Evaluation of cortical granules and viability of canine oocytes during long-term in vitro maturation

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THE morphological appearance of the canine cumulus-oocyte complex (COC) is known to influence the rate of in vitro maturation (IVM) (Farstad 2000). The low rate of IVM in dogs might also be related to the unsuitability of the culture medium to support oocyte viability and development. Canine ovulation of oocytes occurs at the germinal vesicle stage; completion of the maturation process continues in the oviduct, requiring an extended period of two to five days (Renton and others 1991). In order to mimic this process in vitro, it is necessary to use prolonged culture periods, and thus the viability of the gametes becomes an issue. Correct culture conditions have been determined by the preservation of oocyte morphology, and thus sustained oocyte viability (Figueiredo and others 1994).

In addition to nuclear maturation, proper cytoplasmic and membrane maturation are critical for continued viability of oocytes. Most studies on canine oocytes have evaluated nuclear maturation in culture, but there has been little attention given to cytoplasmic maturation. An oocyte that has not completed cytoplasmic maturation is of poor quality, and thus unable to successfully complete the normal developmental processes (Krisher 2004). Cytoplasmic maturation mainly involves the redistribution of cortical granules around the periphery of the oocyte (Sun 2003), which then contribute to the inhibition polyspermy (Hoodbhoy and Talbot 1994). Considering the long culture period required for IVM in dogs, the aims of this study were to evaluate the viability of the plasma membrane of canine oocytes recovered from the ovaries before and during culture, and to identify and observe the distribution of cortical granules before and after culture.

In each experimental replicate, oocytes were obtained from normal bitch ovaries following ovariohysterectomy. In the laboratory, COCs were released by slicing the ovarian cortex. Oocytes with uniform ooplasm and a compact cumulus cell mass were selected. After two washes in phosphate buffered saline (PBS) the COCs were placed in maturation medium (TCM 199; Earle's salt, buffered with 25mM Hepes; Invitrogen), supplemented with 10 per cent fetal calf serum and 2·5 µl/ml pyruvic solution (11·2 mg/ml pyruvic acid), 10 iu/ml of human chorionic gonadotrophin and 5 µl/ml antibiotic solution (12·2 mg/ml penicillin and 20 mg/ml streptomycin) (Sigma Chemical). The cultures were performed at 38·5°C in a humidified atmosphere of 5 per cent carbon dioxide for up to 96 hours (De los Reyes and others 2005).

To verify the viability of oocytes matured in vitro, several oocytes (approximately 15 from each replicate) were submitted for IVM in parallel to the experiment carried out for the cortical granule evaluation. Oocyte viability was assessed before culture and then every 24 hours in culture up to 96 hours. After each culture time the COCs were incubated with 5 µg/ml fluorescein diacetate (FDA; Sigma) in PBS for 5 minutes, and evaluated under an epifluorescence microscope, with green fluorescence indicating cell viability (Barros and others 1982). Evaluation of cortical granules was performed before incubation and after 96 hours of culture. Oocytes were fixed in 2 per cent paraformaldehyde/PBS for 30 minutes, and permeabilised for 5 minutes in PBS containing 0·1 per cent Triton X-100 with 0·3 per cent BSA to block non-specific binding sites. The oocytes were incubated with 20 µg/ml lectin *Lens culinaris* agglutinin (LCA) coupled to fluorescein isothiocyanate, in PBS for 30 minutes. The oocytes were washed and subsequently mounted with Vectashield (Vector Laboratories) under coverslips supported with vaseline/paraffin dots. The samples were evaluated with an epifluorescence microscope (UV emission 480 nm) (Nikon Optiphot II; Nikon).

TABLE 1: Viability of canine oocytes after different culture times in vitro

Time of culture (hours)	Number of oocytes	Viable oocytes (%)
24	67	62 (92·5)*
48	73	63 (86·3)*
72	113	80 (70·8)*
96	69	60 (86·9)*

* P>0·05

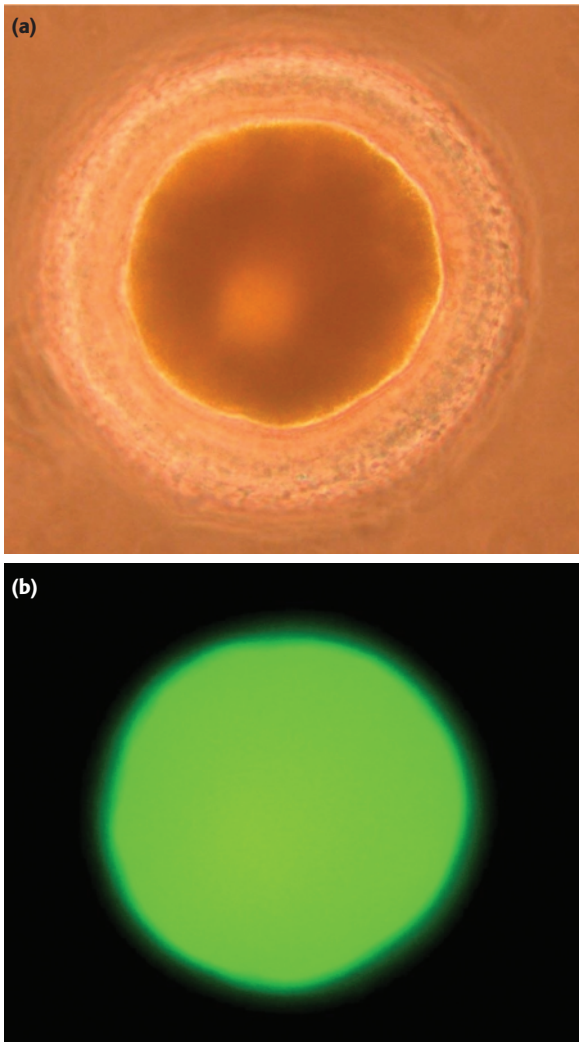

FIG 1: Canine oocyte stained with fluorescein diacetate and studied by (a) phase contrast and (b) epifluorescence microscopy. The green fluorescence in (b) indicates cell viability. X **400**

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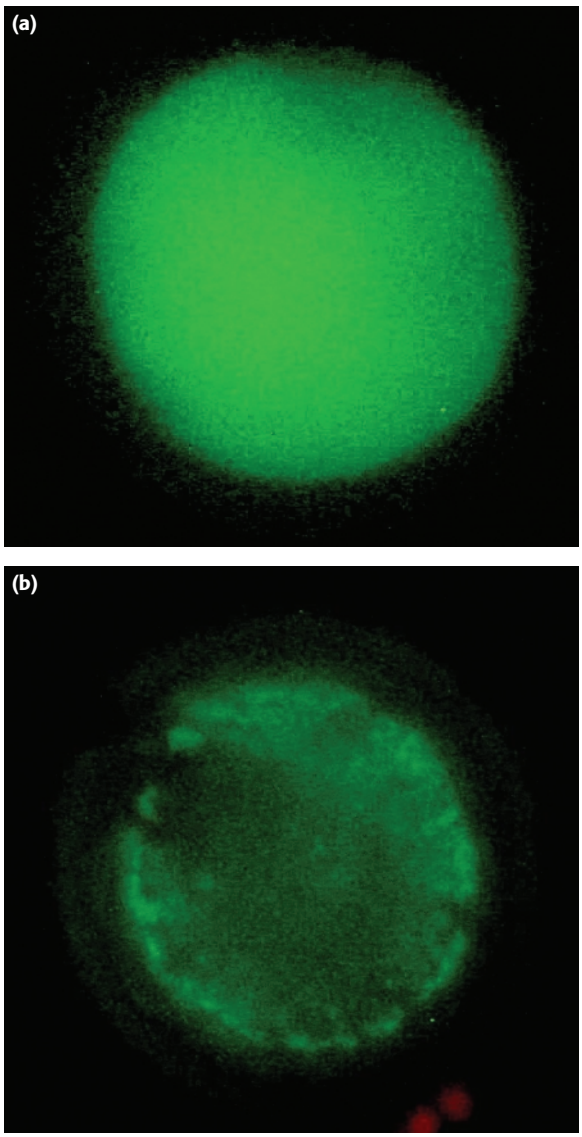

FIG 2: Distribution pattern of cortical granules in canine oocytes incubated with lectin *Lens culinaris* agglutinin coupled to fluorescein isothiocyanate, and evaluated under an epifluorescent microscope. (a) Immature oocyte. (b) Oocyte after 96 hours of in vitro maturation. x **400**

The proportion of oocytes with altered viability was analysed using the Student's *t* test to provide a measure of viable to non-viable oocytes after every 24 hours of culture. In the cortical granule analyses, the distribution patterns between the immature oocytes (before culture) and the in vitro matured oocytes after 96 hours were analysed using a chi-squared distribution comparison. Differences reaching $P \leq 0{\cdot}05$ were considered significant.

The number and percentage of oocytes that displayed fluorescence are shown in Table 1. The viability analysis showed that 399 of 450 (88·7 per cent) oocytes recovered from ovaries before culture were fluorescence positive (Fig 1), indicating that the majority of the oocytes selected for culture were suitable for IVM. During culture, a high proportion of oocytes were also viable, as demonstrated by the fluorescent probe. No differences were found between each culture time-point ($P<0{\cdot}05$) (see Table 1).

The fluorescent probe is clearly a sensitive indicator of oocyte integrity. For a sustained period after removal from the ovaries, a high percentage of the oocytes possessed a plasma membrane with physical integrity, as judged by the retention of fluorescein. Fluorescein diacetate is incorporated into the oocyte, then in the cytoplasm it is converted by non-specific esterases to fluorescein, which is retained and stains the living cells (Barros and others 1982). Thus, the fluorescence displayed by the oocytes is dependent on cytoplasmic esterases that release the fluorescein from the fluorescein diacetate. Therefore, this study shows that esterases remain active for up to 96 hours after culture initiation. Furthermore, similarly treated oocytes have proven to be fertilisable (De los Reyes and others 2005)

Cytoplasmic maturation of the oocytes is essential to allow normal fertilisation and male nucleus decondensation (Raz and others 1998). The intensity of lectin binding to the cortical granules was uniform in immature oocytes (Fig 2a) compared with those oocytes matured in vitro (Fig 2b). Following examination of the LCA-stained oocytes, a significant difference ($P<0{\cdot}05$) in the distribution and staining intensity of cortical granules was observed between oocytes before culture and those oocytes subjected to 96 hours of culture (cortical distribution 19 of 195 v 198 of 198, respectively). The distribution pattern of the cortical granules after 96 hours of culture was typical of that reported in mature oocytes, as shown by LCA labelling (Nicosia and others 1997, Wang and others 1997).

Changes in cortical granule distribution during culture have also been observed in oocytes from other species, suggesting that cytoplasmic maturation may be demonstrated by studying specific changes in the ooplasm during maturation. Here, in both immature and in vitro-matured oocytes, the zona pellucida was unreactive before and after culture. This corroborates the findings of other studies, which, by means of fluorescence microscopy, suggest that LCA lectins can specifically recognise cortical granule contents in oocytes (Jimenez-Movilla and others 2004).

Energy substrates are critical in the control of maturation and viability (Downs and others 1998), with pyruvate being the predominant energy source for maturing oocytes (Roberts and others 2002). It is possible that the exogenous supplementation of pyruvate to culture media could act as a survival factor for oocytes during prolonged culture.

The present study demonstrates long-term survival and cytoplasmic maturation of canine oocytes in vitro. Further studies are required to determine the exact requirements that will enable high numbers of canine oocytes to undergo IVM with continued viability to enable more in vitro fertilisation in dogs.

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