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Effect of fetal bovine serum on *in vitro* maturation and fertilization of bovine oocytes

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Abstract
The present experiment was conducted to investigate the effect of fetal bovine serum on *in vitro* maturation and fertilization of bovine oocytes. The mean percentages of *in vitro* maturation performance of bovine oocytes based on cumulus cells expansion in both serum and serum free basic maturation media in type-I & type-II ovaries were recorded as 76.52 ± 3.95 and 64.81+ 4.60 & 73.33 ± 5.71 and 62.96 ± 6.57 respectively. The mean percentages of *in vitro* maturation based on cumulus cells expansion were recorded as 75.43 ± 3.25 and 64.20 ± 3.77 respectively in serum and serum free culture media irrespective of ovarian types. So, the mean percentage of *in vitro* maturation performance of bovine oocytes based on cumulus cells expansion increased significantly ($P< 0.05$) in serum basic maturation media than in serum free media in both type I and type II ovaries and also irrespective of ovarian types. The mean fertilization rates (%) were found to significantly higher ($P< 0.05$) in serum culture media (70.33 ± 3.21) than the serum free media (55.81 ± 4.33). The mean cleavage percentages recorded from 4-cell to 16-cell stages of embryos in serum culture media were found to be significantly higher ($P< 0.05$) than the serum free media.

Keywords: Blastocyst, cumulus cell, *In vitro* maturation, morula, polar body etc.

Introduction
As documented from inception, the competence of embryonic development is lower in case of *in vitro* matured oocytes compared with *in vivo* matured oocytes because there may be some defects in the cytoplasmic maturation of the former process [1]. Although many attempts have been made to improve culture conditions for oocytes maturation in order to produce higher quality embryos but the pregnancy rate remains lower for embryos derived from *in vitro* matured oocytes compared with *in vivo* matured oocytes. Therefore, to improve success rate of *in vitro* production (IVP), the systems that more closely follow the *in vivo* conditions of embryo production need to be explored. Therefore, inclusion of serum and combination of the hormones have been considered essential for obtaining higher maturation and fertilization rates in buffalo and cattle oocytes [2, 3]. Foetal calf serum (FCS) and foetal bovine serum (FBS) are routinely used in culture media for oocyte maturation and known to enhance the fertilizing capacity of bovine oocytes [4].

Better fertilization rate of oocytes can be obtained by culturing for 20 to 24 h in TCM 199 with 10% buffalo estrus serum alone [5]. The use of serum free and semi-defined media for *in vitro* maturation (IVM) of buffalo oocytes were also reported to have a positive effect [6, 7]. Buffalo oocytes can be developed up to the blastocyst stage in serum-free, semi-defined media containing bovine serum albumin (BSA), follicle stimulating hormone (FSH), epidermal growth factor (EGF) and insulin-transferrin selenium and the efficiency on the production of embryos varies with media and supplements [8]. But fetal calf serum (FCS) is more effective in achieving maturational competence than with buffalo estrus serum and sources of serum might be influencing oocyte maturation [9]. Moreover, the proportion of cleaved embryos developing to blastocyst stage are more with super ovulated buffalo serum than with buffalo estrus serum, fetal bovine serum (FBS) and steer serum [10] and the sources of serum supplement may have various effects on the maturation of bovine oocytes. Therefore, the present experiment was conducted in order to understand the effect of fetal bovine serum on *in vitro* maturation and fertilization of bovine oocytes.

Materials and Methods

Collection of oocytes

Bovine ovaries of unknown reproductive status were collected from local slaughter house and carried to the laboratory in normal saline solution (0.85% NaCl) fortified with gentamicin (50 µg/ml) in a thermo flask at 37-38 °C within 1- 2 h of slaughter. In the laboratory, extraneous tissues were removed and ovaries were thoroughly washed with 70% ethanol followed by three rinses in phosphate buffer saline solution.

Grading of oocytes

Oocytes were aspirated from all the visible non-atretic surface follicles of the ovary by using 10 ml sterile syringe fitted with 18 G needle containing oocytes collection medium after final washing and after that, oocytes were searched in oocytes collection media under stereo zoom microscope and cumulus oocytes complexes (COCs) were recovered. Oocytes possessing a full cumulus mass, unfragmented cytoplasm and intact zona were selected for culture and after that, the COCs were evaluated and graded [11]. Good and excellent quality oocytes having more than 3-5 cumulus cell layers were cultured in 50 µl droplets (20-25 oocytes/droplet) of maturation media in 35 mm sterile petridish.

In vitro maturation (IVM) of bovine oocytes

The excellent (>5 layers) and good (>3 layers) quality of COCs were selected for *in vitro* maturation (IVM). Two different types of maturation and culture media *viz* serum basic maturation media (SBMM) containing modified TCM-199 + serum (10% Fetal Bovine Serum) + Sodium pyruvate + L-glutamine + gentamicin + pFSH + hMG inj + E_2 (estradiol), serum free basic maturation media (SFBMM) containing modified TCM-199 + Polyvinylpyrrolidone (PVP) + Bovine serum albumin (BSA) + Sodium pyruvate + L-glutamine + pFSH + gentamicin + hMG inj + E_2 (estradiol), serum basic culture media (SBCM) containing mCR2aa stock +10% FBS+ Gentamicin, serum free basic culture media) (SFBCM) containing mCR2aa stock + BSA-V+ PVP + Gentamicin were used for *in vitro* maturation and *in vitro* culture of the oocytes. Epidermal growth factor (EGF) (30 ng) and Insulin like growth factor (IGF-1) (100 ng) were added in maturation media as well as embryo culture media singly and in combination in both the groups of media. Frozen bull semen straws of proven fertility were used and prepared for *in vitro* capacitation by density gradient method using B.O. media.

Statistical analysis

All the collected data were analyzed using [12] as per statistical procedures [13] and expressed in Mean ± SE. 'Z' test of SPSS was performed for mean statistical significant difference ($P< 0.05$).

Results and Discussion

The findings related to *in vitro* maturation of bovine oocytes based on cumulus cells expansion and extrusion of polar body in serum and serum free basic maturation media in type I and type II ovaries are presented in Table 1. In the present experiment, a total of 223 cumulus oocyte complexes (COCs) were collected from type-I ovaries and out of 223 nos. cumulus oocyte complexes (COCs), 115 and 108 nos. COCs were subjected to serum and serum free basic maturation media respectively for *in vitro* maturation. Similarly, 114 nos.COCs were also collected from type-II ovaries and out of 114 nos. cumulus oocyte complexes (COCs), 60 and 54 nos. COCs were distributed in serum and serum free basic maturation media respectively for *in vitro* maturation. It was observed that in type-I ovaries, out of 115 nos. COCs, 88 nos. culturable oocytes showed full expansion (+++) in serum basic maturation media whereas in serum free basic maturation media, out of 108 nos. COCs, 70 nos. culturable oocytes showed full expansion (+++). The total mean percentage of *in vitro* maturation performance of bovine oocytes based on cumulus cells expansion in both serum and serum free basic maturation media in type-I ovaries were recorded as 76.52 ± 3.95 and 64.81 ± 4.60 respectively. The total mean percentages of *in vitro* maturation performance of bovine oocytes increased significantly ($P<0.05$) in serum basic maturation media based on cumulus cells expansion than the serum free basic maturation media in type-I ovaries. Similarly in type-II ovaries, out of 60 nos. COCs, 44 nos. culturable oocytes showed full expansion (+++) whereas in serum free group, out of 54 nos. COCs, 34 nos. culturable oocytes showed full expansion (+++). The total mean percentages of *in vitro* maturation performance based on cumulus cells expansion in both serum and serum free basic maturation media in type-II ovaries were recorded as 73.33 ± 5.71 and 62.96 ± 6.57 respectively and the value recorded in serum basic maturation media also increased significantly ($P< 0.05$) than the serum free basic maturation media in type-II ovaries. Thus, based on cumulus cells expansion, the *in vitro* maturation performance of bovine oocytes increased significantly ($P< 0.05$) in serum basic maturation media than serum free media which might be due to addition of bovine serum. The results recorded in the present experiment were in close agreement with the earlier reports [6, 14, 15] where they reported better maturation rates of bovine follicular oocytes than serum free medium. On the contrary, ovarian types (type I without CL and type II with CL) did not have any significant effect on *in vitro* maturation rate based on cumulus cells expansion and nuclear maturation. Addition of fetal calf serum [9], fetal bovine serum [16-18], estrous buffalo serum [2], [10], estrus cow serum [19] to the IVM media, at the rate of 10-20%, improved the maturation rate. But on the contrary, [20] reported that the addition of various serums in maturation media had no effect on IVM and IVF of buffalo oocytes.

Table 1: *In vitro* maturation of bovine oocytes based on cumulus cells expansion and extrusion of polar body in serum and serum free media

Total number of ovaries	Basic maturation media	COCs subjected to IVM (n)	Degree of cumulus expansion			IVM % (Mean±SE)	Matured oocytes denuded (n)	Oocytes showing polar body (n)	IVM % (Mean±SE)
			+	++	+++				
Type I (n=223)	Serum	115	12	17	88	76.52±3.95[a]	33	18	54.55±8.67
	Serum Free	108	13	25	70	64.81±4.6[b]	25	12	48.00±9.99
Type II (n=114)	Serum	60	6	12	44	73.33±5.71[a]	15	8	53.33±12.88
	Serum Free	54	21	16	34	62.96±6.57[b]	12	5	41.67±14.23

[a,b]Means bearing different superscripts in a column differ significantly ($P<0.05$)

In case of type-I ovaries for confirmation of nuclear maturation, 33 and 25 nos. of matured oocytes from serum and serum free basic maturation media were subjected to denudation in which 18 out of 33 and 12 out of 25 nos.

denuded oocytes showed polar body. Though the extrusion of polar body was found to be apparently higher in serum basic maturation media (54.55 ± 8.67%) than that of serum free media (48.00 ± 9.99%) in type- I ovaries but no significant differences were recorded in respect of extrusion of polar body. Likewise, in type-II ovaries also, 15 and 12 nos. of matured oocytes from serum and serum free basic maturation media were subjected to denudation for confirmation of nuclear maturation (detection of polar body) in which after denudation, 8 and 5 nos. of matured oocytes out of 15 and 12 nos. matured oocytes respectively showed polar body. The mean percentage of extrusion of polar body in serum basic maturation media (53.33 ± 12.88) increased non-significantly than that of serum free basic maturation medium (41.67 ± 14.23) in type-II ovaries. The study revealed the beneficial effect of serum on both nuclear and cytoplasmic maturation which might be due to effect of hormones cyclic adenosine monophosphate, catecholamine, vitamins, growth factors, lipids, albumin [21] and other uncharacterized factors in the serum that aid in *in vitro* maturation of oocytes [9]. The reason behind the action of serum that promotes cumulus cells-oocyte uncoupling by retaining the hyaluronic acid within the COCs in a manner that results in cumulus modification and this uncoupling could be responsible for stopping the transfer of oocyte maturation inhibition factor via gap junction [22]. The present findings were in close agreement with the earlier report [23] where they recorded the highest IVM rate on using TCM-199 and 10% steer serum supplemented with 40 or 50 IU/ml of eCG.

The findings related to *in vitro* maturation of bovine oocytes based on cumulus cells expansion and extrusion of polar body in serum and serum free basic maturation media irrespective of ovary types are presented in Table 2. In the present experiment, 115 and 60 nos. COCs were subjected to serum basic maturation media in type-I and type-II ovaries respectively for *in vitro* maturation. Out of 115 nos. cumulus oocyte complexes (COCs) in type-I ovaries, 88 nos. culturable oocytes showed cumulus cell expansion and in type-II ovaries also, out of 60 nos. COCs, 44 nos. culturable oocytes showed cumulus cell expansion in serum basic maturation. Similarly, 108 and 54 nos. COCs were subjected to serum free basic maturation media respectively in type-I and type-II ovaries for *in vitro* maturation. Out of 108 nos. cumulus oocyte complexes (COCs) in type-I ovaries, 70 nos. culturable oocytes showed cumulus cell expansion and in type-II ovaries also, out of 54 nos. COCs, 34 nos. culturable oocytes showed cumulus cell expansion in serum free basic maturation media. The total mean percentages of *in vitro* maturation performance of bovine oocytes based on cumulus cells expansion in both serum and serum free basic maturation media were recorded as 75.43 ± 3.25 and 64.2 ± 3.77 respectively irrespective of ovarian types. However, it was observed that the mean percentages of *in vitro* maturation of bovine oocytes in serum basic maturation media (75.43 ± 3.25) based on cumulus cell expansion increased significantly ($P< 0.05$) than in serum free media (64.2 ± 3.77) irrespective of ovaries which might be due to the effect of growth factors, nutrients and anti-oxidant present in serum (10% FBS) in both type I (without CL) and type II (with CL) ovaries [6, 17, 18] or super ovulated buffalo serum to the IVM media, at the rate of 10-20%, improved the maturation rate [10].

Table 2: *In vitro* maturation of bovine oocytes based on cumulus cells expansion and extrusion of polar body in serum and serum free media irrespective of ovarian types

Basic maturation media	No of oocytes used		IVM %			
			Cumulus cell expansion (Mean±SE)	Matured oocytes denuded (n)	Oocytes showing polar body (n)	Extrusion of polar body (Mean±SE))
Serum	Type I	Type II	75.43±3.25[a]	18	8	54.17±7.19
	88	44				
	115	60		33	15	
Serum Free	70	34	64.2±3.77[b]	12	5	45.95±8.19
	108	54		25	12	

[a,b]Means bearing different superscripts in a column differ significantly ($P<0.05$)

The mean percentages of extrusion of polar body in serum basic maturation media (54.17 ± 7.19) increased apparently than that of serum free basic maturation medium (45.95 ± 8.19) irrespective of ovarian types but statistical analysis revealed no significant differences in respect of extrusion of polar body between them. Similarly, these results were in close agreement with the earlier report [23]. The study revealed the beneficial effect of serum on both nuclear and cytoplasmic maturation which might be due to effect of hormones cyclic adenosine monophosphate, catecholamine, vitamins, growth factors, lipids, albumin and other uncharacterized factors in the serum that aid in *in vitro* maturation of oocytes [9, 21]. The reason behind the action of serum that promotes cumulus cells-oocyte uncoupling by retaining the hyaluronic acid within the COCs in a manner that results in cumulus modification and this uncoupling could be responsible for stopping the transfer of oocyte maturation inhibition factor via gap junction [22].

In Vitro fertilization and early embryonic development

The present findings related to *in vitro* fertilization and early embryonic development of bovine oocytes in serum and serum free media irrespective of ovarian types are presented in Table 3. To study *in vitro* fertilization performance following serum influence, out of 91, 64 nos. serum added *in vitro* matured oocytes and out of 86, 48 nos. non serum added *in vitro* matured oocytes were cleaved respectively and the mean fertilization rates in percentages were found as 70.33 ± 3.21 (64/91) and 55.81 ± 4.33 (48/86) respectively in serum and serum free media and statistical analysis revealed significant differences ($P<0.05$) between serum and serum free media in respect of IVF percentages. The mean cleavage percentages at 4-cell, 8-cell, 16-cell, morula & blastocyst stages of embryos in serum and serum free culture media were recorded as 47.25 ± 4.86 and 31.40 ± 4.99, 31.87 ± 4.99 and 18.60 ± 3.88, 20.88 ± 3.21 and 10.47 ± 2.23, 7.69 ± 4.32 and 3.49 ± 2.23 & 4.40 ± 2.09 and 1.16 ± 2.23 respectively

but the mean cleavage percentages recorded at 4-cell, 8-cell, 16-cell stages of embryos in serum supplemented culture media were found to be significantly higher ($P< 0.05$) than in serum free media and after that the differences were found to be non significant.

Table 3: *In vitro* fertilization and early embryonic development in serum and serum free media irrespective of ovarian types

Basic maturation media	No of oocytes cleaved	IVF (%) (Mean±SE)	Cleavage (%)				
			4 cell	8 cell	16 cell	Morula	Blastocyst
Serum	64	70.33±3.21[a]	47.25±4.86[a]	31.87±4.99[a]	20.88±3.21[a]	7.69±4.32[a]	4.40±2.09[a]
	91						
Serum free	48	55.81±4.33[b]	31.40±4.99[b]	18.60±3.88[b]	10.47±2.23[b]	3.49±2.23[ab]	1.16±2.23[ab]
	86						

[a,b]Means bearing different superscripts in a column differ significantly ($P< 0.05$)

The results recorded in the present experiment were compared with the earlier reports [24, 25]. The findings recorded in the present experiment were in close agreement with the earlier report [26] where they demonstrated that fetal calf serum (FCS) was a superior protein supplement compared with BSA for IVM and IVC of cow and hamster oocytes. On the contrary, more blastocysts were developed in goats per cleaved embryo following maturation in synthetic oviductal fluid (SOF) with BSA than TCM-199 with goat serum but the effect of BSA on post fertilization development was dependent on the proportion of BSA used in maturation medium as such SOF with 2.5 or 8.0 mg/ml BSA yielded more blastocysts than SOF with 20.0 mg/ml BSA or TCM199 with 10% goat serum [27]. However, [28] reported that total blastocyst yield did not differ among CR1-ECS, CR1-BSA and CR1-ECS-BSA (30.9, 33.1 and 32.9%, respectively, $P<0.05$) and reported that it was possible to produce *in vitro* bovine embryos in serum-free culture medium without affecting blastocyst yield and quality.

In our results, explanation for the higher IVF rate, cleavage rate and blastocyst rates in FBS supplemented groups might be related to FBS as a non-defined than a defined serum. As serum is important to add in the *in vitro* maturation medium to prevent hardening of zona pellucida (ZP) which could adversely affect fertilization [29]. The fetuin, a major glycoprotein constituent of fetal calf serum, which prevents hardening of zona pellucida during *in vitro* maturation and acts by preventing the action of proteolytic enzymes originating from precociously released cortical granules and improves the fertilization capacity of oocytes. In this case, the presence of fetuin (an FBS component, inhibiting zona pellucida hardening during oocyte maturation), hormones, growth factors, proteins and some other components in FBS may exert effects on oocyte competence compared with BSA [30]. Additionally, the synthesis and storage of certain forms of mRNA and proteins during IVM and early embryonic development which are necessary for further development may be influenced by the type of serum used [31, 32]. In our study, cleavage rates differed significantly ($P<0.05$) between media supplemented with FBS or with BSA. However, the post cleavage development to the blastocyst stage of *in vitro*-derived bovine embryos was better in media supplemented with fetal bovine serum (FBS) than bovine serum albumin (BSA).

Conclusion

The *in vitro* maturation rate of bovine oocytes was higher in serum basic maturation media than serum free basic maturation media which might be due to the effect of growth factors, nutrients and anti-oxidant present in serum. Therefore, from the present experiment, it can be inferred that FBS was found to be better than BSA-V for *in vitro* maturation of cattle oocytes. However, in regards to cleavage and early embryonic development, serum had an influence up to 16-cells stage and after that, serum did not have any influence.

Conflict of Interest

There is no conflict of interest among authors for this study.

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