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Original article

Proteins in oocytes from calves and adult cows before maturation: relationship with their development capacity

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Summary — In order to improve the culture of oocytes that remain in an immature stage, the protein content before maturation and developmental ability of different groups of oocytes were investigated. The oocytes were classified according to source (adult or calf), the size of the follicle and the cumulus aspect. The influence of cumulus cells on developmental capacities was also analyzed and their protein patterns differ from those obtained with oocytes. The protein patterns differ specifically at 140 kDa and also at 143, 137, 60, 58, 52 and 27 kDa between oocytes from different sources, as does their capacity to develop into morula. Following *in vitro* maturation and fertilization, the oocytes were evaluated for percentage embryo development to the morula stage after 5 d. For cows' ovaries, denuded oocytes and oocytes with a defective cumulus had a percentage development of 2 and 12%, respectively. Cow ovary oocytes that were selected for cumulus compactness of 1–3 mm and > 3 mm had a percentage development of 21 and 25%, respectively. Oocytes from calf follicles had 0% development. These results indicate that the source of oocyte is a major determinant of its developmental capacity.

bovine / *in vitro* oocyte maturation / protein / developmental competence

Résumé — Protéines des ovocytes de veaux et de vaches adultes avant maturation : rapport avec leur capacité de développement. *Dans le but d'améliorer la culture des ovocytes demeurant à un stage immature, des comparaisons du contenu protéique avant maturation et la capacité de développement de différents groupes d'ovocytes ont été analysées. Les ovocytes ont été classés selon la source (veau ou adulte), la grosseur du follicule et l'aspect du cumulus. L'influence des cellules de cumulus sur la capacité de développement a également été étudiée et des différences en protéines ont été observées entre ces cellules et les ovocytes. Les patrons protéiques diffèrent spécifiquement à 140 kDa mais aussi à 143, 137, 60, 58, 52 et 27 kDa entre les groupes d'ovocytes tout comme leurs capacités de développement en morula. Suite à une maturation et une fécondation* in vitro, *les ovocytes provenant d'ovaires de vache : i) dénudés, ii) non sélectionnés, iii) sélectionnés selon l'aspect du cumulus des follicules de 1-3 mm, iv) > 3 mm et des ovocytes aspirés des follicules de veaux donnent un pourcentage de dévéloppement au stade morula évalué à j5 de : 2, 12, 21, 25 et 0% respectivement. Ces résultats corroborent le fait que la source des ovocytes est déterminante de leur capacité de développement.*

bovin / maturation ovocytaire* in vitro / *protéines / capacité de développement

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INTRODUCTION

Mammalian oocytes are arrested at the dictyate stage of the first meiotic prophase before or shortly after birth. Resumption of meiosis normally occurs in the preovulatory follicle as a consequence of the LH (luteinizing hormone) surge or can also be initiated when oocytes are released from their inhibitory follicular influence and cultured *in vitro* (Pincus and Enzmann, 1935; Edwards, 1965). In fact, when oocytes leave the nongrowing pool of female gametes and begin a program of development they acquire a series of abilities of great importance for subsequent resumption of meiosis, fertilization and embryo development. Only a small proportion of oocytes will result in a developing embryo after *in vitro* maturation. The acquisition of developmental competence in oocytes of domestic species occurs during the periovulatory period and, in bovine oocytes, it is related to follicle size (Tan and Lu, 1990; Pavlok *et al,* 1992) and the morphology of oocytes and their cumulus (De Loos *et al,* 1989). The age of the animal can also influence the acquisition of these abilities. In fact, induction of superovulation in prepubertal calves allows the growth of numerous follicles and ovulation, but the quality of the eggs and the resulting embryo obtained by such treatment are not satisfactory (Seidel *et al,* 1971). The poor quality of the calf embryos might be caused by an abnormal cytoplasmic maturation of the oocyte (absence or deficiency of some proteins) which prevents an adequate response to maturation signals. It might also be caused by oviduct or uterine non-reponsiveness. To know whether calf oocytes possess similar developmental competence to cow oocytes, a surgical transfer of *in vivo* matured calf oocytes to oviducts of an inseminated cow should be performed, but such transfers are not very successful (Sirard and Lambert, 1986). *In vitro* fertilization represents an alternative and *in vitro* maturation of oocytes from follicles of a similar size could be used to evaluate the potential without simultaneous evaluation of the endocrinological capacity of the calf ovary. The production of embryos from oocytes of prepubertal mammals is of great interest from many viewpoints: the ovary of the female calf contains many antral follicles (Erickson, 1966), the number of which reaches a maximum before puberty (Henrickson and Rajakoski, 1959); using calf oocytes would also mean a shorter time period between generations.

For the production of good embryos, it is necessary that the oocytes have undergone or can undergo normal nuclear and cytoplasmic maturation. The characterization of the relationship between protein content and capacity for the later biological events may allow a better control of maturation and thus acquisition of developmental competence. In order to evaluate this relationship, constitutive proteins in oocytes classified by follicular size, origin and cumulus aspect were investigated. Moreover, because of the potential role of cumulus cells in the promotion or inhibition of the maturation, their influence on development was also evaluated using *in vitro* maturation, fertilization and development.

MATERIALS AND METHODS

Recovery of oocytes

Ovaries were collected at a slaughterhouse and transported in saline (NaCl, 0.9% w/v with the following additives: penicillin 100 IU/ml, streptomycin 0.1 mg/ml; amphotericin B 0.25 μg/ml) to the laboratory within 2 h of collection at a temperature of 28–30°C. Oocytes were obtained by aspirating small (1–3 mm) or medium-large (≥ 3 mm) follicles of cow ovaries or all the follicles of calf ovaries. The calves were less than 40 d old. Aspiration was performed with an 18-gauge needle and a 10-ml syringe. The follicular contents were transferred to 50-ml conical tubes and allowed to sediment for about 15 min. The oocytes

were recovered under a stereomicroscope. The cumulus-enclosed oocytes (COCs) were further classified into 2 groups according to their cumulus aspect. The first group (selected) was composed of cow oocytes completely surrounded by unexpanded cumulus. The second group included oocytes with dark or expanded cumulus or not fully surrounded by cumulus cells (defective).

Electrophoresis of constitutive proteins in COC

The COCs were washed 3 times in PBS and then some oocytes from the first group (selected) were denuded of their cumulus cells by vortexing. The completely naked oocytes were washed again and recovered under a stereomicroscope. The naked oocytes obtained were washed in Tris-Cl (0.1 M), transferred with a minimum amount of fluid into a microtube and then frozen at –20°C until use. The buffer (Tris-Cl) used to wash the oocytes in the preceding steps and cumulus-containing cells were pooled in a microtube and centrifuged and the supernatant was discarded. The pellet (cumulus cells) was also frozen at –20°C. The COCs or their respective cumulus cells were analyzed immediately after collection. The proteins were extracted by 2–3 freeze-thaw cycles. The quantity of protein were determined by Bradford reaction for each group (4 catagories of COC: cow defective occytes, selected oocytes of follicle of 1–3 or > 3 mm, and calf oocytes), following a sonification of 10 s. The reagents for this reaction and for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, Ca). Electrophoresis was performed on 5–12% linear gradient SDS-PAGE, according to Laemmli (1970). For each group, 30 µl SDS lysis buffer was added and about 7 µg total protein was placed in each well (corresponding to approximately 130 adult cow oocytes and 260 calf oocytes). The gel was silver staining prior to Coomassie staining (Dzandu *et al*, 1984).

Evaluation of developmental capacities of oocytes

Five groups of oocytes (the same as those described in the analysis of constitutive protein plus naked oocytes) were matured as previously described (Sirard *et al*, 1988; Gagné *et al*, 1991) in 50 µl droplets of a maturation medium consisting of: TCM–199 with Earles salts; $NaCO_3$ (2.2 g/l Gibco Lab, Grand Island, NY); heat-treated FCS (10%); gentamycin (0.05 mg/ml); pyruvate (0.2 mM); FSH (0.5 µg/ml National Institute of Diabetes and Digestive and Kidney Diseases); LH (5 µg/ml NIDDK); and estradiol (1 µg/ml Sigma Chemical Co, St Louis, Mo). After 24 h maturation, they were fertilized in fertilization medium droplets of 45 µl composed of modified tyrode lactate medium (Bavister and Yanagimachi, 1977), BSA FAF (0.6%), pyruvic acid (0.2 mM), heparin (10 µg/ml) and gentamycin (0.05 mg/ml) and allowed to develop *in vitro* (same as maturation medium without hormones) for 5 d post-fertilization. The embryos were then transferred onto glass slides. The slides were immersed in a fixative solution (ethanol/acetic acid, 3:1) for a minimum of 24 h. They were stained with 1% aceto-orcein and the number of nuclei was counted with phase contrast (Sirard *et al*, 1988). Each treatment was replicated with the proper controls for each experiment and the significance of individual comparison was evaluated by Chi-square evaluation (Snedecor and Cochran, 1980).

RESULTS

Patterns of constitutive proteins in oocytes

Figure 1 illustrates the constitutive proteins in oocytes and cumulus cells upon recovery from the ovaries. Changes in protein patterns of oocytes observed during follicular growth (small follicles of 1–3 mm to medium-large of ≥ 3 mm) were found to resemble the difference occurring between selected and defective oocytes. This is true for most analyzed bands with the exception of a 140 kDa protein. The synthesis of this protein increases with follicular growth. Although a specific production has not been visualized, the relative amount of proteins of 143, 137, 60, 58, 52 and 27 kDa seems to increase whereas some large molecular weight bands are much less apparent (180, 158, 131, 71 kDa) in the oocytes coming from follicles > 3 mm. In general, calf oocytes presented a

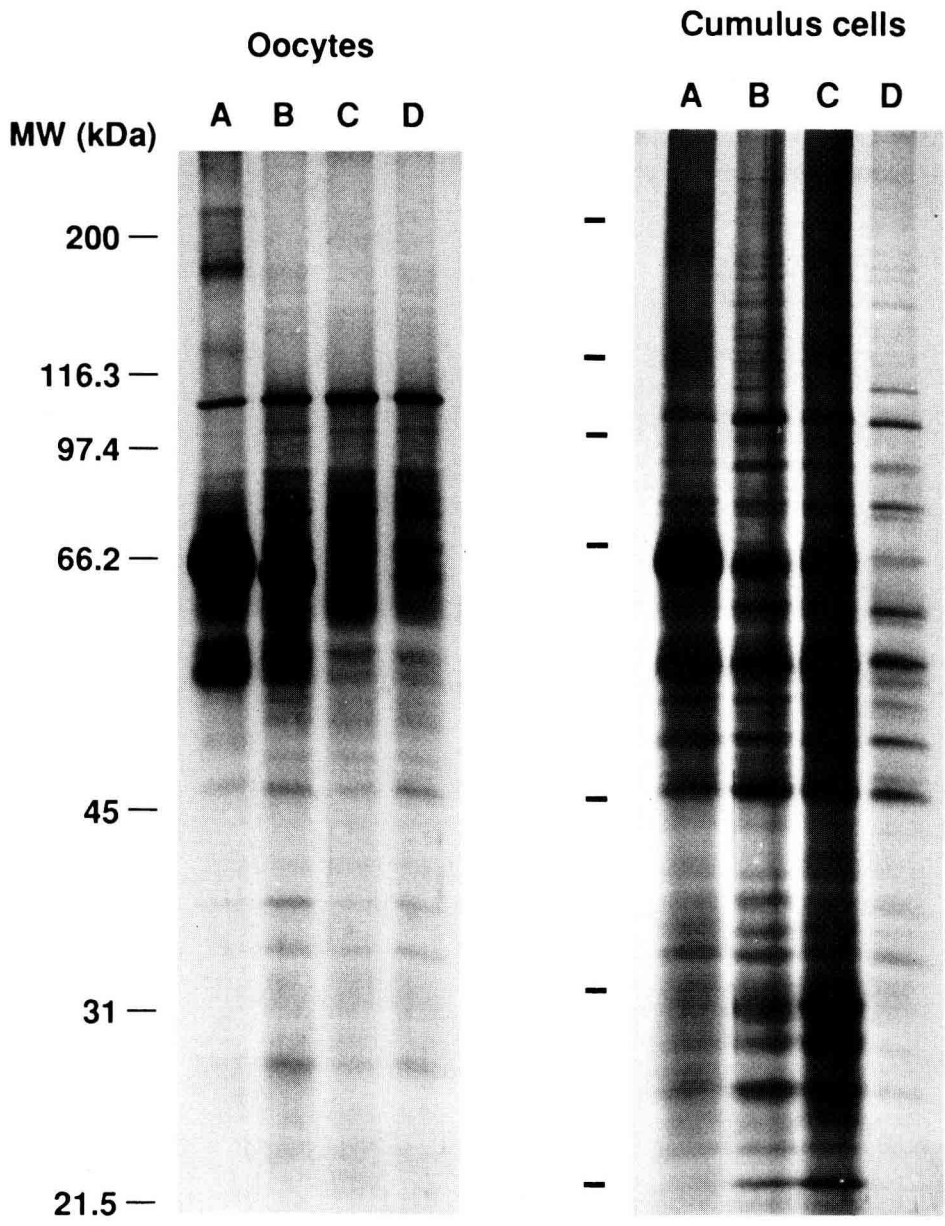

Fig 1. Patterns of constitutive proteins in oocytes or respective cumulus cells at collection. A: non-defective oocytes or respective cumulus cells from cow follicles of 1–3 mm. B: non-defective oocytes or respective cumulus cells from cow follicles of ≥ 3 mm. C: defective oocytes or cumulus cells from cow follicles. D: non-defective oocytes or cumulus cells from calf follicles. The dashes on the right-hand side represent the same molecular weight as on the left.

protein pattern similar to defective oocytes. Some proteins (180, 159, 117, 107 and 84 kDa) were present and more easily visualized in oocytes than in cumulus cells. In fact, the 107 and 84 kDa bands might be glycoproteins of the zona pellucida. The protein pattern of cumulus cells differ from oocytes in numerous ways. The proteins present in cumulus but not in oocytes were: 191, 188, 185, 156, 152, 136, 129, 126, 105, 89, 71, 63, 61, 54, 50 42 and 24 kDa. Moreover, the protein pattern from the cumulus of calf oocytes differed from other groups of cumulus cells, mainly by the absence or the decrease in the content of proteins of molecular weight 191, 185, 159, 69, 67 and 25 kDa. No single band seemed to be increased in the calf cumulus cells compared with the cumulus surrounding the oocytes of cycling cows.

Effects of follicle origin and presence of cumulus cells on developmental capacity of oocytes

The capacity of oocytes to develop into embryos was evaluated. After *in vitro* maturation and fertilization, cleavage was obtained at high levels in all groups (50–86%) except for the calf oocytes (19%). The maturation rate was not evaluated but we did not observe immature stage oocytes in calves when fertilization was examined. The capacity to develop to 8 or 16 cells differed between groups (table I). Although oocytes from cow follicles > 3 mm in diameter showed a slightly higher rate of development than oocytes from smaller follicles, the difference was not significant. All other groups of oocytes displayed a significantly lower capacity to develop compared to these 2 groups. Moreover, calf oocytes were never able to reach the morula stage (table I).

DISCUSSION

The constitutive protein profile has been compared for defective and non-defective oocytes and from follicles of different origins and sizes. A small contamination of oocyte proteins by cumulus prolongations embedded in the zona should be expected (Thibault *et al*, 1987). Surprisingly, the protein pattern of defective oocytes showed many similarities to those of calf oocytes and could account for their lower develop-

Table I. Developmental competence of different categories of oocytes cultivated 5 d after *in vitro* fertilization.

Category	*N*	In vitro *development (%)*	
		No embryos with 8–16 nuclei	*No embryos with > 16 nuclei*
Calf oocytes	189	12 (6) [a]	0 (0) [a]
Cow oocytes			
Defective	242	45 (19) [a]	30 (12) [a]
Non-defective			
1–3 mm	378	95 (25)	79 (21)
> 3 mm	211	50 (24)	53 (25)
Denuded	59	16 (27)	1 (2) [a]

[a] Statistically different from oocytes from cow follicles 1–3 mm in diameter (χ^2, $p < 0.02$).

mental capacity. The effect of follicular size has recently been reported to affect the developmental potential of the oocytes (Tan and Lu, 1990; Pavlok *et al,* 1992), but unfortunately is not related to the appearance of a visible specific product with the methodology used here. However, the relative amount of some proteins (143, 137, 60, 58, 52 and 27 kDa) seems to increase and some large molecular weight bands are much less apparent (180, 158, 131, 71 kDa) in the oocytes coming from follicles > 3 mm. Many constitutive proteins of cycling cow cumulus cells differ from those of oocytes and several proteins are simply absent in calf cumulus cells. These proteins may be of tremendous importance for the initiation of a cascade of events necessary for calf oocytes to proceed through development or for a proper response to ovulatory signals.

There was a difference in embryonic development (morula) among the different categories of oocytes. We failed to obtain development (> 16 cells) from fertilized calf oocytes in contrast with a few reports in which blastocysts were obtained. The calves used in this study were less then 40 d old and the dimensions of the ovaries were about 1 cm. These facts could account for their poor potential to develop. In addition, only a small fraction (2%) of denuded oocytes were able to reach this stage. These results are in accordance with those of Yang and Lu (1990) in which no development to blastocyst stage was obtained with naked oocytes, and those of Sirard *et al* (1988), in which a significantly lower developmental capacity was observed with denuded oocytes. The highest percentage of morula (25%) came from oocytes of medium-large follicles (> 3 mm). Although this result was not significant in our study, given the limited availability of large follicles, it is possible that the size of follicles is related to embryonic development competence as shown significantly by Tan and Lu (1990) and Pavlok *et al* (1992). Moreover, the quality and especially the presence of cumulus cells also affected the developmental capacities of oocytes in embryos. Bovine GVBD (germinal vesicle breakdown) does not necessitate the presence of cumulus cells in the current study, in contrast to rabbit COCs, which are dependent upon early transcription and translation events in the cumulus cells (Motlík *et al,* 1989). Furthermore, the low rate of development of calf oocytes and denuded cow oocytes could result from abnormalities in maturation divisions. In fact, a cytologic study by Foote and Thibault (1969) on calf oocytes revealed unusual spindle formation. Similarly, the presence and quality of cumulus cells may be helpful for normal maturation and developmental potential since the capacity of naked oocytes is largely reduced compared with COCs and is also observed for defective oocytes (Sirard *et al,* 1988; Stubbings *et al,* 1988).

The evidence presented here suggests that the protein content of bovine oocyte at collection differs according to follicular origin, size and cumulus aspect and is associated with the capacity to develop. The presence of cumulus cells can also modulate the ability of oocytes to undergo the events necessary for the production of embryos.

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