JOURNAL OF EXPERIMENTAL ZOOLOGY 311A:448–452 (2009)

A Journal of Integrative Biology

Changes in Testosterone or Temperature During the In Vitro Oocyte Culture Do not Alter the Sex Ratio of Bovine Embryos

CARMEN DÍEZ[1]*, PABLO BERMEJO-ALVAREZ[2], BEATRIZ TRIGAL[1], JOSÉ NÉSTOR CAAMAÑO[1], MARTA MUÑOZ[1], IRENE MOLINA[1], ALFONSO GUTIÉRREZ-ADÁN[2], SUSANA CARROCERA[1], DAVID MARTÍN[1], AND ENRIQUE GÓMEZ[1]

[1]*SERIDA, Área de Genética y Reproducción Animal, Gijón, Spain*

[2]*Dpto de Reproducción Animal y Recursos Zoogenéticos, INIA, Madrid, Spain*

ABSTRACT High follicular testosterone levels have been associated with a skew in the sex ratio in favor of males following in vitro fertilization, whereas egg incubation temperature has been found to influence sex ratio in some reptiles. The incubation temperature interferes with the aromatase activity, resulting in a sex determination mechanism thought to be lost in mammals. In this work we aimed to test the effects of testosterone on sex ratio of bovine embryos produced in vitro and to determine whether effects of sex and temperature are effectively decoupled in mammals. Bovine oocytes were in vitro matured for 22 hr in TCM199, PVA, FSH and LH after a 22 hr meiotic arrest in TCM199, PVA and roscovitine 25 μM. Matured oocytes were in vitro fertilized and cultured up to Day 3, and embryos having three or more cells were sexed. In the first experiment, testosterone (0, 30, 300 and 1,500 nM), present both during meiotic inhibition and subsequent in vitro maturation (IVM), did not affect development rates or embryonic sex ratio. In the second experiment, increasing incubation temperatures (38, 39 or 40°C) during meiotic inhibition and subsequent IVM, reduced embryo development, but did not change the sex ratio. Under our experimental conditions, testosterone does not promote a preferential selection of Y-chromosome bearing spermatozoa by the oocyte, and temperature and sex ratio seems to be decoupled in mammals. *J. Exp. Zool. 311A:448–452, 2009.*

How to cite this article: Díez C, Bermejo-Alvarez P, Trigal B, Caamaño JN, Muñoz M, Molina I, Gutiérrez-Adán A, Carrocera S, Martín D, Gómez E. 2009. Changes in testosterone or temperature during the in vitro oocyte culture do not alter the sex ratio of bovine embryos. J. Exp. Zool. 311A:448–452.

The ability to preferentially produce either male or female offspring would be of enormous economic benefit to the livestock industry. However, mechanisms for a satisfactory system of adaptive control of the sex ratio in mammals remain unexplained (Williams, '79; Reiss, '87; Hardy, '97; Krackow, 2002; Jiménez et al., 2003). In many reptile species, sexual differentiation of gonads is sensitive to temperature (temperature-dependent sex determination) during a critical period of embryonic development (thermosensitive period, TSP) (Lance, 2008). Experiments carried out with different models, including turtles, crocodilians and lizards, have demonstrated the implication of estrogens and the key role played by aromatase (the enzyme that converts androgens to estrogens) in the ovary differentiation during TSP and in the ovary structure maintenance after TSP (Pieau et al., 2001). In some of these experiments, the occurrence of various degrees of gonadal intersexuality is related to weak differences in aromatase activity, suggesting that environmental temperature can affect the transcription level of the aromatase gene.

Grant sponsor: CAJASTUR; Grant numbers: AGL2008-01530; RTA2008-0082; Grant sponsors: MICIN; Fondo Social Europeo.

*Correspondence to: Carmen Díez, SERIDA, Camino de los Claveles 604, Somió. 33205 Gijón, Spain. E-mail: mcdiez@serida.org

Received 6 February 2009; Revised 20 March 2009; Accepted 24 March 2009

Published online 21 April 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.540

On the other hand, the mammalian female may exert some control over the sex of the offspring she conceives (Grant et al., '96, '98, 2001, 2007; Sheldon and West, 2004). Follicular steroids concentrations widely vary between individuals (Henderson et al., '82) and fluctuate over time within the oestrous or menstrual cycles (McNatty et al., '84). In fact, high levels of follicular testosterone in vivo associate to more male embryos resulting from in vitro fertilization (Grant et al., 2008), and high maternal serum testosterone concentrations seem to be associated with the conception of male offspring (Grant and France, 2001; Grant and Irwin, 2005; Grant et al., 2008). Within antral follicles, testosterone acts primarily as a precursor for the production of oestrogen (Henderson et al., '82).

In this work we aimed to test the effects of testosterone on sex ratio of bovine embryos produced in fully in vitro conditions and to determine whether effects of sex and temperature are effectively decoupled in mammals.

MATERIALS AND METHODS

All chemicals were purchased from Sigma (Madrid, Spain), unless otherwise indicated.

Collection of cumulus oocyte complexes (COCs)

Ovaries recovered from slaughtered cows were placed in NaCl solution (9 mg/mL) with antibiotics (penicillin, 100 UI/mL and streptomycin sulphate, 100 μg/mL) and maintained at 25–30°C until COCs collection. Follicles 2–7 mm in size were aspirated through an 18-gauge needle connected to a syringe, and the contents filtered (Comextrade, Tarragona, Spain) for obtaining COCs that were rinsed three times with holding medium (HM: TCM199—Invitrogen, Barcelona, Spain, 25 mM Hepes and PVA 0.1 mg/mL).

Oocytes enclosed in a compact cumulus with evenly granulated cytoplasm were washed twice in basic medium (BM: TCM199+PVA 0.1 mg/mL) containing roscovitine 25 μM (Gómez et al., 2004). Meiotic arrest was performed to mimic in vivo intrafollicular conditions before in vitro maturation (IVM), and was accomplished by culturing COCs in this medium for 22 hr. Subsequent IVM was carried out in BM containing, FSH (1 μg/mL) and LH (5 μg/mL) for 22 hr at 39°C in 5% CO_2 under air and high humidity.

In vitro fertilization

Sperm separation was carried out using a swim-up procedure similar to that reported by Parrish et al. ('86). After IVM, COCs were washed twice in HM, twice in pre-equilibrated fertilization medium (Fert-TALP), and placed in four-well culture dishes containing Fert-TALP medium with heparin (10 μg/mL, Calbiochem, La Jolla, CA). Spermatozoa were then added to a concentration of 2×10^6 cells/mL. In vitro fertilization was accomplished by incubating oocytes and sperm cells for 18–20 hr at 39°C in 5% CO_2 under high humidity.

Embryo culture

Presumptive zygotes were slightly passed through a small gauge pipette to eliminate cumulus cells and sperm, washed three times in HM and twice in culture medium. Embryo culture was performed up to Day 3 in droplets (volume: 1–2 μL/embryo; up to 45 embryos/microdrop) of modified synthetic oviduct fluid containing amino acids, citrate and myo-inositol (Holm et al., '99) and 6 g/L of BSA. Incubations were carried out at 39°C, 5% CO_2, 5% O_2 and 90% N_2.

Embryo sexing by PCR

After embryo incubation into 5 mg/mL pronase in PBS for 1 min to remove the zona pellucida and any attached spermatozoa, embryos were washed 3–4 times in PBS+PVA 0.1 mg/mL, individually snap frozen in liquid nitrogen in 0.2 μL Eppendorf tubes and stored at −80°C until analysis. For increasing PCR efficiency, embryos were digested overnight with 8 μL of a 100/mL proteinase K solution at 55°C. After digestion, proteinase K was inactivated at 95°C for 10 min. Two sets of PCR primers were used to determine embryo sex: Y-chromosome-specific primers (BRY1a), and bovine-specific satellite sequence primers (Sat1). The PCR reactions were conducted in a total volume of 25 μL containing 8 μL of the proteinase K digested sample, 1 × Gotaq Flexi buffer, 1 IU of Gotaq (Promega, Madison, WI), 1.25 mM $MgCl_2$, 0.1 mM dNTP, 1 ng/μL BRY primers and 0.2 ng/μL Sat primers. PCR was performed with a first cycle (94°C for 3 min, 60°C for 40 sec and 72°C for 15 sec) followed by 35 cycles (94°C for 15 sec, 60°C for 30 sec, 72°C for 15 sec; final elongation 72°C for 5 min). Products were visualized on an ethidium bromide stained 2% agarose gel. The gel was visualized under ultraviolet illumination for the positive 300 bp band of BRY 1a and 216 bp of the

satellite sequence. Samples that exhibited both bands were assigned as male, whereas samples exhibiting only a satellite sequence band were assigned as female. Every PCR was carried out with three controls: male genomic DNA, female genomic DNA and a negative control (Bermejo-Alvarez et al., 2008).

Experimental design

The first experiment analyzed the effect of testosterone on sex ratio of bovine embryos produced in vitro. Four testosterone (Sigma T-86500) concentrations (0, 30, 300 and 1,500 nM) were tested during meiotic inhibition and subsequent IVM.

The second experiment aimed to determine whether effects of sex and temperature were effectively decoupled. Immature COCs were cultured at 38, 39 or 40°C during meiotic inhibition and subsequent IVM.

In both experiments embryos having three or more cells were sexed on Day 3 (70–72 hr post-fertilization).

Statistical analysis

Data were analyzed using SAS v. 8.2 software (SAS Institute Inc., '99; Cary, NC). Treatment and replicate were tested by their influence on dependent variables (development stage and embryonic sex). Those factors identified as significant were used to produce a linear model using the general linear models procedure (GLM; SAS software) Duncan's multiple-range test was used to compare the raw means calculated for the main effects. Data are presented as LSM ± SE percentages.

RESULTS

In Experiment 1 (Table 1), a total of 737 immature bovine COCs were cultured; out of them, 252 were fixed and sexed in 5 replicates. Testosterone did affect neither developmental rates nor sex ratio, and no interaction was detected between developmental stage and sex ratio.

In experiment 2 (Table 2), 142 embryos were sexed in 3 replicates, accounting for 502 COCs cultured (3 replicates). Embryo development on Day 3 decreased as incubation temperature increased. However, no interaction was detected between temperature and sex ratio.

DISCUSSION

In this work, the presence of testosterone at several concentrations throughout meiotic arrest and IVM had no effect on sex ratio or embryo development. Similarly, COCs incubation at 38, 39 or 40°C during the same period had no effect on the sex ratio. However, a dose–response effect of temperature was noted, with the lowest development rates being observed for COCs cultured at 40°C.

TABLE 1. In vitro development and male to female ratio of Day 3 bovine fertilized oocytes after a roscovitine-mediated meiotic inhibition and maturation in the presence of several concentrations of testosterone

(Testosterone)	*N*	% 3–4 cells	% ≥5 cells	Sexed	Male to female percentage
1,500 nM	182	40.6 ± 5.2	24.5 ± 4.0	63	57.6 ± 8.0
300 nM	187	45.8 ± 5.2	27.3 ± 4.0	81	53.6 ± 6.0
30 nM	190	37.8 ± 5.2	21.3 ± 4.0	52	42.6 ± 8.0
0 nM	178	47.7 ± 5.2	25.3 ± 4.0	56	53.0 ± 8.0

Data are LSM ± SE percentages from oocytes submitted to meiotic inhibition and subsequent in vitro maturation and in vitro fertilization (*N*). Five replicates.

TABLE 2. In vitro development and male to female ratio of Day 3 bovine-fertilized oocytes after a roscovitine-mediated meiotic inhibition and maturation under temperatures of 38, 39 or 40°C

Temperature	*N*	% 3–4 cells	% ≥5 cells	Sexed	Male to female percentage
38°C	167	67.3 ± 4.6*x*	44.6 ± 4.0*a*	64	0.45 ± 0.07
39°C	171	43.3 ± 4.6*y*	19.1 ± 4.0*b*	62	0.49 ± 0.07
40°C	164	15.7 ± 4.6*z*	5.9 ± 4.0*c*	16	0.47 ± 0.14

Data are LSM ± SE percentages from oocytes submitted to meiotic inhibition and subsequent in vitro maturation and in vitro fertilization (*N*). Superscripts express significant differences: *x*, *y*, *z* ($P < 0.01$); *a*, *b*, *c* ($P < 0.05$). Three replicates.

Currently, the only consistent approach to achieve offsprings of a determined sex is the use of sex-sorted sperm; however, the low throughput of sperm sorting limits its widespread use (Seidel, 2007; Garner and Seidel, 2008). When using unsorted sperm, several factors have been shown to influence sex ratio in cattle, including timing of insemination (Martínez et al., 2004), maturation stage of the oocyte at the time of insemination in vitro (Gutiérrez-Adán et al., '99; Rizos et al., 2008), length of gamete co-incubation in vitro (Kochhar et al., 2003), and postfertilization culture conditions in vitro (Gutiérrez-Adán et al., 2001).

Mammalian females might exert some control over the sex of the offspring she conceives. Eberhard ('96) described a number of ways by which the mother may influence the offspring sex, both pre- and postconception. Among these, maternal dominance measured around the time of conception has been associated with a higher proportion of males in the offspring (Sheldon and West, 2004). Dominance has been shown to be a behavioral trait determined by testosterone in mammals (Bouissou, '78), including humans (Grant and France, 2001). Higher female testosterone levels within follicular fluid have been associated to increased proportions of male embryos in culture (Grant et al., 2008), which has been explained as a possible oocyte sex selection mechanism, a theory that has been recently challenged (Bermejo-Alvarez et al., 2008). In our work, we submitted the oocytes to a period of meiotic inhibition to mimic in part in vivo intrafollicular conditions before IVM. However, we found no effect of testosterone on embryonic sex ratio, which is inconsistent with findings from Grant et al. (2008). These authors reported that a wide range of testosterone concentrations exists in follicular fluid (11–977 nM), and found that follicles containing more that 300 nM testosterone, produced oocytes that were mainly fertilized by male sperm (17 out of 20 fertilized oocytes were male embryos). It is in this context, we selected concentrations ranging between 30 and 1,500 nM to test. The observed discrepancy between our results and the above cited work may lie on the presence of exogenous testosterone in our work, as only endogenous testosterone was analyzed by Grant et al. (2008). Therefore, comparisons with the Grant et al. (2008) study should be carefully interpreted. In our work, COCs were maintained in meiotic arrest for 22 hr with roscovitine before IVM, whereas in Grant et al.'s study COCs readily fertilized COCs upon follicle release. In addition, within in vivo conditions oocytes may interact with other follicular components affecting testosterone that could not have been present in the COC culture. Contrary to follicular fluid, where a final testosterone concentration was measured upon oocyte release, we are unaware of possible changes in testosterone concentration as a consequence of its metabolism in culture, whether they exist; it is likely that changes in intrafollicular testosterone concentration are a consequence rather than a cause in sex determination mechanisms.

Temperature differences around IVM did not affect sex ratio, although development decreased in a dose-dependent way. A number of theories explaining how the temperature may exert its effect on the embryonic sex have been proposed, but most were described as inconsistent (Deeming and Ferguson, '89). Pieau and Dorizzi (2004) hypothesized that incubation temperature during the TSP affects the aromatase gene regulation, and that the subsequent oestradiol levels determines the sex of the embryo. Although in some amphibians and reptiles aromatase is affected by the temperature (Deeming and Ferguson, '89; Deeming, 2004; Crews, '94; Pieau et al., '99, 2001; Endo et al., 2008), it seems that mammalian aromatase does not to vary, at least within a temperature window compatible with the oocyte ability to be fertilized and to start embryo development. To the best of our knowledge, we did not find published data on termosensitivity of aromatase in mammalian species.

If aromatase (or one or several of its transcription factors) were regulated by temperature within a physiological range, perhaps testosterone or oestradiol concentrations could be modified by incubation temperatures, which in turn would modify sex ratio in conditions under which testosterone were involved.

Our results provide evidence that mammals do not conserve reptilian reminiscences of a temperature control on sex ratio, and testosterone did not alter sex ratio under in vitro conditions.

ACKNOWLEDGMENT

Beatriz Trigal is supported by CAJASTUR (AGL2008-01530; RTA2008-0082). Marta Muñoz is supported by MICIN (Programa Ramón y Cajal) and David Martín is supported by MICIN and Fondo Social Europeo.

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