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EFFICIENCY OF FATTY ACID-FREE DEFINED SYSTEM FOR *IN VITRO* MATURATION OF BUFFALO OOCYTES

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ABSTRACT

The evaluation of medium for *in vitro* maturation of buffalo oocytes has progressed towards more defined conditions for the studies evaluating effect of specific components in the medium. Therefore, aim of the present study was to evaluate the effect of fatty acid free-bovine serum albumin (FAF-BSA) alone or supplemented with epidermal growth factor (EGF) in the *in vitro* maturation medium on *in vitro* maturation of oocytes and subsequent embryo development. The cumulus oocyte complexes (COCs) were collected from ovaries (900) of slaughtered animals by aspirating follicles, and incubated in maturation medium for 24 h. Three types of maturation media (MM) supplemented with 1) 10% fetal calf serum (FCS), 2) 6 mg/ml FAF- BSA or 3) 6 mg/ml FAF- BSA + 10.0 ng/mL epidermal growth factor (EGF) were evaluated. After maturation, Tyrode's albumin lactate pyruvate (TALP) and synthetic oviductal fluid (SOF) media were used for fertilization and culture environments, respectively. The percentage of COCs with expanded cumulus was higher ($P < 0.05$) in MM supplemented with FBS and FAF-BSA+EGF compared with that having FAF-BSA alone. No difference ($P > 0.05$) was observed in meiotic resumption and nuclear maturation rate in all three types of maturation media. Percentage of embryos crossing 8-16 cell stage and reaching up to 32 cell stage (morula) after IVC was lower ($P < 0.05$) in MM supplemented with FAF-BSA, however with addition of EGF, embryo development was improved (statistically non significant) and was comparable ($P>0.05$) with development in MM supplemented with FCS. In conclusion, serum free defined system having FAF-BSA+EGF was comparable to the MM containing FCS and can be used to replace serum supplemented media for the *in vitro* maturation of buffalo oocytes and further embryo development.

Key words: *In vitro* maturation, serum free medium, defined medium, epidermal growth factor.

INTRODUCTION

Buffalo represents an integral part of the agricultural economy in Pakistan contributing high quality animal protein, both milk and meat. However, the production of milk in buffalo is lower than its potential (Bilal *et al.*, 2006). The production potential can be improved by genetic selection of superior animals using assisted reproductive technologies. Success rate of the reproductive biotechnologies viz., artificial insemination, super ovulation and embryo transfer is limited in buffalo as compared to cattle, due to few primordial follicles, smaller number of recruitable follicles, high level of follicle atresia, poor estrus detection and poor response to superovulation (Madan, 1990). Therefore, the emphasis has now been shifted towards *in vitro* production of high quality buffalo embryos (IVEP) for research, biotechnology and commercial settings (Hansen, 2006) utilizing germ plasm of both male and female animals simultaneously (Barakat *et al.*, 2012).

During IVEP, *in vitro* maturation is a crucial step for the generation of quality oocytes capable of being fertilized and developed into normal embryos (Wang *et al*., 1997). The process of meiotic maturation and acquisition of developmental competence determines the ability of the oocyte to undergo successful fertilization, cleavage, and embryonic development. The culture conditions in which oocytes develop are critical; especially the composition of media affects nuclear and cytoplasmic maturation of oocytes and ultimately the early embryonic development (Gordon, 2003). It is relevant to mention that the maturation medium and its protein supplements play an important role in oocyte maturation and *in vitro* development following IVF (Bavister *et al*., 1992). Serum is commonly used constituent of maturation media that promote *in vitro* maturation of oocytes and post cleavage development of embryos (Kim *et al*., 2001). It contains essential components like proteins, fatty acids, vitamins, trace elements, hormones, and growth factors (Harper and Brackett, 1993). Although serum has an inevitable ability to support oocyte maturation and embryo development *in vitro*, there are certain disadvantages. Due to its complex and undefined nature, it may contain varying concentrations of unknown molecules and batch to batch variation exists in its activity (Takagi *et al*., 1991). Furthermore, being blood derivative, serum can induce contamination and infection of oocytes and gametes (Freshney, 2000). Therefore, recently, research efforts

have focused at replacement of serum with alternative compounds including polyvinyl pyrrolidone (PVP) or polyvinyl alcohol (PVA), chicken egg albumin, BSA-V, purified BSA, essentially fatty acid-free BSA (Ali and Sirard, 2002; Collado *et al*., 2014) particularly in studies evaluating effect of specific components in the media (Korhonen *et al*., 2010).

Bovine serum albumin (BSA) is an alternative to serum and is a relatively pure fraction than serum (Bavister, 1995). The constituents of BSA include albumin, estradiol, low molecular weight substrates and growth factors (Mingoti *et al*., 2002). Essentially fatty acid-free bovine serum albumin (FAF- BSA) is another form of BSA that can also replace serum as protein source to obtain more defined IVM environment (Ali and Sirard, 2002). It is pertinent to mention that undefined source of lipids in maturation media results in accumulation of lipid droplets in *in- vitro* produced bovine embryos that increase their sensitivity to cryopreservation (Rizos *et al*., 2003). However, the factors that induce lipid accumulation are removed during purification (Yotsushima *et al*., 2004) and had not been identified in fatty acid free- BSA. The requirement of a fatty acid free defined media is further intensified if the effect of certain fatty acids is to be investigated.

Along with the merits of fatty acid free defined media, there is also a drawback in its use. It has been investigated in bovine that embryo yield is compromised in culture medium supplemented with FAF-BSA compared to serum supplemented group (Korhonen *et al*., 2010). It is therefore needed to reinforce the fatty acid free defined media to acquire full advantage during IVM of oocytes. It is known that growth factors present in the serum contribute towards increased proportion of matured oocytes and embryos developed to blastocyst stage (Chauhan *et al*., 1999; Kumar and Purohit, 2004; Sadeesh *et al*., 2014). Among these, epidermal growth factor (EGF) is reported to resume meiosis in various mammalian species (Gall *et al*., 2004). It was therefore hypothesized that serum in the *in vitro* maturation media can be replaced with FAF-BSA if fortified with epidermal growth factor.

The objective of the present study was to evaluate the effect of replacing serum with fatty-acid free BSA (FAF-BSA) alone or with epidermal growth factor (EGF) on *in vitro* maturation of oocytes and subsequent development of the embryos.

MATERIALS AND METHODS

Unless otherwise stated, all reagents were purchased from Sigma.

Collection of ovaries: During breeding season, buffalo ovaries were collected from slaughtered animals and transferred to the IVF laboratory, Buffalo Research Institute (BRI), Pattoki, within two hours in sterilized phosphate buffered saline (PBS) kept at 33-35°C. Fresh PBS was used to wash the ovaries immediately after arrival. A total of 900 ovaries were collected in five replicates of the study.

Collection of oocytes: Sterile disposable plastic syringe (10 ml) fitted with 18 gauge needle was used for aspiration of follicular fluid with immature cumulus-oocyte complexes (COCs) from 2-8 mm follicles. Searching for COCs in the follicular fluid was done in a 35 mm petri dish under stereomicroscope and collected. The COCs were classified as grade A, B, C and D, on the basis of their cumulus investment and ooplasm homogeneity (Sabasthin *et al*., 2013). Only type A and B COCs were selected for IVM.

***In vitro* Maturation (IVM):** Selected COCs were washed twice in PBS (37ºC) and twice in pre-equilibrated maturation medium (MM: TCM-199 supplemented with 0.02 IU/mL FSH, 1 µg/mL estradiol-17β (E2), 50 µg/mL gentamicin and 1) 10% fetal calf serum (FCS), 2) 6 mg/ml FAF- BSA or 3) 6 mg/ml FAF- BSA + 10.0 ng/mL epidermal growth factor (EGF). The washed COCs were randomly allocated into 100 µl of respective culture droplets covered with sterile mineral oil and matured *in vitro* in an incubator at 38.5°C under 5% CO_2 in air with 95% relative humidity for 24 hours. All the media and culture dishes were equilibrated at 38 °C in CO_2 incubator for at least 1-2 h before experiment. Data were collected in five independent repeats.

Assessment of cumulus cell expansion: After 24 hours of maturation, cumulus cell expansion was assessed by visual assessment using stereomicroscope as 1) not expanded 2) Partially expanded or 3) Fully expanded (Kobayashi *et al*., 1994). Criterion is shown in Plate 1. Data on cumulus cells expansion of oocytes after 24 hours of maturation, from five (5) replicates are pooled and presented.

Nuclear maturation assessment: For determination of nuclear stage in meiosis, COCs were completely denuded, stained with 1% aceto-orcein and examined for GV (Germinal vesicle), GVBD (Germinal vesicle break down), MI (Metaphase I) and MII (Metaphase II) stage under a phase contrast microscope (Yadav *et al*., 1997). Criterion is shown in Plate 2. Data on nuclear stage of oocytes after 24 hours of maturation, from five (5) replicates are pooled and presented.

***In vitro* fertilization (IVF):** Motile spermatozoa were separated by swim up technique (Parrish *et al.*, 1986) in Tyrode's albumin lactate pyruvate (TALP) medium. Oocytes were washed in fertilization media and were placed in the 50 µl droplet of pre warmed fertilization media under mineral oil with final sperm concentration of 2×10^6 mL^{-1}. The oocyte and spermatozoa were incubated

at 38.5 °C under 5% CO_2 with 95% humidity for 20 h (Gasparrini *et al*., 2008).

***In vitro* Embryo Culture (IVC):** After 20 h of fertilization, cumulus cells were removed by vigorous pipetting in PBS. After one time washing with PBS and two times with culture media; synthetic oviductal fluid medium (SOF), presumptive zygotes were transferred to 25 µl of culture droplet under mineral oil in four- well dish and cultured at 38.5°C in a humidified incubator with 5% CO_2 in air.

Cleavage Rate and Early embryo development: On day 2 of development (Day 0 = day of insemination) the cleavage rate (number of oocytes cleaved/total COCs incubated × 100) was observed. The culture was continued and further developmental stages were recorded every other day.

Statistical Analysis: Differences in cumulus expansion, nuclear maturation of oocytes after 24 h after maturation and further developmental stages were analyzed by one-way analysis of variance (ANOVA) at 5% level of significance. Least Significant Difference (LSD) test was used to compare treatment means.

RESULTS

Cumulus Expansion: Data on cumulus mass expansion of the oocytes matured in different media are presented in Table 1. Higher ($P < 0.05$) number of COCs with fully expanded cumulus mass was recorded in IVM medium supplemented with serum compared to medium supplemented with FAF-BSA. However, the difference between IVM medium supplemented with serum and the medium with FAF-BSA+EGF (10ng/mL) was non-significant. Percentage of COCs with partial cumulus expansion remained similar among treatment groups, while COCs with non-expanded cumulus mass were lower ($P < 0.05$) in IVM supplemented with FCS and FAF-BSA + EGF treatment groups compared to the COCs submitted to maturation medium supplemented with FAF-BSA.

Nuclear Maturation: The data on nuclear maturation of buffalo oocytes matured in the media supplemented with FCS, FAF-BSA or FAF- BSA + EGF are shown in Table 2. The nuclear maturation rate assessed in terms of GV, GVBD, MI and MII stage of oocytes submitted to IVM in the presence of FCS, FAF-BSA or FAF- BSA + EGF were similar ($P > 0.05$) among experimental groups.

Early Embryonic Development: Data on development of embryos after maturation of oocytes in the media supplemented with FCS, FAF-BSA or FAF-BSA + EGF are given in Table 3. The cleavage rate and percentage of embryos reaching 4-8 cell stage after IVC were similar ($P > 0.05$) among treatment groups. However, number of embryos crossing 4-8 cell block and reaching 8-16 cell stage were lower ($P < 0.05$) when COCs were submitted to *in vitro* maturation media supplemented with FAF-BSA compared with IVM medium supplemented with FCS, though with addition of EGF, embryo development was improved (non-statistically) and was comparable with development in MM supplemented with FCS. Similar ($P > 0.05$) trend was seen for embryos reaching 32 cell stage (Morula).

Table 1. Cumulus expansion rate of buffalo oocytes after 24 hours of maturation in IVM medium supplemented with FCS, FAF-BSA or FAF-BSA+EGF.

Treatments	Cumulus Expansion (Mean percentage±SEM)			
	N	Fully Expanded N (%)	Partially Expanded N (%)	Not Expanded N (%)
FCS	129	88 (68.21±1.74)[a]	30 (23.25±1.36)[a]	11 (8.50±0.86)[b]
FAF-BSA	129	70 (54.26±1.26)[b]	34 (26.35±0.67)[a]	25 (19.37±1.10)[a]
FAF-BSA+EGF	129	84 (65.11±2.04)[a]	31 (24.03±0.79)[a]	14 (10.85±1.41)[b]

Data were collected in five independent repeats.
[a,b]The values with different superscripts in the same coloumn differ significantly ($P < 0.05$).
N: Total number of COCs
FCS: Fetal Calf Serum
BSA: Bovine Serum Albumin
FAF-BSA: Fatty acid free BSA
EGF: Epidermal Growth Factor

Table 2. Nuclear maturation status of buffalo oocytes after 24 hours of maturation in IVM medium supplemented with serum, FAF-BSA or FAF-BSA+ EGF.

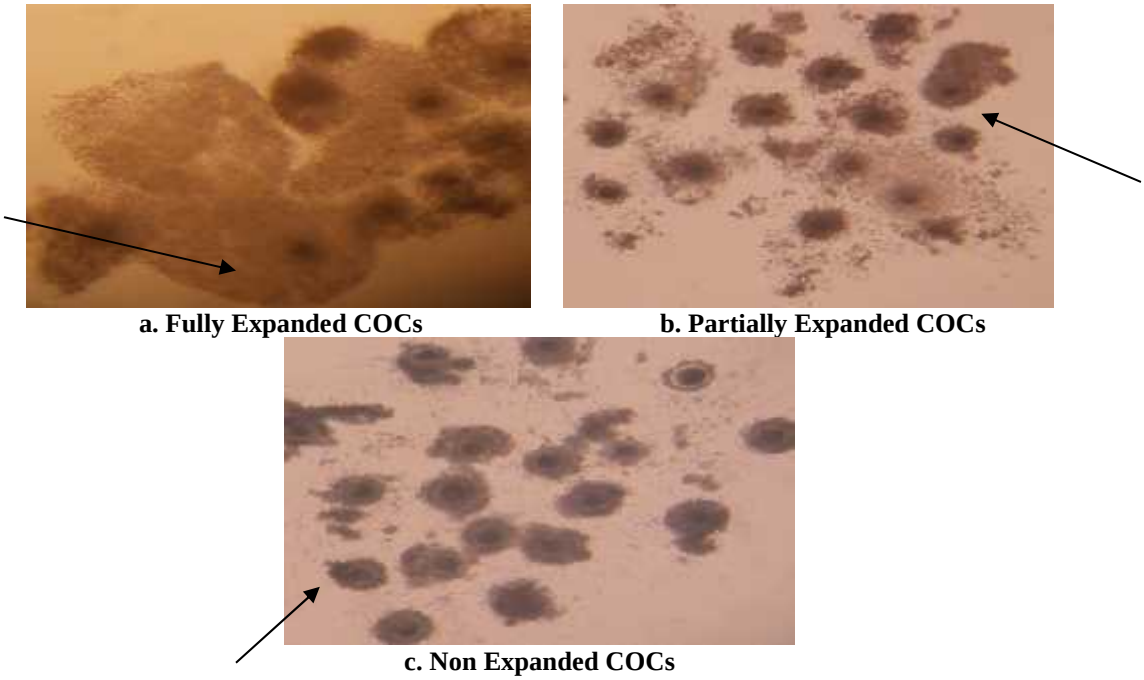
Treatments	Nuclear Stage in Meiosis (Mean percentage±SEM)				
	N	GV N (%)	GVBD N (%)	MI N (%)	MII N (%)
FCS	129	3 (2.32±0.94)	28 (21.71±1.25)	19 (14.73±0.83)	79 (61.24±1.71)
FAF- BSA	129	2 (1.55±0.92)	29 (22.48±1.59)	21 (16.28±0.93)	77 (59.68±1.26)
FAF- BSA+EGF	128	2 (1.56±0.92)	29 (22.65±1.17)	20 (15.62±1.18)	77 (60.15±1.57)

Data were collected in five independent repeats.
All the values were statistically non-significant among treatments (P > 0.05).
N: Total number of COCs; FCS: Fetal Calf Serum; BSA: Bovine Serum Albumin; FAF-BSA: Fatty acid free BSA; EGF: Epidermal growth Factor; GV: Germinal Vesicle; GVBD: Germinal Vesicle Breakdown; ; MI: Metaphase I; MII: Metaphase II

Table 3. Effect of supplementation of IVM medium with FCS, FAF-BSA and FAF-BSA+EGF on the cleavage rate and subsequent development of buffalo oocytes *in vitro*.

Treatments	Developmental stages (Mean percentage±SEM)				
	N	Cleaved N (%)	4-8 cell embryos N (%)	8-16 cell embryos N (%)	32 cell embryos (Morula) N (%)
FCS	129	75 (58.1±1.58)^{a}	60 (46.37±3.52)^{a}	35 (27.12±1.24)^{a}	23 (17.79±0.74)^{a}
FAF- BSA	129	69 (53.48±1.12)^{a}	53 (41.01±1.43)^{a}	22 (17.05±0.93)^{b}	10 (7.70±1.24)^{b}
FAF- BSA+EGF	128	71 (55.04±0.49)^{a}	56 (43.69±1.93)^{a}	30 (23.41±1.64)^{ab}	18 (14.06±0.94)^{ab}

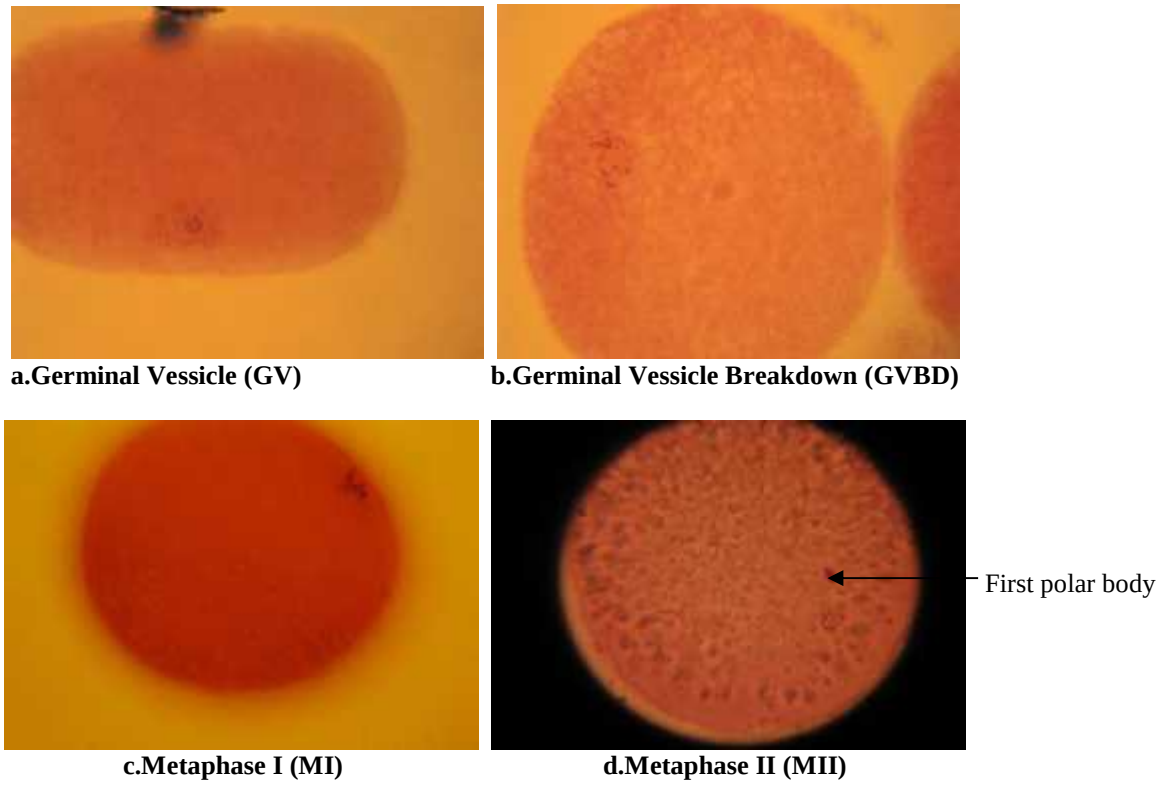
Data were collected in five independent repeats.
^{a,b,c}The values with different superscripts in the same coloumn differ significantly (P < 0.05).
N: Total number of COCs
FCS: Fetal Calf Serum
BSA: Bovine Serum Albumin
FAF-BSA: Fatty acid free BSA
EGF: Epidermal growth Factor



a. Fully Expanded COCs **b. Partially Expanded COCs**

c. Non Expanded COCs

Plate 1. Cumulus cells expansion of matured COCs after 24 h of maturation
a. Expansion of all layers of cumulus cells
b. Expansion of outer layers of cumulus cells
c. No expansion observed



a.Germinal Vessicle (GV) **b.Germinal Vessicle Breakdown (GVBD)**

c.Metaphase I (MI) **d.Metaphase II (MII)**

Plate 2. Nuclear maturation status of buffalo oocytes after aceto-orcein staining

a. Oocyte nucleus with a nucleolus and filamentous chromatin
b. Oocyte without nucleolus, nuclear membrane and shortening of chromosomes
c. Chromosomes look as thick dots arranged at metaphase plate
d. Complete segregation of chromosomal sets and release of first polar body

DISCUSSION

To obtain serum free IVM medium, bovine serum albumin (BSA) is an alternate source for oocyte maturation (Wydooghe *et al.*, 2014). In present study, BSA alone in the IVM medium could not initiate proper protein synthesis for embryo development compared to serum supplemented IVM medium. Therefore, EGF was supplemented along with BSA in the maturation media. It is relevant to mention that oocyte utilizes EGF as a source of protein and it also alters the pattern of proteins neosynthesis during IVM (Lonergan *et al*., 1996). Receptors for EGF are reported on cumulus cells of oocytes that are the major site of action of the growth factors for regulation of oocyte maturation (Downs *et al*., 1988).

In the present study, when FAF-BSA was used to replace fetal calf serum, the cumulus expansion was compromised during IVM. These results are in line with previous studies that reported reduced cumulus cell expansion when serum was substituted with bovine serum albumin (Cetica *et al*., 1999). However, with the addition of EGF, cumulus expansion was improved compared to FAF-BSA alone and was comparable with expansion obtained in MM supplemented with FCS. This improvement in cumulus expansion of the oocytes matured in the media having FAF-BSA+EGF may be attributed to the reinforcement of FAF-BSA alone treatment group with epidermal growth factor. The present study is in line with the previous reports that buffalo COCs respond well towards EGF and resulted in improved meiotic resumption, increased proportion of matured oocytes and embryos developed to blastocyst stage (Chauhan *et al*., 1999; Kumar and Purohit, 2004; Sadeesh *et al*., 2014).

In the present study, the nuclear maturation rate was not compromised when serum was substituted with FAF-BSA alone or supplemented with EGF, although there was significant lower cumulus expansion when serum was substituted with FAF-BSA in IVM medium. Similar results have been reported by Saeki *et al*. (1991) in bovine where the nuclear maturation rate of oocytes did not differ among media supplemented with variable protein sources (FCS or PVP). The results of nuclear maturation in our study were independent of the cumulus expansion rate and it has previously been reported that the rate of oocyte nuclear maturation or fertilization does not seem to depend on the extent of cumulus expansion (Sirard *et al*., 1988).

The developmental competence of an oocyte is determined by its nuclear and cytoplasmic maturation rate *in vitro*. During maturation period, transcription factors, mRNAs and proteins are stored, which are necessary for maturation, fertilization and the beginning of early embryonic development (Ferreira *et al*., 2009). In the current study, percentages of embryos crossing 8-16 cell stage and reaching up to 32 cell stage (morula) after IVC was lower ($P < 0.05$) in MM supplemented with FAF-BSA compared to development in MM supplemented with FCS, however with addition of EGF, embryo development was improved, although it was statistically non significant and was comparable to the development in MM supplemented with FCS. Compromised nature of FAF-BSA alone experimental group was improved (non-significant) by reinforcing it with EGF, which might have enhanced the oocyte maturation through changing the pattern of protein synthesis, as has been reported in cattle (Park *et al*., 1999; Lonergan *et al*., 1996).

Conclusion: In conclusion, serum free defined system having FAF-BSA+EGF was comparable to the MM containing FCS and can be used to replace serum supplemented media for the *in vitro* maturation of buffalo oocytes and further embryo development.

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