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Effect of two temperatures on *in vitro* nuclear maturation of bitch oocytes: relation to time culture intervals

Efeito de duas temperaturas na maturação *in vitro* de ovócitos de cadelas: relação com tempos de cultivo

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Abstract

In the present study, two commonly incubation temperatures used on *in vitro* oocyte maturation in canine species (*Canis familiaris*) were analysed with the aim to verify the rate of nuclear maturation of bitch oocytes after three culture intervals (48, 72 and 96 h). The culture medium was TCM 199, supplemented with 25mM Hepes/l (v/v), with 10% heat inactivated estrous cow serum (ECS), 50mg/mL gentamicin, 2.2mg/mL sodium bicarbonate and 22mg/mL pyruvic acid, 1.0mg/mL oestradiol (E 8875 Sigma), 0.5mg/mL FSH (Folltropin-V, Vetrepharm Inc. Ont, Canada) and 0.03IU/mL hCG (Profasi HP, Serono, Aubonne, Switzerland). Oocytes were recovered from ovaries of bitches at random estrous cycle stages by routine ovariohysterectomy (n=14) or by therapeutical ovariohysterectomy (n=8) at the veterinary hospital of the Universidade Federal do Rio Grande do Sul (UFRGS), Brazil. There was no statistical difference in the rate of meiosis of oocytes matured at 37ºC or at 39ºC at any time point. However, the proportion of oocytes that reached the metaphase II (MII) stage at 37º C after 72 h of *in vitro* culture (16/123; 13%), tended to be higher (P=0.064), when compared to that matured at the 39ºC (6/148; 4,1%). The results demonstrate that temperatures of 37ºC and 39ºC are equally effective for the *in vitro* culture of bitch oocytes.

Keywords: canine/ oocyte/ *in vitro* maturation/ temperature/ culture time.

Resumo

No presente estudo, duas temperaturas de incubação habitualmente usadas na maturação *in vitro* ovocitária na espécie canina (*Canis familiaris*) foram testadas com o objetivo de verificar o índice de maturação nuclear de ovócitos submetidos a três tempos de cultivo (48, 72 e 96 horas). O meio de maturação usado foi TCM-199, suplementado com 25mM Hepes/l (v/v), adicionado de 10% de soro inativado de vaca em estro (SVE), 50mg/mL de gentamicina, 2,2mg/mL de bicarbonato de sódio, 22mg/mL de ácido pirúvico, 1,0mg/mL de estradiol (E 8875 Sigma), 0,5mg/mL de FSH (Folltropin-V, Vetrepharm Inc. Ont, Canada) e 0,03UI/mL de hCG (Profasi HP, Serono, Aubonne, Switzerland). Os ovócitos foram retirados de cadelas submetidas à ovario-histerectomia em caráter eletivo (n=14) ou terapêutico (n=8) no hospital veterinário da Faculdade de Veterinária da Universidade Federal do Rio Grande do Sul (UFRGS), Brasil. Não foi encontrada diferença estatística significativa no índice de meiose de ovócitos maturados a 37ºC ou a 39ºC nos diferentes intervalos de tempo testados. No entanto, a proporção de ovócitos que alcançaram o estádio de metáfase II (MII) a 37ºC após 72 horas de incubação (16/123; 13%) mostrou uma tendência estatística à significância (p=0,064), quando comparada com a proporção de ovócitos maturados a 39ºC (6/148; 4,1%). Os resultados mostram que temperaturas de 37ºC e 39ºC são equivalentes na indução da maturação *in vitro* de ovócitos caninos.

Palavras-chave: canino, ovócito, maturação *in vitro*, temperatura, tempo de cultivo.

Introduction

During the last years, little progress has been reported towards understanding the basic requirements of the *in vitro* oocyte development in the bitch.

Parallel studies developed with various mammalian species, particularly cattle, mice and even humans, have been traditionally used as models to study canine oocyte maturation and *in vitro* fertilization.

An elementary aspect, but not emphasized in previous studies, concerns the influence of culture temperature for oocyte development. Temperature modulates the physical properties of the lipids in biological membranes, together with changes in the lipid composition of the membrane (Quinn, 1985).

The lack of information and the need for the standardization of a specific temperature is therefore a very important feature of the *in vitro* reproductive program in the bitch, which still needs to be defined.

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The aim of this study was to compare the effect of two different incubation temperatures on the nuclear *in vitro* oocyte maturation in the bitch. The relation to three different time culture intervals was additionally investigated.

Materials and methods

Ovaries and collection of oocytes

Ovaries were harvested from purebred (n=11) and crossbred (n=11) bitches (aged 2.5-120 months). Oocytes were recovered from ovaries of bitches at random stages of the estrous cycle by routine ovariohysterectomy (n=14) or by therapeutical ovariohysterectomy following vaginal hyperplasia (n=1), cystic endometrial hyperplasia- piometra (n=3), abortion (n=1), dystocia (n=2), vaginitis (n=1) at the veterinary hospital of the Universidade Federal do Rio Grande do Sul (UFRGS), Brazil. Oocytes were recovered by slicing the ovaries in modified phosphate-buffered saline (PBS) (Whittingham, 1971) supplemented with foetal calf serum (FCS) (1:100) at 37ºC to release cumulus oocyte complexes (COCs). Oocytes were graded according to the criteria reported by Hewitt et al. (1998). Grade 1 (darkly pigmented and completely surrounded by one or more layers of cumulus cells) and Grade 2 COCs (lightly pigmented with incomplete layers of cumulus cells) were selected for culture (n=523) and rinsed in HEPES-buffered TCM 199 before maturation.

In vitro maturation

The culture medium was TCM-199, supplemented with 25mM Hepes/l (v/v), supplemented with 10% heat inactivated estrous cow serum (ECS), 50µg/mL gentamicin, 2.2µg/mL sodium bicarbonate and 22µg/mL pyruvic acid, 1.0µg/mL oestradiol (E-8875 Sigma), 0.5µg/mL FSH (Folltropin-V, Vetrepharm Inc., Ont, Canada) and 0.03 IU/mL hCG (Profasi HP, Serono, Aubonne, Switzerland). Selected oocytes were randomly distributed to 37ºC and 39ºC temperatures and matured at culture intervals of 48, 72 and 96 hours in 100µl droplets (up to 25 oocytes per drop) under mineral oil in a 100% humidified atmosphere containing 5% CO_2 in air.

Assessment of nuclear stage of maturation

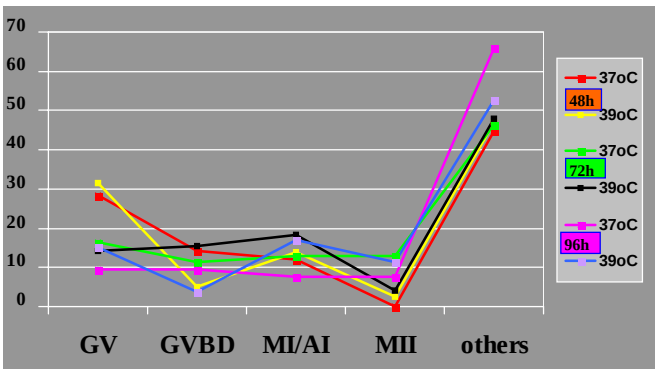
At final time of *in vitro* culture (48, 72 or 96 h), oocytes were denuded of cumulus cells, permeabilized in Triton X (Sigma, St Louis, MO, USA), fixed in a 3.7% paraformaldehyde solution and stained with the deoxyribonucleic acid (DNA) specific stain Hoechst 33342 (10µg/ml) (Sigma, St Louis, MO, USA) for fluorescence microscopic analysis of the nuclear maturation stage.

Nuclear morphology was classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase/anaphase I (MI/AI), metaphase II (MII), unidentified or degenerated (others).

Treatment effects were evaluated by Chi-Square analysis with adjusted residual. The data of meiotic resumption were analysed by Goodness-of-fit chi-square test for an uniform distribution. Effects were considered significant with P< 0.05 as level of significance.

Results and discussion

There was no statistical difference between the nuclear meiotic rate of oocytes matured at 37ºC or at 39ºC, suggesting that the outcome of *in vitro* oocyte maturation in the domestic canine species is similar at the tested temperatures. However, the proportion of oocytes maturing to the MII stage at 37ºC after 72 h of *in vitro* culture (16/123; 13.0%) tended to be higher (P=0.064), when the data were compared to the combined temperatur of 39ºC (6/148; 4.1%) (Graphic 1).



Graphic 1: Two combined temperatures and three time culture intervals on the *in vitro* nuclear maturation of bitch oocytes

Meiotic resumption was similar at the combined temperatures at each time point.

Likewise, viability maintenance was similar at the tested temperatures into culture intervals.

Meiosis rate showed a high individual variability with the percentage of MII stage achievement ranging from 0% to 22% among bitches. The majority of oocytes maturing to MII stage were retrieved from bitches aged between 1 to 5 years.

Oocytes retrieved from three bitches submitted to therapeutical ovariohysterectomy following abortion, dystocia and vaginitis matured to MII at rates of 8.5% (3/35), 15.6% (5/32), and 22% (2/9), respectively.

At 37ºC and 72 h time culture and at 39ºC and 96 h time culture, an increase of oocytes reaching the MII stage (16/123; 13%) and the MI/AI stages (9/53; 17%) achievement was respectively observed (Graphic 1), suggesting an improvement of the meiotic nuclear acquisition at extended time of *in vitro* culture.

In vitro incubation temperatures of bitch oocytes still do not follow an established pattern. Thus, during the last years, several temperatures have been used on the *in vitro* maturation of bitch oocytes. Oocyte development in canine species has already been attempted at 37ºC (Mahi and Yanagimachi, 1976), at 37.5ºC (Otoi et al., 2000), at 38ºC (Srsen et al., 1998), at 38.5ºC (Saint-Dizier et al., 2001) and at 39ºC (Hewitt et al., 1998), with variable rates of success.

Temperature and time culture used on *in vitro* studies with oocytes retrieved from ovarian follicles of domestic animals are based on the *in vivo* knowledge of specific physiologic and reproductive mechanisms.

In the bitch, oocytes are ovulated at the prophase of the first meiotic division (Farstad, 2000), 24 to 50 hours after the LH

peak (Concannon et al., 1989; Hase et al., 2000). Ovulation is a synchronous phenomenon confined to a narrow time window up to 12-24 h (Dieterich, 1994; Meisl, 1998).

In contrast, 48 to 72 hours are required to complete oocyte maturation to the MII stage in the final portion of the oviduct (Concannon et al., 1989), a finding observed on previous *in vitro* studies (Fujii et al., 2000; Hewitt et al., 1998), as well as in the present experiment.

Body temperature is about 38ºC (100.5ºF) in the bitch. However, thermoregulation mechanisms before parturition, induced by the drop in the plasma progesterone concentrations, lead to temperature decline bellow 37.5ºC (99.5ºF) (Allen, 1992).

Experimental data have shown good results from *in vitro* matured an *in vitro* fertilized bitch oocytes at temperatures equal or slightly higher than 37ºC (Yamada et al., 1992; Otoi et al., 2000), as well as at temperatures above 38ºC (Saint-Dizier et al., 2001; England et al., 2001).

In cattle, ambient temperature influences embryonic development (Zeron et al., 2001), which is the ultimate goal of an *in vitro* program. Therefore, studies conduct to examine oocyte development should also be commited with oocyte competence after *in vitro* fertilization.

It seems that temperatures between or at the physiologic body extremes, do not adversely influence oocyte development in the canine species. Despite that, further research is required to evaluate in which way temperatur is capable to favorise embryonic development of advanced stages in the bitch.

The results herein demonstrate that temperatures of 37ºC and 39ºC are equally adequate to support the nuclear *in vitro* maturation requirements of bitch oocytes.

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