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Sperm Nuclear Decondensation Induction Capacity of *In Vitro* and *In Vivo* Matured Canine Oocytes

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Contents

The objectives of this study were to evaluate the sperm nuclear decondensation capacity of ovulated and *in vitro*-matured (IVM) canine oocytes during different culture times and correlate this decondensation ability with the state of oocyte nuclear maturation *in vitro* and *in vivo*. Fresh ejaculates from three dogs were used for *in vitro* fertilization (IVF). Dog spermatozoa were cocultured with ovulated or IVM oocytes after each culture period (0, 48, 72 and 96 h) for 24 h. The nuclear stage of the oocytes and the appearance of the sperm nucleus were determined, and data were analysed with a chi-square test. The rates of decondensation and meiotic development in IVM oocytes increased up to 72 h of culture. In contrast, almost all *in vivo*-matured oocytes showed MII nuclear stage and sperm chromatin decondensation. The percentages of oocytes at MII stage were much lower ($p < 0.05$) in all IVM groups compared with ovulated oocytes; the rate of sperm chromatin decondensation was higher in ovulated oocytes than in those matured *in vitro*. Thus, IVM canine oocytes are able to decondense the sperm chromatin during IVF, and this ability increases with time. Nevertheless, sperm chromatin decondensation is less efficient than in ovulated oocytes and may not be completely synchronized with nuclear development as it occurs *in vivo*.

Introduction

Sperm chromatin decondensation occurs when a spermatozoon enters the oocyte during fertilization. Before that, sperm DNA is compactly bound to protamines, which replaces somatic histones. These protamines must be removed from the DNA by a process in which their disulphide bonds are reduced (Perreault et al. 1984), which is the first step of sperm nucleus decondensation. As the links are cleaved, the coiled chromatin loops unfold and enable oocyte factors to further decondense the chromatin. When this happens, the sperm nucleus becomes much larger, and the DNA combines with oocyte histones, which later on constitutes the male pronucleus (Calafell et al. 1991). The decondensation of the sperm nucleus is therefore a critical process and is closely connected with oocyte maturation. In fact, one of the criteria for oocyte cytoplasmic maturation is the ability to decondense the sperm chromatin.

Several investigators have described the limited capacity of development in canine oocytes matured and penetrated by sperm *in vitro*. Despite continuing research effort, the overall efficiency of *in vitro* maturation in canine oocytes remains low, and neither clinical nor laboratory procedures can be considered routinely feasible as in other species. The challenges for *in vitro* maturation in canine are therefore to improve oocyte maturation potential to produce maturation rates that are at least equivalent to those obtained in other species. The ability of canine oocytes to induce sperm chromatin decondensation is not fully known, and this decondensation capacity may change during *in vitro* conditions. Thus, the objectives of this study were to evaluate the sperm nuclear decondensation capacity of ovulated and *in vitro*-matured (IVM) canine oocytes during different culture times comparing this ability with the meiotic progression.

Material and Methods

The experiments described in this study were performed in accordance with the protocols approved by the Chilean Bioethics Committee of the National Commission for Scientific and Technological Research (FONDECYT).

In all experiments, oocytes were obtained from mix breed bitches, aged 1–6 years, following ovariohysterectomy. Ovaries were collected and rapidly transported to the laboratory in physiological saline solution (0.9% NaCl) at 37°C.

Oocyte preparation for IVM

Cumulus-oocyte complexes (COCs) for IVM were obtained from ovaries at different stages of the oestrous cycle. COCs were selected according to size (>100 μm), compactness of cumulus cells and the presence of a homogeneous dark cytoplasm, and then incubated for IVM in TCM-199 Earle's salt, 25 mM HEPES (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FCS, 0.25 mM pyruvate, 10 IU ml^{-1} of hCG, 300 IU ml^{-1} of penicillin, and 20 mg ml^{-1} of streptomycin, for 0, 48, 72 and 96 h at 38.5°C and 5% CO_2 (De los Reyes et al. 2011).

Ovulated oocytes

In vivo-matured oocytes (ovulated) were obtained from nine bitch ovaries, 50–72 h after ovulation. The time of ovulation was estimated before ovariohysterectomy, by vaginal smears and progesterone concentrations, assessed by enzyme-linked immunosorbent assay (ELISA), (PHomo Microplate Reader®; Autobio Labtec Instruments, Zhenghaidong, China) with a P_4 canine kit (Prog ELISA Kit, MyBioSource®; San Diego, CA, USA). Ovulation was assumed to have occurred when serum progesterone concentration rose to 5 ng/ml. Follicular growth and ovulation were also monitored by Color-Doppler Ultrasonography (Sonosite M-turbo;

Sonosite Inc, Bothell, WA, USA) from the first day of proestrus. Oviducts and the tip of the uterine horns were transported to the laboratory in saline solution at 37°C. Ovulated oocytes were collected by flushing the oviducts with 15 ml of TCM 199 supplemented with 10% foetal calf serum (FCS) (De los Reyes et al. 2011).

Sperm preparation and *in vitro* fertilization

Fresh ejaculates from three adult dogs were used for *in vitro* fertilization (IVF) experiments. Semen was examined for motility, morphology and acrosome integrity as previously described (De los Reyes et al. 2009). In each replicate, a normospermic semen specimen was centrifuged at 300 × ***g*** for 10 min, and the remaining sperm pellet was suspended in Fert-TALP medium. Six to seven ovulated oocytes or around 10–12 IVM oocytes (at each culture time) were transferred separately to 100-μl droplet of Fert-TALP with 2.5×10^6 spermatozoa ml^{-1} under paraffin oil for IVF. Gamete coculture was performed for 24 h, at 38.5°C, 5% CO_2 in high humidity.

Oocyte evaluation

Ovulated oocytes, non-matured and IVM oocytes after coculture, were washed free of sperm, denuded from cumulus cells, and then fixed in 3% paraformaldehyde. The nuclear stage of the oocytes and the appearance of the sperm nucleus in the cytoplasm were determined by incubating with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) staining in PBS for 15 min at room temperature (21°C).

The samples were evaluated with an inverted epifluorescence microscope, Olympus IX71, equipped with an IX2-RFA lamp and a ProgRes-Capture Pro camera (Tokyo, Japan).

Statistical analysis

To determine whether IVM oocytes at different stages of nuclear maturation have the capability to decondense sperm chromatin after IVF compared with those that had undergone meiotic maturation *in vivo*, totally 138 non-matured oocytes, 379 IVM after different culture periods, and 30 *in vivo*-matured oocytes were analysed. Experiments were replicated five times. The comparison of decondensing ability of oocytes at different stages of maturation was performed with a chi-square (χ^2) test. Differences $p < 0.05$ were considered significant.

Results

Oocytes at different stages of nuclear development were penetrated by spermatozoa that could decondense their chromatin after coculturing for 24 h. Analysis by fluorescence microscopy showed a cytoplasmic blue fluorescence (DAPI staining that forms fluorescent complexes with double-stranded DNA) in all stained oocytes.

Meiotic development was classified as previously described (De los Reyes et al. 2011) and the rates of decondensation were the sum of both moderately (Fig. 1A) and grossly decondensed sperm heads (Fig. 1B). Polyspermic oocytes were also examined and assessed for the presence of sperm chromatin dispersion, similar to the monospermic oocytes. Abnormal oocytes were discarded.

Table 1 shows the number of oocytes at different stages of meiotic progression and those with sperm head decondensed in their cytoplasm within each maturation group. Sperm decondensation ability was a time-dependent process as well as nuclear maturation progression; however, the two processes were not synchronized throughout time. The rates of sperm chromatin decondensation increased after 48 h of culture ($p < 0.05$), irrespective of meiotic stage.

Oocytes at germinal vesicle (GV) stage was observed in all *in vitro*-maturated groups, but mainly in those non-matured, which showed the lowest rates of sperm decondensation. This GV stage decreased significantly during *in vitro* culture. In contrast to non-matured oocytes, few IVM oocytes displaying sperm decondensation were blocked at GV.

Oocytes before germinal vesicle breakdown (GVBD) matured *in vitro* were capable of decondensing sperm nuclei, and after the MI stage, the rates of sperm nuclear decondensation significantly increased up to 72 h of culture. Considering all maturation groups, the highest percentage of sperm decondensation was observed by the *in vivo*-matured oocytes, with nearly 97% of them with decondensed sperm chromatin in the cytoplasm.

Regarding nuclear development, the MI–MII stage increased with time of culture; however, the

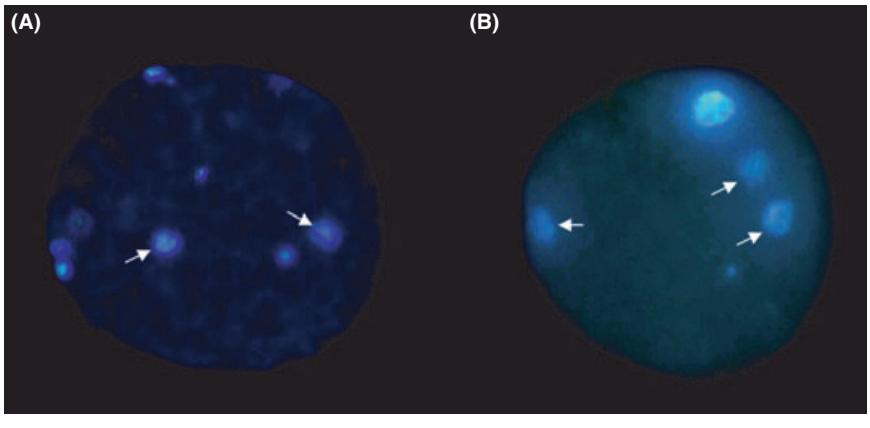

Fig. 1. *In vitro*-fertilized canine oocytes stained with DNA-specific binding dye, DAPI (4′-6-diamidino-2-phenylindole) and visualized under UV light. Fertilizing sperm nuclei in different degrees of de-condensation. (A) Moderately decondensed and (B) Fully decondensed (arrows). Magnification × 200

Table 1. Canine oocytes at different maturation groups and meiotic development and number of oocytes with sperm decondensation in each group after 24 h of IVF

Oocytes	GV n = 43	GVBD n = 110	MI-TI n = 291	MII n = 103	Total decondensed (%) n = 547
Non-matured (n = 138)	$32/1^{aA}$	$68/7^{aA}$	$38/9^{aA}$	–	12.3
IVM 48 h n = 108	$8/8^{bB}$	$18/17^{bB}$	$75/42^{bB}$	$7/4^{aA}$	65.7
IVM 72 h n = 118	$1/1^{bB}$	$11/7^{cC}$	$82/65^{cB}$	$24/22^{bB}$	80.5
IVM 96 h n = 153	$2/2^{bB}$	$13/5^{dC}$	$96/82^{cB}$	$42/41^{bB}$	85
Ovulated n = 30	–	–	–	$30/29^{bC}$	96.7

Nuclear stages: GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, first metaphase; MII, second metaphase.
[a–d]Within a column (sperm decondensation), numbers without a common superscript differed ($p < 0.05$).
[A–D]Within a column (meiotic progression), numbers without a common superscript differed ($p < 0.05$).
IVF, *in vitro* fertilization; IVM, *in vitro* matured.

percentages of MII stage were much lower ($p < 0.05$) in all *in vitro*-maturing groups at any time of culture, in comparison with ovulated oocytes. Nevertheless, the rates of chromatin decondensation in MII oocytes were similar between 72, 96 h IVM and *in vivo*-matured oocytes.

Discussion

The present study demonstrates that canine oocytes of differing maturation stages were able to decondense dog sperm chromatin as previously reported in early studies of gamete interaction in dogs (Mahi and Yanagimachi 1976). However, this capability was not equal in all types of oocytes, as the highest percentages of sperm decondensation were related to longer periods of culture and greater degree of meiotic maturation in comparison with non-matured GV oocytes, which showed the lowest percentages of decondensation. Indeed, *in vitro*, the arrest of sperm heads at a condensed stage has been related to oocyte immaturity (Calafell et al. 1991). The results obtained herein suggest that the decondensing ability of the cytoplasm of canine oocytes seems to be acquired during the earliest phases of maturation. It has been described that decondensing factors are present in the interphase nuclei and are released into the cytoplasm during nuclear envelope breakdown in most mammals (Maeda et al. 1998), which would be related to the fact that GV-intact oocytes lack sufficient reducing power to affect sperm nuclear decondensation (Perreault et al. 1984). However, our observation revealed that, even though decondensation was low in GV oocytes, around 28% of them could display a swollen sperm head in the cytoplasm. This indicates that such a factor may begin to be active even before the GVBD or may become only partly active just before GVBD, and its activity is increasing with time. Nevertheless, although the resumption of meiosis brought an increase in the ability to decondense the fertilizing sperm nucleus in IVM oocytes, it seems that the factors affecting sperm chromatin decondensation depends upon the maturational state of the cytoplasm, which is directly related to culture time, rather than meiotic changes. In fact, nuclear and cytoplasmic maturation are not synchronized in time in IVM dog oocytes (De los Reyes et al. 2011).

Nuclear maturation also increased throughout time; however, the percentage of MII stage was much lower than that of chromatin decondensation in all IVM groups. On the contrary, almost all *in vivo*-matured oocytes reached MII stage and induced sperm chromatin decondensation. The differences between *in vitro* and *in vivo*-matured oocytes might be related to developmental competence, most IVM oocytes that could support sperm chromatin decondensation were only partially competent to undergo nuclear maturation. But those *in vitro* that could reach the MII stage were also able to decondense sperm chromatin similar to those *in vivo*.

The differences found between culture times of IVM oocytes and ovulated oocytes may also be related to reducing agent levels, such as a glutathione (GSH), during maturation. Indeed, *in vivo*-matured dog oocytes have significantly higher GSH concentrations compared with IVM oocytes (Kim et al. 2007). Furthermore, ejaculated dog sperm chromatin is extremely condensed, as dog sperm contain only protamine 1 (Johnson et al. 1988). This makes dog sperm chromatin more stable than sperm chromatin of those species that have two types of protamines and thus may require more oocyte GSH for decondensation.

Even though the rates of sperm chromatin decondensation in activated IVM oocytes was not equal throughout time, its morphological changes appeared to be the same as those observed in the sperm chromatin of *in vivo*-maturing oocytes. Whether sperm chromatin decondensation in IVM oocytes depends on similar or different mechanism than those in ovulated oocytes remains to be verified.

In conclusion, these results suggest that canine oocytes matured *in vitro* are able to decondense the sperm chromatin during IVF, and this ability increased up to 72 h of culture. Nevertheless, sperm chromatin decondensation, in canine oocytes matured *in vitro* may not be completely synchronized with nuclear development as it occurs *in vivo*. Factors important in regulating the onset of oocyte maturation, the temporal aspects of meiotic progression and sperm pronucleus formation after sperm decondensation are not clearly defined and merit further attention.

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Conflicts of interest

None of the authors have any conflicts of interest to declare.

Author contributions

MDLR designed the study, coordinated the roles of the authors, performed analysis and wrote the manuscript. JP was responsible for

oocytes evaluation and performed the statistical analysis; VHP was responsible for data analysis, and JV was responsible for semen collection, semen processing and participated in the laboratory protocols. All authors read and approved the final manuscript.

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