

The impact of using newborn bovine serum as fetal calf serum substitute in the in vitro bovine embryos production system

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Abstract

This study was conducted to assess the effects of addition of 10% newborn bovine serum (NBS) in the maturation and culture media of bovine system on oocytes maturation and developed morula and blastocyst rates. For this purpose, 10% NBS was added to in vitro maturation (IVM) medium alone (Experiment I), in vitro culture (IVC) (Experiment II), and in IVM + IVC (Experiment III). In vitro bovine oocytes maturation and developmental rates of bovine embryos were then compared with control group where the basal maturation or culture media were supplemented with 10% fetal calf serum (FCS). Results revealed that maturation, cleavage, morula, and blastocyst formation rates of in vitro fertilized embryos were not significantly different in NBS and control groups in the 3 experiments. In conclusion, NBS is an efficient macromolecule in bovine system when added either to IVM or to IVC or to both stages. Thus, NBS could provide cheaper and more available treatment to be used as FCS substitute in the IVC system of bovines.

Abbreviations: COCs = cumulus oocyte complexes, ECS = estrus calf serum, EES = estrus ewe serum, FCS = fetal calf serum, HCG = human chorionic gonadotropin, IVC = in vitro culture, IVF = in vitro fertilized, IVM = in vitro maturation, NBS = newborn bovine serum, PECS = pro-estrus calf serum, PMSG = pregnant mare serum gonadotropins, SOF = synthetic oviduct fluid.

Keywords: blastocyst, cleavage, in vitro culture, in vitro maturation

Introduction

Numerous protocols and a wide variety of culture media were studied in order to improve the rates of in vitro mammalian oocytes maturation and embryos development.¹ Most of these protocols use the basic media that are supplemented with hormones and different concentration of sera. Sera are usually added to the in vitro maturation (IVM) medium by the rate of 10% to 20%.² Supplementation of in vitro embryo cultures with fetal calf serum (FCS) is crucial as it contains many hormones, vitamins, transport proteins, growth factors that optimize oocyte maturation. Also, it avoids hardening of the zona pellucida. Moreover, it was reported that FCS had a potent antioxidant activities which assist in chelating free radicals and protect oocytes from oxidative stress conditions.³ For this purpose, about 1 million of bovine fetuses have to be harvested to get about

500,000 L of FCS annually, which represents a big economic loss. Therefore, scientists developed many approaches to minimize or replace the requirement for FCS in cell culture media.⁴

The maturation medium and the selection of protein supplements in IVM play an important role in the subsequent development of in vitro fertilized (IVF) embryos.¹ Heat inactivated serum can be added to culture medium as a source of protein, which is essential for oocytes maturation.⁵ Several types of sera were evaluated, FCS, estrus calf serum (ECS), pro-estrus calf serum (PECS), steer serum, and maternal serum have been used in bovine IVM. The authors found that oocyte maturation rates did not differ significantly when the IVM medium either supplemented with these different sera or with FCS.⁶ Also, in many experiments, maturation medium was supplemented with estrous goat serum,⁷ estrous sheep serum,⁷ ECS,⁸ and FCS⁸ as protein supplements. In addition, another study showed that ECS supplementation in IVM media increased the cleavage rates of IVF embryos. Moreover, the authors reported 62.9% maturation rate when FCS was added as a supplement to the IVM medium and this was significantly lower than the rates obtained when the IVM medium supplemented with either bovine serum obtained at D0 (estrus), D1 (metestrus), D10 (diestrus), or D20 (proestrus).⁹ In addition, D20 and/or D0 bovine sera may contain factors that increase the developmental competence of oocytes during IVM. Similarly, the highest fertilization rate was shown when PECS was added to the media at the standing estrus.⁵ In conclusion, the previous studies clarified that in comparison to FCS, supplementation of IVM medium with FCS alternatives such as ECS achieved higher maturation rates.

In addition, in cattle, it was reported that addition of estrus serum to the synthetic oviduct fluid (SOF) (culture medium) at different periods after fertilization either at 20 hours (control) or 42 hours (experiment) resulted in similar cleavage rates. However, blastocyst and hatched blastocyst formation rates

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were higher in experiment group than the control one.¹⁰ Moreover, addition of 20% estrus ewe serum (EES) to the fertilization medium significantly ($P < .05$) enhanced the cleavage rate ($78.0 \pm 4\%$) compared with 10% ($72.6 \pm 6\%$) or 5% ($73.9 \pm 2\%$) EES-supplemented groups. Moreover, it was noticed that blastocyst development rates were not affected among the different groups.¹¹

Thus, due to the shortage of information about the use of newborn bovine serum (NBS) in the *in vitro* embryo production system, the present study was designed to examine the effects of addition of NBS as FCS alternative in either IVM medium alone or *in vitro* culture (IVC) alone or both of them on the *in vitro* bovine oocytes maturation and the potential bovine embryos developmental rates.

Materials and methods

Preparation of media and buffers

All reagents used for media or buffers preparation were purchased from Sigma Chemical Co (St Louis, MO), unless otherwise indicated.

Preparation of NBS

NBS was prepared by centrifugation of venous blood from newborn calves of Holstein breed (48 h after birth) at $4000 \times g$ for 20 minutes. Then, the NBS was filtered sequentially using 0.2 μm syringe and stored in aliquots at -20°C until use.

Collection of the ovaries

Bovine ovaries of slaughtered apparently healthy and none pregnant mature cows that have functional ovarian structures (cyclic follicles and/or corpus luteum) were collected from Belifia abattoir in Beni-Suef, Egypt. Ovaries were separated from slaughtered animals within 15 min of slaughter and then transported to the laboratory in a thermos containing sterile (25°C) normal saline "0.85%" supplemented with 100 $\mu\text{g}/\text{mL}$ gentamycin sulfate (Memphis Co Pharm and Chem Ind, Egypt) and 100 IU/mL penicillin G.¹² In the laboratory, the collected ovaries were carefully dissected from other tissues and washed thrice with presterilized warm normal saline to ensure removal of the adhering blood then rinsed again till the recovery of oocytes.¹³

Recovery and selection of immature primary oocytes

The immature cumulus oocyte complexes (COCs) were harvested from the collected ovaries using the slicing method.¹⁴ According to Ganguli *et al.*,¹⁵ the recovered oocytes were classified, based upon their morphological criteria, into 3 categories; oocytes with evenly granulated cytoplasm and completely surrounded by multiple layers of cumulus cells (grade I), oocytes which were surrounded by scanty layers of cumulus cells (grade II) and nude oocytes that were devoid of cumulus cells (grade III). Grade I and II oocytes were included to be cultured while nude oocytes were excluded.

In vitro maturation of oocytes

COCs were washed twice in TCM-199 supplemented with 10% FCS, 50 $\mu\text{g}/\text{mL}$ gentamycin sulfate and 5 $\mu\text{L}/\text{mL}$ L-glutamine¹⁶ then transferred to 50 μL of the maturation medium supplemented with 0.2 IU pregnant mare serum (PMSG) (Folligon, Intervet, Netherlands, EU), 2.0 IU human chorionic gonadotropin (hCG) (Pregnyl, Intervet), and 1.0 μg Estradiol/ mL "E₂, Sigma Chemical

Co."^{17,18} Oocytes containing droplet (10 cells) was covered with 4 mL sterile mineral oil to prevent evaporation. The cells were incubated for maturation in the CO₂ incubator for 24 hours¹³ after which the oocytes were examined under stereomicroscope (100 \times) for evaluation of cumulus mass expansion.¹⁹

In vitro fertilization of oocytes

Motile spermatozoa were selected using swim-up technique and allowed to be capacitated in modified Sperm-Tyrod's Albumin Lactate Pyruvate medium modified by the addition of N-[2-hydroxyethyl] Piperazine-N-[2-ethanesulfonic acid] at concentration of 2.3 mg/mL, sodium bicarbonate (160 $\mu\text{g}/\text{mL}$), sodium pyruvate (112 $\mu\text{g}/\text{mL}$), sodium lactate "98%" (1.84 $\mu\text{L}/\text{mL}$), gentamycin sulfate (50 $\mu\text{g}/\text{mL}$), bovine serum albumin fraction-V "essential fatty acid-free" (6 mg/mL), and heparin sodium salt (200 IU/mL).²⁰ For this purpose, 2 straws of frozen buffalo bull semen, received from Animal Reproduction Research Institute, Agriculture Research Center, El-Haram, Egypt, were used. The capacitated was resuspended in 1.0 mL of Fertilization Tyrod's, Albumin, Lactate, Pyruvate medium (F-TALP).²¹ Sperm concentration was measured by hemocytometer and a sufficient medium was added to yield the final concentration of 1×10^6 sperm/mL. Following maturation, good and excellent mature COCs were washed thrice by F-TALP medium then transferred to 50 μL droplets of the same medium (5 oocytes/droplet). The oocytes were covered with warm sterile mineral oil then incubated in CO₂ incubator for an hour after which the oocytes were inseminated with sperm suspension (2 $\mu\text{L}/\text{droplet}$).

In vitro culture

Twenty-four hours following fertilization, the fertilized oocytes were cultured Hammam *et al.*²² and cleavage of resulted embryos was identified and evaluated according to the protocol previously published Linder and Wright.²³

Experimental design

The present study includes 3 experiments:

- (1) Experiment 1: It includes addition of FCS by 10% to the basal maturation medium (control group) or 10% NBS to the basal maturation medium (NBS group).
- (2) Experiment 2: It includes addition of 10% FCS or 10% NBS in the basal culture medium.
- (3) Experiment 3: It includes addition of 10% FCS or 10% NBS in the maturation and afterwards in the basal culture media.

Statistical analysis

Throughout the current study, the obtained data (absolute values) subjected to statistical analysis using SAS Program. All values were reported as mean \pm standard error. The means were compared using 1-way analysis of variance followed by multiple comparison tests. P value $< .05$ was considered significant.^{24,25}

Results

Effect of addition of NBS in maturation medium on maturation and bovine embryo developmental rates

The effect of supplementation of 10% FCS and NBS in maturation media on the maturation rate of bovine oocytes is

Table 1**Effect of addition of newborn bovine serum in maturation medium on the maturation rates (mean \pm SE)**

| Groups | No. of oocytes | Maturation rates, % | Significant value (<i>P</i>) |
|----------------------------|----------------|-------------------------------|--------------------------------|
| Control group | 240 | 84.20 \pm 1.87 ^a | >.05 |
| Newborn bovine serum group | 261 | 81.43 \pm 1.63 ^a | >.05 |

Values with identical letters revealed none significant difference from each other ($P > .05$). These results for 5 replicates.
SE=standard error.

Table 2**Effect of addition of newborn bovine serum in maturation medium on cleavage and developmental rate of morulae and blastocysts**

| Groups | No. of mature oocytes | Cleavage rates, % | Morula rates, % | Blastocyst rates, % | Significant value (<i>P</i>) |
|----------------------------|-----------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------|
| Control group | 222 | 47.36 \pm 1.53 ^a | 34.45 \pm 0.74 ^a | 20.56 \pm 1.34 ^a | >.05 |
| Newborn bovine serum group | 216 | 46.23 \pm 1.24 ^a | 31.70 \pm 0.96 ^a | 18.45 \pm 0.90 ^a | >.05 |

In the same column, values with identical letters revealed none significant difference from each other ($P > .05$). These results for 5 replicates.

given in Table 1. The results revealed that there are no significant ($P > .05$) differences in maturation rate when the maturation media was supplemented with either FCS or NBS (84.20 \pm 1.87 or 81.43 \pm 1.63, respectively). Also, the results in Table 2 showed that there are no significant ($P > .05$) differences in cleavage, morula, and blastocyst formation rates among the groups.

Effect of addition of NBS in culture medium on cleavage and developmental rates of morulae and blastocysts

The results in Table 3 revealed that addition of NBS in culture medium did not induce any significant differences ($P > .05$) on cleavage, morula, and blastocyst formation rates when compared with corresponding values of FCS group (48.25 \pm 0.79, 36.93 \pm 1.13, 23.35 \pm 0.35 vs 45.78 \pm 0.35, 33.91 \pm 1.01, 18.58 \pm 0.63, respectively).

Effect of addition of NBS in both maturation and culture medium on maturation and bovine embryos developmental rates

The results in Table 4 exhibited that addition of NBS in maturation medium and then in the culture medium resulted in a

similar cleavage, morula, and blastocyst formation rates in comparison to the FCS group ($P > .05$) (45.13 \pm 0.79, 32.52 \pm 1.44, 18.51 \pm 0.38 vs 42.65 \pm 1.45, 29.17 \pm 1.81, 15.86 \pm 0.77, respectively).

Discussion

In the current study, IVM and IVC media were supplemented with either FCS or NBS to assess their effects on the bovine oocytes maturation, cleavage, morula, and blastocyst formation rates either when they were added in the basal maturation medium only (Experiment 1) or in the basal culture medium only (Experiment 2) or in both the maturation and culture media (Experiment 3).

Serum supplementation in IVM medium was found to have a great effect on the in vitro embryo development in different species. In this regard, numerous reports studied how to improve the culture media of in vitro produced embryos in domestic animals via addition of many supplements as sera, hormones, and somatic cells.²⁶ Serum supplementation in maturation media was found to have many benefits as the serum contains a number of growth factors that play crucial role in the regulation of oocyte maturation. Also, it has antioxidant properties and prevents the

Table 3**Effect of addition of newborn bovine serum in culture medium on cleavage and developmental rates of morulae and blastocysts (mean \pm SE)**

| Groups | No. of matured oocytes | Cleaved rate, % | Morula rates, % | Blastocyst rate, % | Significant value (<i>P</i>) |
|----------------------------|------------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------|
| Control | 224 | 48.25 \pm 0.79 ^a | 36.93 \pm 1.13 ^a | 23.35 \pm 0.35 ^a | >.05 |
| Newborn bovine serum group | 250 | 45.78 \pm 0.35 ^a | 33.91 \pm 1.01 ^a | 18.58 \pm 0.63 ^a | >.05 |

In the same column, values with identical letters revealed none significant difference from each other ($P > .05$). These results for 5 replicates.
SE=standard error.

Table 4**Effect of addition of newborn bovine serum in maturation and culture medium on cleavage and developmental rate of morulae and blastocysts (mean \pm SE)**

| Groups | No. of matured oocytes | Cleavage rates, % | Morula rates, % | Blastocyst rates, % | Significant value (<i>P</i>) |
|----------------------------|------------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------|
| Control group | 235 | 45.13 \pm 0.79 ^a | 32.52 \pm 1.44 ^a | 18.51 \pm 0.38 ^a | >.05 |
| Newborn bovine serum group | 238 | 42.65 \pm 1.45 ^a | 29.17 \pm 1.81 ^a | 15.86 \pm 0.77 ^a | >.05 |

In the same column, values with identical letters revealed none significant difference from each other ($P > .05$). These results for 5 replicates.
SE=standard error.

hardening of the zona pellucida.²⁷ Also, serum is useful to supply the media with many essential elements including hormones, minerals, trace elements, lipids, and detoxifying factors.²⁸

The results of the current study revealed that there are no significant ($P > .05$) difference among FCS and NBS on the rates of maturation of bovine oocytes as well as on cleavage, morulae, and blastocysts formation rates either when the sera were added in basal maturation medium only or in basal culture medium only or in both the maturation and culture media. These data met agreement with the study of Lee et al²⁹ who reported that supplementation of IVM medium with newborn canine serum enhanced the developmental rate of canine oocytes to the MII stage and the maturation rate was found to be higher than the rates obtained when IVM medium supplemented with either estrus bovine serum, FBS, or bovine serum globulin (14.6% vs 10.4%, 8.8%, and 3.3%, respectively). In addition, a study of Son et al³⁰ reported that supplementation of IVM medium with different types of sera including newborn pig serum, prepubertal gilt serum, estrus sow serum, or pregnancy sow serum resulted in similar cleavage and blastocyst formation rates among all groups. Thus, the authors proved that newborn pig serum had a pronounced effect on IVM as estrus sow serum. These results coincided with the current results of Experiment 1 and indicated that the NBS is an efficient substitute to FCS in IVM medium.

Moreover, the results of the current study come in accordance with the study of Allen et al³¹ who found that supplementation of Hams F-10 tissue culture medium with 5% or 10% NBS was effective than FCS ($P < .05$) in promoting the in vitro development of bovine morulae. Also, a study of Watson et al³² reported that the supplementation of tissue culture medium with NBS resulted in greater blastocyst rates than when cultured in SOF medium. These results come in agreement with the current results of Experiment 2 and clarified that the NBS is an efficient serum alternative to FCS in the culture medium.

The reasons that augment the current results and potentiate the use of NCS instead FCS in culture media are the high ethical concerns, which should be followed at the slaughterhouse during serum collection from the fetuses of pregnant cows.³ In addition to the ethical and scientific concerns, there are many factors contribute to the unsteady supply of FBS which consider a big problem including environmental factors, such as drought and policies that control governmental farm.³³ Recently, due to the limitation of its availability, the requirement for FBS in many countries has increased significantly and the price of it has increased by >300% in the past few years.³⁴ Because of the current shortage and high price of FBS, many scientists are motivated to study many alternatives to cells and tissue culture. The main FCS alternatives are bovine calf serum-based products especially the NBS, which has more similar constituents as FCS and this allow mitigation of the ethical rules applied for the use of FBS.⁴ In addition, NBS provides a stable and available serum source and it decreased the cost greatly, and induced good uniformity between different lots.³⁵ All of these points augment the results of the current study and give a final judgment that FCS is an optimal cheaper serum alternative in the culture of the in vitro embryo production system.

Conclusion

Thus, it could be concluded that FCS and NBS have the same effect for IVM of bovine oocytes as well as cleavage, morula, and blastocyst formation rates. Thus, NBS might be used as cheaper serum substitute for maturation of oocytes and bovine embryos development.

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Conflicts of interest

The authors declare no conflicts of interest.

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