryonic stage of development at 72 h of gestation of 7 AA, 80 AG and 72 GG were analyzed using the following model:

$$y_{ijklmno} = \mu + YS_i + FH_j + I_k + O_l + G_m + U_n + e_{ijklmno}$$

where U_n was the effect of presence or absence of embryos in the uterus. It is more probably to loose embryos when the embryos are within oviducts and uterine horns than when those are only in oviducts, thus this effect was considered when analyses at 72 h of gestation were performed. The other effects were described before. The EES was analyzed with the same model, including OR as a covariate.

Traits were analyzed using a Bayesian approach. Data are conditionally distributed as: $y \mid b, \sigma^2_e \sim N(Xb, I\sigma^2_e)$ where b contains the effects to be estimated. The know incidence matrix is X and I is the identity matrix. Bounded uniform priors were used for all unknown parameters. Marginal posterior distributions of all unknowns were estimated using Gibbs sampling. A chain of 120,000 samples with a burn-in period of 20,000 was used. Convergence was tested using Z criterion of Geweke (Sorensen and Gianola, 2002) and Monte Carlo sampling errors were computed using time-series procedures described by Geyer (1992).

An advantage of the Bayesian approach trough MCMC procedures is the possibility of easy construction of all kinds of confidence intervals and probability computation. Thus, it is possible to calculate the shortest interval containing the true value with a probability of 95% ($HPD_{95\%}$). In our case, we are interested in estimating differences between genotypes, thus we can calculate the probability of this difference being higher than zero or higher than a relevant value (Pr in Table 1). A relevant value (R) is a quantity under which this difference has no biological or economical meaning. We also estimate the probability of this difference being lower in absolute value than relevant value (i.e. the probability of both genotypes being similar in biological or economical terms, Ps in Table 1). When the marginal posterior mean of the difference between genotypes is higher than zero, Pr is the probability that the difference is higher than R. However, if the marginal posterior mean of the difference between genotypes is lower than zero, Pr is the probability that the difference being lower than –R. The Pr and Ps allow us to distinguish the case in which there is no difference between genotypes from the case in which we do not find difference between genotypes because the precision is low. In the last case, Ps and Pr are low and $HPD_{95\%}$ is high.

RESULTS AND DISCUSSION

Features of the estimated marginal posterior distributions of the differences (D_m) between AA and GG genotypes of *TIMP1* gene for OR, FR, EES and embryo development are presented in Table 1. The AA and GG genotypes were respectively more frequent in animals belonging to the H and L lines of the divergent selection experiment on uterine capacity (Merchán, 2007). Marginal posterior distributions were approximately normal, thus mode, mean and median were similar, and only the posterior mean of the difference is showed. All Monte Carlo standard errors (MCse) were very small and lack of convergence was not detected by the Geweke test.

Table 1: Features of the estimated marginal posterior distributions of the differences between AA and GG genotypes of the A>G SNP *TIMP1* gene in ovulation rate (OR), fertilization rate (% FR), early embryo survival (EES) and percentage of early morulae (% EM), compacted morulae (% CM) and blastocysts (% B) in F2 population at 48 and 72 h of gestation

	D_m	$HPD_{95\%}$	P (%)	R	Ps (%)	Pr(%)	MCse	Z
OR	0.38	-0.78 , 1.50	74	0.5	52	41	0.006	-0.022
48 h of gestation								
%FR	0.94	-2.82 , 4.39	70	3.5	91	8	0.020	-0.169
EES	-0.01	-0.92 , 0.89	51	0.25	42	29	0.004	0.625
%EM	-3.28	-22.73 , 17.66	62	8	53	33	0.087	-0.388
72 h of gestation								
%FR	3.40	-2.51 , 8.73	88	3.5	49	50	0.011	0.902
EES	0.88	-0.55 , 2.21	90	0.25	12	83	0.006	-0.999
%EM	-16.33	-39.40 , 5.96	92	8	22	76	0.113	0.902
%CM	19.85	-7.68 , 46.44	92	8	17	81	0.135	0.902
%B	3.52	-16.78 , 24.01	56	8	25	43	0.009	-0.671

D_m: posterior mean of the difference between AA and GG genotypes; $HPD_{95\%}$: highest posterior density region at 95%; P: P(D>0) when D>0 and P(D<0) when D<0; R: assumed relevant difference between genotypes; Ps: probability of similarity (probability of the absolute value of D_m being lower than R); Pr: probability of relevant (P(D>R) when D>0 and P(D<R) when D<0); MCse: Monte Carlo standard error; Z: Z-score of the Geweke test

We consider a difference of 0.5 ova for OR and 3.5% for FR as relevant value. This corresponds to 0.5 embryos, half of difference for the number of embryos found between H and L lines at 72 h of gestation by Mocé *et al.* (2004). The AA genotype showed higher OR than GG genotype (P(D>0)=74%, Table 1), however, this difference was not relevant (D_m=0.38 ova), although the precision was low. At both stages of gestation, there were no relevant differences in FR among the homozygote genotypes (D_m=0.94% and D_m=3.4% at 48 and 72 h of gestation respectively). These results agree with previous results in the H and L lines, where no relevant differences in OR and FR were found (Mocé *et al.*, 2004; Peiró *et al.*, 2007a).

For ESS, 0.25 embryos is proposed as a relevant difference. This corresponds to half of the relevant value for the number of embryos at 62 h of gestation established in the paper of Peiró *et al.* (2007a). The relevant value for all the embryonic stage of development was 8%. This value was established as one third of the phenotypic standard deviation of the trait (Peiró *et al.*, 2007b). At 48 h of gestation, there was no difference between AA and GG genotypes for EES (D_m=-0.01 embryos), although the estimation had a low precision. In concordance with this result, Peiró *et al*. (2007a) obtained no relevant difference in EES at this stage of gestation when comparing the H and L lines. Besides, both homozygote genotypes also showed similar embryonic stage of development. At this stage of gestation, the H line showed a more advanced embryonic development than the L line (Peiró *et al.*, 2007a).

At 72 h of gestation, AA genotype had a higher EES than GG genotype (P(D>0)=90%) and the difference was relevant (D_m=0.88 embryos, Pr=83%). Embryonic development was more advanced in AA genotype, showing this genotype a lower percentage of early morulae (P(D<0)=92%, D_m=-16.33% and Pr=76%), a higher percentage of compacted morulae (P(D>0)=92%, D_m=19.85% and Pr=81%), and similar percentage of blastocysts (D_m=3.52%). These results agree with more advanced embryonic stage of development and higher embryo survival reported by Mocé *et al.* (2004) in the H line at 72 h of gestation. In AA genotype, a more advanced embryonic stage of development seems to be associated with a higher embryo survival at 72 h of gestation (0.88 embryos) and at implantation (2 embryos; Merchán, 2007). However, no difference between both homozygote genotypes was found at birth (Merchán, 2007).

CONCLUSIONS

The diallelic 1423A>G SNP found in the promoter region of *TIMP1* gene seems to be involved in development of embryo and its survival at 48 and 72 h of gestation.

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