Anim. Reprod., v.2. n.1, p.70-73, Jan./March 2005

The effects of the number of oocytes and the volume of maturation medium in bovine *in vitro* embryo production

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Abstract

For *in vitro* embryo production following ovum-pick-up groups with different number of oocytes and volume of medium may be required to maintain an individualized production of each cow. Bovine oocytes were randomly allocated in a 3x3 factorial design in order to evaluate the effect of number of oocytes (5, 10 and 20) and maturation medium volume ratios (1:1, 1:5 and 1:10 oocyte/µl) on embryo production. After 22-24h maturation in TCM-199, *in vitro* fertilization was performed in FERT-TALP medium, for 18-22h. Groups of 20 presumptive zygotes were cultured in 200µl of SOF medium for eight days. There was no effect of interaction between number of oocytes and volume of maturation medium (P>0.05) on embryo development. Cleavage rates were not affected either by number of oocytes nor by volume of medium (P>0.05). Blastocyst rates on D7 (19, 26 and 28%; D0=day of fertilization) and D9 (23, 33 and 33%) were lower (P<0.05) in groups of 5 oocytes compared with groups of 10 and 20 oocytes, respectively. Blastocyst rates on D7 (22, 24 and 27%) and D9 (26, 31 and 32%), were similar (P>0.05) for ratios 1:1, 1:5 and 1:10 oocytes/µl, respectively. Hatching rate with a 1:1 volume ratio (26%) was lower (P<0.05) than that obtained with a 1:5 volume ratio (44%). Maturation in larger groups increases embryo production. The volume of maturation medium does not influence the blastocyst production, however a low oocyte/µl medium ratio reduces the hatching rate.

Keywords: *In vitro* maturation, bovine, medium volume, oocyte.

Introduction

Transvaginal ovum pick-up has been used commercially to improve *in vitro* embryo production (IVP) from cows with high genetic merit for milk or beef production. There are many advantages in IVP, such as the fact that it can be used in females with acquired infertility and also in those up to three months of pregnancy. The protocols for IVP from oocytes aspirated of live females, have been adjusted constantly in order to achieve better and more consistent results.

Several factors may influence the commercial embryo production rates, such as individual variation of the oocyte donor, semen, medium and culture conditions employed during each phase of *in vitro* embryo production. Efficient transport systems for oocytes and embryos as well as the volume of medium for *in vitro* production are equally important. In commercial programs, the number of oocytes per drop of medium is much less than the number used in research programs. Furthermore, oocyte recovery rates by *in vivo* aspiration are relatively low, with an average of 10 to 12 oocytes per female (Galli *et al*., 2001; Dayan *et al.*, 2002) which could demand for low volumes of maturation medium.

Although it is known that the culture in groups, after fertilization, has a beneficial effect on development to blastocyst stage (Donnay *et al*., 1997; Ahern and Gardner, 1998; Ward *et al*., 2000), the number of oocytes matured or the number of embryos cultured per drop of medium have been used empirically for many years without considering the volume of medium per drop. Currently, the number of oocytes/embryos used in *in vitro* fertilization programs is still variable. On the other hand, bovine oocytes have also been cultured individually for obtaining more precise information on their requirements for fully developmental competence (Oyamada and Fukui, 2004) which can contribute to improve blastocyst yield from ovum-pick-up oocytes.

The purpose of this study was to determine the best volume of medium per drop for *in vitro* maturation with reduced number of oocytes**.**

Materials and Methods

The ovaries of slaughtered cows were transported to the laboratory within four hours at 22-25ºC (Yang *et al*., 1990) in NaCl 0.9% solution with 100mg of streptomycin and 50.000IU of penicillin per liter of solution. Cumulus-oocyte complexes were aspirated from follicles with 2 to 8mm of diameter. Aspirated oocytes were maintained in follicular fluid while they were searched and selected under a

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Received: October 22, 2004
Accepted: March 3, 2005

stereomicroscope. Selection was based on morpho-ological aspects according to criteria described by De Loos *et al.* (1989). Unless stated otherwise, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Selected oocytes with excellent and good quality scores were randomly allocated to nine treatments, in a 3x3 factorial design, using three levels of two factors: number of oocytes (5, 10 and 20) and maturation medium volume ratios (1:1, 1:5 1:10 oocytes/µl). Seven replicates were performed. Oocytes were matured in different volumes of medium so that groups of different number of oocytes (20, 10 and 5) were distributed in all proportions of medium (1:10, 1:5 and 1:1 oocytes/µl). Thus, in treatments 1, 2 and 3, groups of 20 oocytes were matured in drops of 200, 100 and 20µl of medium. For treatments 4, 5 and 6, groups of 10 oocytes were matured in drops of 100, 50 and 10µl, respectively. In treatments 7, 8 and 9, groups of 5 oocytes were matured in 50, 25 and 5µl of medium. In order to have the same number of oocytes for all treatments, two and four drops of maturation medium were used for groups of 10 and 5 oocytes, respectively, in each replicate.

Maturation was accomplished in TCM-199 (Gibco, BRL, USA) plus 2.2mg/ml of sodium bicarbonate, 5.95mg/ml of HEPES, 0.025mg/ml sodium pyruvate, 0.01 IU of rFSHh/ml (Serono Pharma, Italy) and estrus cow serum (ECS). Oocytes were matured for 22 and 24 hours in an incubator at 39ºC, under 5% of CO_2 in air and saturated humidity. All drops of maturation medium were covered with mineral oil.

Incubation of oocytes with spermatozoa was conducted in groups of 20 oocytes in 200µl of Fert-Talp medium with 6mg/ml of BSA, 0.022mg/ml sodium pyruvate and 30µg/ml of heparin, covered with mineral oil. The fertilization period was 18 to 22 hours in an incubator at 39ºC, with 5% of CO_2 in air and saturated humidity. Thawed semen from a *Bos taurus* bull was submitted to the swim-up process in Sperm-Talp medium with 6mg/ml of BSA and 0.11 mg/ml of sodium pyruvate. The insemination dose was 1 x 10^6 spermatozoa/ml.

After the fertilization period, presumed zygotes were denuded by vortex agitation and cultured for 8 days in 200µl of SOF_{aaci} culture medium (Holm *et al.*, 2000) containing 5% of estrus cow serum, under mineral oil, in an incubator at 39°C, with 5% of CO_2 in air and saturated humidity.

Evaluations of cleavage, blastocyst and hatching rates were performed on days 2 (D2), 7 (D7) and 9 (D9), respectively, considering the date of insemination as day zero (D0). Arcsine square root transformation was performed on the percentages of cleavage and blastocyst rates before being submitted to analysis of variance (SAS, 1998). The mean percentages were compared by Tukey´s test, at a 5% significance level. Hatching rates were compared by the Chi-Square test.

Results

There was no effect of the interaction between the number of oocytes and the volume of medium (P>0.05; Table 1 and 2) on embryo development. Maturation in groups of 10 oocytes was not different (P>0.05) in terms of cleavage, blastocyst rates at D7, D9 and of hatching rates at D9, if compared to groups of 20 oocytes (Table 1). Although maturation in groups of 5 oocytes resulted in lower rates (P<0.05) of blastocysts production at D7 and D9, no differences in cleavage and hatching rates were observed in comparison to groups of 10 and 20 oocytes. Volume of medium had no effect on cleavage and blastocyst production at D7 and D9, but the maturation of 1 oocyte in 1µl drop resulted in a hatching rate lower (P<0.05) than 1 oocyte in 5µl (Table 2).

Table 1. *In vitro* production of bovine embryos with different numbers of oocytes regardless of maturation medium volume

Number of oocytes per group	Presumed zygote n	Cleavage n (%)	Embryos D7 n (%)	Embryos D9 n (%)	Hatching* n (%)
5	393	319 (81.2)[a]	73 (18.6)[a]	92 (23.4)[a]	28 (30.4)[a]
10	388	334 (86.1)[a]	102 (26.3)[b]	128 (33.0)[b]	43 (33.6)[a]
20	405	352 (86.9)[a]	115 (28.4)[b]	135 (33.3)[b]	56 (41.5)[a]

Different letters in the same column indicate significant difference (P<0.05)
*Considering D9 embryos

Table 2. *In vitro* production of bovine embryos with different volumes of maturation medium regardless of number of oocytes matured

Volume of medium per oocyte	Presumed zygote n	Cleavage n (%)	Embryos D7 n (%)	Embryos D9 n (%)	Hatching* n (%)
1µl	394	326 (82.7)[a]	88 (22.3)[a]	106 (26.9)[a]	28 (26.4)[a]
5µl	396	335 (84.6)[a]	95 (24.0)[a]	123 (31.1)[a]	54 (43.9)[b]
10µl	396	344 (86.9)[a]	107 (27.0)[a]	126 (31.8)[a]	45 (35.7)[ab]

Different letters in the same column indicate significant differences (P<0.05)
*Considering D9 embryos

Discussion

Cattle in vitro embryo production seems to be directly affected by the number of oocytes/embryos cultured. Groups with less than 20 structures, during the whole IVP, resulted in lower rates of production than those cultured in groups with a larger number of structures (Donnay *et al*., 1997; O'Doherty *et al*., 1997; Fukui *et al*., 2000). This also occurs with IVP in other species (Salahuddin *et al*., 1995), probably due to the stimulatory effect of autocrine and paracrine factors produced by embryos (O'Doherty *et al*., 1997).

Many studies were successfully performed using individual culture or culture of small groups of embryos, but maturation and fertilization have been predominantly performed in larger groups (Ferry *et al*., 1994; Vajta *et al*., 2000). It has been demonstrated that individual oocyte maturation result in a reduced production of blastocysts (O'Doherty *et al*., 1997), despite fertilization and culture being performed in groups (Jewgenow *et al*., 1999). Recently, embryonic development of oocytes matured individually was considered unsatisfactory compared with the blastocyst yielded from groups of 5 to 10 oocytes (Omayada and Fukui, 2004). In previous reports, maturation in groups of 5 and 10 oocytes resulted in similar blastocyst rates (O'Doherty *et al*., 1997; Ward *et al*., 2000), which is in contrast with the observed in the present study. Despite of the fertilization and culture being performed in groups of 20 structures, a decrease in blastocyst production was observed when oocytes were matured in groups of five compared to 10 or 20 oocytes per group. The fact that smaller groups of oocytes resulted in lower production of embryos may be connected to the hypothesis that beneficial interactions among oocytes, such as the production of growth factors, may occur during maturation (O'Doherty *et al*., 1997). Indeed, frequency of polar body extrusion in bovine oocytes derived from small follicles ($\leq$3mm) increased when maturation was accomplished in the presence of EGF or EGF + IGF-I (Sakaguchi *et al*., 2002). Furthermore, the addition of EGF to the maturation medium had a beneficial effect on fertilization rate and developmental competence of bovine oocytes individually matured in a chemically defined medium (Oyamada and Fukui, 2004).

The volume of medium used for IVP is quite variable among laboratories and there is no specific recommendation of volume for each phase of production. A reduction of the volume of medium per embryo, during *in vitro* culture, is suggested to increase their cooperation (Ahern and Gardner, 1998). It is known that oocyte maturation conditions can result in deficient maturation which may lead to low embryo development or even to a lower cryoresistance of blastocysts produced (Oyamada and Fukui, 2004). In the present study, maturation in a small volume of medium per oocyte (1µl per oocyte) resulted in reduced hatching rate without affecting the development up to blastocyst stage. However, it is important to emphasize that the remaining phases of *in vitro* production were accomplished in a proportion of 10µl of medium for each oocyte or embryo probably avoiding the possible deleterious effect of the 1:1 proportion on the cleavage and blastocyst formation if all steps were performed using this proportion of medium. Hashimoto *et al*. (1998) achieved successful maturation using oocytes only with the corona radiata cells in a reduced volume of medium (1:1). Cumulus cells have a beneficial effect during maturation, but if they are in high concentration they can compete with oocytes for nutrients. Using low volumes of medium (1 to 2µl), a high density of cumulus cells ($\geq$ 8.0 x 10^6 cell/ml) and 10 to 11 oocytes per group, Hashimoto *et al*. (1998) observed a decrease in maturation, fertilization, cleavage and blastocyst rates in comparison to volumes of 5 and 10µl, which allowed densities of 3.2 and 1.6 x 10^6 cells/ml, respectively. In this study, oocytes had a normal number of cumulus cells layers ($\geq$5), probably providing a high concentration of cells per volume of medium in the 1:1 proportion. This fact may have contributed to a lower ability of hatching in blastocysts derived from oocytes matured in a low proportion of medium.

Investigations of certain parameters such as pH, oxygen and ammonia concentration could help to understand the mechanisms by which a reduction in the number of oocytes or in the volume of maturation medium affects the embryonic development. These responses would maximize the results of IVP protocols.

Production of bovine blastocysts increases when oocytes are matured in groups of 10 and 20 instead of 5 oocytes. The proportion of medium for each oocyte, during maturation, does not affect cleavage or blastocyst production; however it does affect the hatching rate.

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