Effect of Buffalo Follicular Fluid Alone and in Combination with PMSG and M199 on *in vitro* Buffalo Oocyte Maturation

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ABSTRACT : The effect of replacement of *in vitro* maturation medium completely with the buffalo follicular fluid (buFF) on *in vitro* oocyte maturation of buffalo oocytes was studied. 5 to 8 buffalo cumulus oocyte complexes were cultured in a single drop with each of the eight media studied i.e., M199+steer serum (10% v/v), M199+steer serum (10% v/v)+PMSG, M199+buFF (10% v/v), M199+buFF (10% v/v)+PMSG, M199+buFF (50% v/v), M199+buFF (50% v/v)+ PMSG, buFF (100%) and buFF+PMSG at 39℃ and 5% CO_2 in air for 24 h. Supplementation of M199 with Steer serum alone resulted in IVM rate of 35% only. When the above medium was supplemented with PMSG, the maturation rate rallied to 82%. Significant increase in the maturation rates were observed when M199 was supplemented with increasing levels of buFF. A further increase in the maturation rate was also obtained when PMSG was incorporated into the medium of M199 supplemented with buFF. The rate of maturation was to the tune of 91% when oocytes were matured in buFF alone which was increased non significantly on the addition of PMSG. Highest maturation rate (97%) obtained with M199+buFF (50%v/v)+PMSG did not differ significantly from that obtained by either M199+buFF (10%v/v)+PMSG or buFF +PMSG. It is suggested that buFF alone without any supplementation can form the effective in vitro maturation medium for buffalo oocytes. *(Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 5 : 693-696)*

Key Words : Follicular Fluid, Oocytes, IVM, Buffalo

INTRODUCTION

In recent times, laboratory production of embryos is drawing keen attention because of its applications in the emerging technologies like cloning and transgenic animal production apart from embryo transfer technology. Since the cost of production of embryos is very high, several efforts are being made by various research workers to economize the production by way of replacing the expensive components of culture media with less expensive ones. In order to reduce the cost of this technology, conventionally used fetal bovine serum (FBS), an important and expensive component in the in vitro maturation medium of oocytes is being successfully replaced with steer serum, cow serum or follicular fluid. Follicular fluid (FF) is being successfully incorporated in IVM media of i.e cattle (Romero-Arredondo and Seidel 1994, Choi et al., 1998), buffaloes (Chauhan et al., 1997, Tajik et al., 2000), sheep (Larocca et al., 1993), pigs (Zak et al., 1997). Use of FF has been common in the IVM of pig oocytes than in cattle due to the conflicting reports on its effect on IVM in cattle (Dostal et al., 1996).

The advantages of incorporation of follicular fluid on in vitro oocyte maturation are due to the presence of growth factors, Follicle stimulating hormone (FSH), Leutinizing hormone (LH) and several nutrients in it whereas the disadvantage is being the presence of oocyte maturation inhibitory factor. Use of follicular fluid as an IVM medium was attempted as early as in 1969 (Sreenan, 1969). In cattle, up to 50% replacement of generally used IVM medium with follicular fluid has resulted in good maturation rate (Ocana Quero et al., 1997) but replacement at 100% level had inhibited the maturation rate (Ayoub and Hunter, 1993). Oocytes maturation rates were observed higher when cultured in 100% follicular fluid collected from large follicles than from small follicles in cattle (Choi et al., 1998). In buffaloes, the replacement level was restricted to 40% (Chauhan et al., 1997). It is assumed that hundred percent replacement of IVM medium with follicular fluid obtained from the same species may simulate the conditions of *in vivo* maturation thereby improve the in vitro maturation rates of oocytes in buffaloes. Hence this study was conducted to test the efficacy of buffalo follicular fluid from large follicles on IVM of buffalo oocytes if used at 100% level.

MATERIALS AND METHODS

All media and chemicals were procured from Sigma chemical, St. Louis, MO, USA unless other wise stated. Pregnant mare serum gonadotrophin (FOLLIGON®) was purchased from Intervet, International B.V., Boxmeer-Holland.

Preparation of buffalo follicular fluid (buFF)

Buffalo ovaries were collected immediately after slaughter of the animals of unknown reproductive status at the local abattoir and transported in normal

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saline (0.9% NaCl) supplemented with gentamycin (50 μg/ml) to the laboratory within 1 h. At the laboratory, they were rinsed thoroughly with fresh normal saline supplemented with gentamycin (50 μg/ml). Follicular fluid was aspirated from large surface follicles (>5 mm diameter) of the ovaries using 18 gauge needle and was then transferred in to a sterile glass beaker for heat inactivation at 50℃ for 30 minutes in a water bath. The buFF was sterilized by filtering through the 0.22 μm syringe driven filter (Millipore Corporation, USA) and then was stored in sterile micro centrifuge tubes of 1.5 ml capacity (Tarsons, India) at -20℃ for subsequent use for IVM. Same batch of pooled follicular fluid was used for all the trials.

Preparation of steer serum

Serum was harvested from the blood collected from a cross bred Holstein-Friesian steer and was heat inactivated and later filtered as described above for follicular fluid and stored at -20℃ till used for IVM. Same batch of pooled serum was used for all the trials.

Collection of cumulus oocyte complexes (COCs)

Follicular fluid was collected as described above into a syringe pre-loaded with aspiration medium consisting of M199 and Dulbeccos phosphate buffer saline supplemented with 0.3% bovine serum albumin in 1:1 ratio and was screened for oocytes under stereo zoom microscope (Olympus, Japan). Only oocytes with homogenous cytoplasmic granulation and with more than three compact cumulus cell layers were taken for IVM study.

***In vitro* maturation**

In each trial of the total 10 trials conducted, 7 to 13 COCs were cultured in a single drop with each of the eight media studied I) M199+steer serum (10% v/v), II) M199+steer serum (10% v/v)+PMSG, III) M199+buFF (10% v/v), IV) M199+buFF (10% v/v)+ PMSG, V) M199+buFF (50% v/v), VI) M199+buFF (50% v/v)+PMSG, VII) buFF (100%) and VIII) buFF+ PMSG. In this study, PMSG was used at uniform level i.e. 40 IU/ml wherever it was supplemented. All culture media were supplemented with gentamycin (50 μg/ml). M199 was supplemented with glutamine at 100 μg/ml. The COCs were washed thrice in the respective culture media and then put for culture in sterile petri dishes (Falcon, Becton & Dickinson Labware, USA) in 50 μl of media drops under mineral oil at 39℃ in 5% CO_2 in air for 24 h. Maturation was assessed by examining the expansion of cumulus cell mass as well as identification of the first polar body in the perivitelline space after denuding them by repeated pipetting. Cumulus expansion was evaluated by the classification scheme of degree of expansion as described by Kobayashi et al (1994). Briefly, Degree 2 (Full cumulus cells expansion) : All cumulus cells homogeneously spread; Degree 1 (Moderate cumulus cells expansion) : 70% cumulus cells homogeneously spread and Degree 0 (Slight or no expansion): Cumulus cells were slightly adherent to the zona pellucida. Oocytes with degree 1 and degree 2 cumulus expansion and with extruded first polar body in the perivitelline space were considered as matured.

Statistical analysis

Difference in the effect of various maturation media was tested by Analysis of Variance. The maturation rate was recorded as the total number of oocytes that matured over the total number of oocytes cultured (also expressed as a percentage). In vitro maturation rate obtained by different media was compared by chi-square test (Snedecor and Cochran, 1967) with a probability level of $p<0.05$ being considered significant.

RESULTS AND DISCUSSION

The difference in the rate of maturation of oocytes obtained by different media (table 1) was significant ($p<0.05$). Significant increase in *in vitro* maturation rates could be obtained with the increase in the level of supplementation of buFF with M199. Buffalo follicular fluid (buFF) without any supplementation could induce 91% IVM rate in buffalo oocytes. When buFF was supplemented with PMSG (40 IU/ml), no significant ($p>0.05$) increase in the in vitro maturation rate was observed. The combination of M199+buFF (50% v/v)+PMSG resulted in highest (97%) *in vitro* maturation rate. When M199 was supplemented with only steer serum (10% v/v), maturation rate was significantly low (35%) in comparison to all other media. When this medium was supplemented with PMSG (40 IU/ml), a spurt in the maturation rate(81%) was observed. Similar maturation rate of 82% could be obtained by supplementing M199 with only buFF at 50% v/v without any additional hormonal supplement. Supplementation of M199 with buFF at 10% v/v instead of steer serum yielded significantly ($p<0.005$) higher maturation rate of 64%. No significant ($p>0.05$) difference in the maturation rates was obtained by using M199+buFF (10% v/v)+PMSG and buFF+PMSG.

Literature on the effect of follicular fluid on IVM of buffalo oocytes is scarce. Generally used IVM media for oocytes in cattle or buffaloes consists of M199, serum and FSH. Replacement of M199 with increasing levels of follicular fluid (50% and 100%) without additional supplementation resulted in signi-

Table 1. Effect of replacement of serum with buffalo follicular fluid (buFF) in the medium for *in vitro* oocyte maturation of buffalo oocytes

Treatment	No. of oocytes cultured	No. of oocytes matured	Maturation rate, % ± SE
M1999+Steer serum	72	25	34.7 ± 1.4^{a}
M199+Steer serum+PMSG	124	102	82.3 ± 1.9^{b}
M199+buFF (10%)	84	54	64.3 ± 0.9^{c}
M199+buFF (10%)+PMSG	118	109	92.4 ± 1.1^{de}
M199+buFF (50%)	91	74	81.3 ± 1.2^{b}
M199+buFF (50%)+PMSG	113	110	97.3 ± 0.8^{e}
buFF (100%)	119	108	90.8 ± 1.7^{d}
buFF+PMSG	105	97	92.4 ± 1.2^{de}

[a,b,c,d,e] Values with different superscripts differ significantly in the last column ($p<0.05$, χ^2 test) (e>d>b>c>a).
Data from 10 trials were pooled together.
PMSG: Pregnant Mare Serum Gonadotropin (at 40 IU/ml).
buFF: Buffalo follicular fluid.

ficantly increasing rates of maturation in this study (table 1) unlike in cattle where there was a non significant difference in maturation rates was observed between 10%, 20% and 50% FF supplementation (Ocana-Quero et al., 1997). However, maturation rates were found to be significantly higher than those obtained with the supplementation of M199 with fetal bovine serum (FBS) (10% v/v) or FBS+FSH (Chauhan et al., 1997). In a recent study too, Tajik et al. (2000) could not observe any significant difference in the in vitro maturation rates of buffalo oocytes when M199 was supplemented with either bovine or buffalo follicular fluid at 10% or 20% level. Moreover, addition of bovine follicular fluid (bFF) at 30% v/v to M199 without any additional gonadotropin resulted in very high in vitro maturation rates (92%) in cattle (Larocca et al., 1995), but in present study addition of buFF at 50% level to M199 without PMSG resulted in only 81% maturation rate. Addition of buFF at 60% v/v level inhibited oocyte maturation in cattle suggesting that fibrin like substances in the follicular fluid might have been involved in the coagulation of cumulus cell mass of oocytes resulting in reduced maturation rate (Kim et al., 1996). To the contrary in the present study, buFF at 100% level resulted in very high maturation rates (>90%) unlike in cattle wherein bFF was reported to have inhibited the maturation rate of oocytes (<15%) (Sirad and First, 1988; Bevers et al., 1992; Ayoub and Hunter, 1993). These differences may be due to the fact that the follicular fluid contains a mixture of both stimulatory and inhibitory factors like maturation inhibiting factor, inhibin, follicular regulatory protein (an aromatase enzyme inhibitor), growth factors, hormones like FSH, FSH receptor binding inhibitor, meiosis-activating sterol (MAS), other nutrients etc., and it is likely that the differences between these two species in the content/ratio of these substances in the follicular fluid might influence altogether different responses in terms of oocyte maturation rates in cattle and buffaloes. Since this is the first report on the effect of 100% replacement of IVM medium with buFF on in vitro maturation of buffalo oocytes, no data is available in the literature for comparing our results with the homologous species.

Administration of buffalo follicular fluid (Kumar, 1997) or isolated buffalo follicular fluid proteins (Ghosh, 1998) increased the ovulation rate in goats indicates its beneficial effects on in vivo oocyte maturation. But the results can not be readily applied to in vitro conditions due to the involvement of many factors during *in vivo* maturation rather than during the in vitro maturation. When FF at 30% v/v level was used in IVM medium, it had increased the fertilization rate and percentage of morula/blastula obtained (Larocca et al., 1993), but such type of additive effect was not observed by Chauhan et al. (1997). Elaborate studies are need to be conducted to know the effect of inclusion of FF in IVM medium on embryo yield in buffaloes. In the present study, the highest maturation rate could be obtained by the medium containing M199+FF (50% v/v)+PMSG followed by M199+buFF (10% v/v)+PMSG and buFF +PMSG. These rates of maturation were nearer to that obtained by buFF alone. Since buFF alone could induce very high maturation rate reported in the present study, it is also suggested that detailed studies needs to be undertaken to estimate the levels of various factors present in the ovarian follicular fluid influencing the oocyte maturation.

CONCLUSIONS

It is concluded that buffalo follicular fluid alone without any supplementation can induce good maturation in buffalo oocytes and it can be used as economic and easily available *in vitro* maturation medium in this species.

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