Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2021; 9(1): 1924-1929

Received: 18-11-2020
Accepted: 28-12-2020

Dipannita Baishya
Veterinary Officer, BCPP, Khanapara, Guwahati, Govt. of Assam, India

Arundhati Bora
Professor, Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Anubha Baruah
Professor & Head, Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

DJ Dutta
Professor, Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Champak Barman
Assistant Professor, Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Shantanu Tamuly
Assistant Professor, Department of Veterinary Biochemistry, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Corresponding Author:
Champak Barman
Assistant Professor, Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Effect of insulin-like growth factor-I on *in vitro* maturation, fertilization and early embryonic development of cattle oocytes

Dipannita Baishya, Arundhati Bora, Anubha Baruah, DJ Dutta, Champak Barman and Shantanu Tamuly

DOI: https://doi.org/10.22271/j.ento.2021.v9.i1aa.8415

Abstract
The present experiment was conducted to investigate the effect of insulin-like growth factor-I on *in vitro* maturation, fertilization and early embryonic development of cattle oocytes. A total of 318 nos. bovine ovaries were collected from slaughter houses for one year and 1089 nos. culturable oocytes were collected. 357 out of 1089 nos. culturable oocytes, were subjected to IGF-I supplemented serum and serum free basic maturation media. There was no significant difference in respect of maturation, fertilization and embryonic development between IGF-I supplemented serum and serum free basic maturation as well as culture media. The mean percentage of *in-vitro* maturation of bovine oocytes (IVM) based on cumulus cell expansion was found to be significantly higher ($P<0.05$) in serum basic maturation media without IGF-I (75.43 ± 3.25) than the serum free basic maturation media with IGF-I (67.27 ± 6.33) respectively. The mean cleavage percentages at 4-cell, 8-cell, 16-cell, morula & blastocyst stages of embryos in serum culture (without IGF-I) and serum free culture media (with IGF-I) were recorded as 47.25 ± 4.86 and 57.14 ± 4.83, 31.87 ± 4.99 and 45.71 ± 4.86, 20.88 ± 3.21 and 37.14 ± 4.72, 7.69 ± 4.32 and 27.62 ± 4.36 & 4.40 ± 2.09 and 4.76 ± 2.08 respectively but the mean cleavage percentages recorded at 4-cell, 8-cell, 16-cell and morula stages of embryos in IGF-I supplemented serum free culture media were found to be significantly higher ($P<0.05$) than in serum culture media (without IGF-I) and after that the differences were found to be statistically non-significant. Similar findings were also observed in serum free culture media with or without IGF-I. From the present experiment, it can be inferred that addition of IGF-I in serum free maturation media had little beneficial effect than serum basic maturation media. Though the addition of IGF-I in serum and serum free culture media had beneficial effect on post fertilization period but the *in vitro* blastocyst production in IGF-I supplemented serum free culture media was comparable to serum free media without IGF-I.

Keywords: Insulin-like growth factor-I, culture media, morula, blastocyst etc.

Introduction
Improvement of *in vitro* embryo production is important for the production of embryos of high quality for use in animal biotechnology and biochemical research [1]. Among various factors in bovine embryo production, one of the most important factors regulating the number and quality of oocyte maturing *in vitro*, is the culture system used for *in vitro* maturation (IVM). Inclusion of serum and combination of the hormones have been considered essential for obtaining high maturation and fertilization rates in buffaloes and cattle oocytes [2-4]. Growth factors (GF) have been shown to play a regulatory role in the functioning of the ovary [5] and of the uterus [6], resulting in a trophic effect on the endometrium and embryo. Growth factors are present *in vivo*, act on embryo receptors, are anti-apoptotic and increase development rates. Insulin-like growth factor-I (IGF-I) improves the survival of embryos and serves as an anti-apoptotic factor during bovine oocyte maturation and embryo development [7]. Further it has been reported that IGF-I protects pre implantation embryos against heat shock [8] and also regulates oocyte maturation by acting synergistically with FSH as autocrine and paracrine modulators of granulosa cells, thereby, promoting mitogenesis, steroidogenesis and protein synthesis [9]. The addition of IGF-I to maturation media may also have a beneficial effect on oocyte maturation, fertilization and embryo development to blastocyst stage [10]. *In vitro* produced embryos are exposed to *in vitro* sub-optimal conditions that greatly differ from the *in vivo* environment. The efficiency of an in vitro embryo production system is assessed based on cleavage of fertilized oocytes and their further development into blastocysts.

More recent observations imply that the effect of growth factor in the enhancement of maturation of oocytes is retarded by serum [11]. It is indisputable that adding serum supplement to a non-defined media leads to desirable blastocyst yields. However, serum culture medium contains unknown factors; therefore to simplify and avoid contamination it is preferable to develop an *in vitro* culture (IVC) system of defined composition, free of blood components or cell constituents. Supplementing specific paracrine and endocrine components during *in vitro* maturation (IVM) of bovine cumulus-oocyte complexes (COCs) improves the success of *in vitro* embryo production and maximize embryonic competency to be useful in oocytes maturation and growth. Although similar works have been conducted in different media, however literatures about the effect of growth factors on serum and serum free media for optimization of embryo production in bovine are very much limited and needs further study. Moreover embryos that will be produced have to be remained more viable without infection and has to be protected from cryo preservation damage. Henceforth, the effect of growth factors on serum and serum free media requires further study in relation to the early embryonic development of cattle 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 a 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 [12]. 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 modifiedTCM-199 + serum (10% Fetal Bovine Serum) + Sodium pyruvate+L-glutamine+ gentamicin + pFSH+ hMGinj+ 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 + hMGinj + 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. Insulin-like growth factor-I (IGF-I) (100 ng) were added in maturation media as well as embryo culture media. Frozen bull semen straws of proven fertility were used and prepared for *in vitro* capacitation by density gradient method using B.O. media.

Insulin-like growth factors (50 µg): It is recommended to reconstitute the lyophilized IGF-I in sterile water not less than 100 µg. For long term storage it is recommended to add a carrier protein (0.1% HAS / BSA).
To study the effect of IGF-I in *in-vitro* maturation and culture, a concentration of 100 ng/ml was used.

Statistical analysis

All the collected data were analyzed using [13] as per statistical procedures [14] 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 the polar body in serum and serum free basic maturation mediawith IGF-I are presented in Table 1. A total of 318 nos. ovaries were collected from slaughter houses for one year and 2082 nos. cumulus oocyte complexes (COCs) were aspirated. 1089 nos. culturable oocytes out of 2082 nos. cumulus oocyte complexes were collected. 357 out of 1089 nos. culturable oocytes subjected to IGF-I supplemented serum and serum free maturation media and cultured in IGF-I supplemented serum and serum free culture media. In the present experiment, 192 and 165 nos. of culturable oocytes were subjected to *in vitro* maturation in serum and serum free basic maturation media supplemented with IGF-I. 138 out of 192 and 111out of 165 culturable oocytes were fully matured (+++) in IGF-1 supplemented serum and serum free basic maturation media. So the mean percentage of *in vitro* maturation in respect to IGF-I supplementation in serum and serum free basic maturation media were recorded as 71.88 ±5.62 and 67.27± 6.33 respectively. 54 out of 138 and 30 out of 111 nos. of matured cocs were denuded to see the polar body in IGF-I supplemented serum and serum free media respectively. 30 out of 54 and 15 out of 30 matured oocytes showed extrusion of polar body in serum and serum free media supplemented with 100 ng IGF-I. The mean percentage of *in vitro* maturation of denuded oocytes based on the polar body extrusion was recorded as 55.56±11.71 and 50.00±15.81 in IGF-I supplemented serum and serum free basic maturation media respectively. Z test revealed that there was no significant difference between IGF-I supplemented serum and serum free basic maturation media based on cumulus cells expansion and extrusion of polar body.

Table 1: *In vitro* maturation of cattle oocytes based on cumulus cells expansion and extrusion of polar body in IGF-I supplemented serum and serum free media

Basic Maturation Media	COCs subjected to IVM (n)	Degree of cumulus expansion			IVM % (Mean±SE)	Total matured oocytes denuded (n)	Denuded oocytes showing polar body (n)	IVM % (Mean±SE)
		+	++	+++				
SERUM + IGF-I	192	18	36	138	71.88±5.62^{NS}	54	30	55.56±11.71 ^{NS}
SERUM FREE + IGF-I	165	30	24	111	67.27±6.33^{NS}	30	15	50.00±15.81 ^{NS}

^{NS}Non-significant

The present findings recorded in the experiment were in close agreement with the earlier reports [10, 15-17]. This is related to the mechanism of IGF action which does not act via cumulus cell or interferes with the production of an expansion factor secreted by the oocyte [17, 20]. Maturation with IGF-I is initiated upon activation of the membrane receptor for this growth factor and requires tyrosine dephosphorylation of p34, the kinase component of maturation promoting factor (MPF).

The findings related to *in vitro* maturation of cattle oocytes in serum (without IGF-I) and serum free basic maturation media (with IGF-I) are presented in Table 2. In the experiment, 175 and 165 nos. culturable oocytes were subjected to *in vitro* maturation in serum (without IGF-I) and serum free basic maturation media (with IGF-I) respectively. From the experiment, it can be revealed that 132 out of 175 and 111 out of 165 culturable oocytes were fully matured (+++) in serum and serum free basic maturation media**.** The mean percentage of *in-vitro* maturation of bovine oocytes (IVM) based on cumulus cell expansion was found to be significantly higher (P<0.05) in serum basic maturation media without IGF-I (75.43±3.25) than the serum free basic maturation media with IGF-I (67.27±6.33) respectively. Similarly, 48 out of 132, and 55 out of 111 nos. of matured cocs were denuded to see polar body in serum (without IGF-I) and serum free media (with IGF-I). Then 26 out of 48 and 39 out of 55 denuded matured oocytes showed extrusion of polar body in serum basic maturation media (54.17±7.19) without IGF-I and serum free media (50.00±15.81) supplemented with IGF-I. Though the mean *in vitro* maturation of bovine oocytes increased based on polar body extrusion in serum basic maturation media (without IGF-I) than the serum free media supplemented with IGF-I but Z test revealed no significant difference between serum and serum free basic maturation media based on extrusion of polar body.

Table 2: Comparison of *in vitro* maturation of cattle oocytes in serum basic maturation media (without IGF-I) and serum free basic maturation media (with IGF-I)

Basic Maturation Media	Nos. of COCs subjected to IVM (n)	Degree of cumulus expansion (n)			IVM % (Mean±SE)	Total mature oocytes denuded (n)	Denuded oocytes showing polar body (n)	IVM % (Mean±SE)
Serum	175	18	29	132	75.43±3.25^{a}	48	26	54.17±7.19^{NS}
Serum Free+Igf-I	165	30	24	111	67.27±6.33^{b}	55	39	50.00±15.81^{NS}
$^{a, b}$ Means bearing different superscripts in a column differ significantly (P<0.05), ^{NS}Non-significant								

The present findings recorded in the experiment were in close agreement with the earlier reports [10, 15, 16]. This is related to the mechanism of IGF action which does not act via cumulus cell or it interferes with the production of an expansion factor secreted by the oocyte [15, 17, 18]. The kinetics of activation of MPF can be assessed by H1 kinase activity. H1 kinase activity showed a rapid increase in correlation to IGF-I supplement in media and the rapid meiotic progress of oocytes indicated by polar body extrusion [11]. *In vitro* studies have shown that IGF-I synergizes with FSH to regulate the aromatase activity of granulosa cells. Other *in vitro* effects of IGF-I include enhanced secretion of follistatin, inhibin-A, activin-A in granulosa cells increased androstenedione production from theca cells and protection from apoptosis in oocytes and granulosa cells.

The present findings related to *in vitro* maturation of cattle oocytes in serum free maturation media with or without IGF-I are presented in Table 3. In the experiment, 162 and 165 nos. culturable oocytes were subjected to *in vitro* maturation in serum free (without IGF-I) and serum free basic maturation media (with IGF-I) respectively. 114 out of 162 and 111 out of 165 culturable oocytes were fully matured (+++) in both media. So the mean percentage of *in vitro* maturation based on cumulus expansion were recorded as 64.20 ±3.77 and 67.27± 6.33 respectively in serum free (without IGF-I) and serum free basic maturation media (with IGF-I). Simultaneously, 37 out of 114, and 30 out of 111 nos. of matured cocs were denuded to see polar body in both media. 17 out of 37 and 15 out of 30 matured oocytes showed extrusion of polar body in serum free without IGF-I (45.95±8.19) and serum free media with IGF-1 (50.00±15.81).

Table 3: Comparison of *in vitro* maturation of cattle oocytes in serum free basic maturation media with or without IGF-I

Basic Maturation Media	Nos. of COCs subjected to IVM (n)	Degree of cumulus expansion			IVM % (Mean ± SE)	Nuclear maturation (n)	No. of polar body (n)	IVM % (Mean ± SE)
		+	++	+++				
Serum Free	162	19	37	114	64.20±3.77^{NS}	37	17	45.95±8.19^{NS}
Serum Free + Igf-I	165	30	24	111	67.27±6.33^{NS}	30	15	50.00±15.81^{NS}

^{NS} Non-significant

Z test revealed that there was no significant difference in between serum free basic maturation media with or without IGF-I based on cumulus cells expansion and extrusion of polar body. The present findings recorded in the experiment were in close agreement with the earlier reports [10, 17].

The present findings related to *in vitro* fertilization and early embryonic development of cattle oocytes in IGF-I supplemented serum and serum free culture media are presented in Table 4. Following *in vitro* maturation and denudation, a total of 84 and 81 *in vitro* matured oocytes in serum and serum free media supplemented with IGF-I were fertilized and out of 84, 52 and out of 81, 51 nos. *in vitro* matured oocytes were cleaved respectively and the mean fertilization rates (%) were found as 61.90 ± 3.20 and 62.96 ± 3.08 respectively in IGF-I supplemented serum and serum free media and statistical analysis revealed no significant differences between IGF-I supplemented serum and serum free media in respect of IVF percentages.

Table 4: Effect of serum and serum free basic culture media supplemented with IGF-I on *in vitro* fertilization and early embryonic development of cattle oocytes

Culture Media	Total COCS subjected to IVF (n)	IVF (%) (Mean±SE)	Cleavage (%)				
			4-CELL% (Mean±SE)	8-CELL% (Mean±SE)	16-CELL% (Mean±SE)	Morula % (Mean±SE)	Blastocyst % (Mean±SE)
Serum + Igf-I	84	61.90±3.20^{NS}	53.40±4.99^{NS}	44.02±3.42^{NS}	34.01±4.14^{NS}	21.0 ± 4.13^{NS}	5.46±2.12^{NS}
Serum Free + Igf-I	81	62.96±3.08^{NS}	57.14±4.83^{NS}	45.71±4.86^{NS}	37.14±4.72^{NS}	27.62±4.36^{NS}	4.76±2.08^{NS}

^{NS} Non-significant

The mean cleavage percentages at 4-cell, 8-cell, 16-cell, morula & blastocyst stages of embryos in IGF-I supplemented serum and serum free media were recorded as 53.40 ± 4.99 and 57.14 ± 4.83, 44.02 ± 3.42 and 45.71 ± 4.86, 34.01 ± 4.14 and 37.14 ± 4.72, 21.00 ± 4.13 and 27.62 ± 4.36 & 5.46 ± 2.12 and 4.76 ± 2.08 respectively but the mean cleavage percentages recorded at 4-cell, 8-cell, 16-cell and morula stages of embryos were found to be statistically non-significant between IGF-I supplemented serum and serum free culture media. Similar findings were also reported by earlier workers [10, 18]. The present results showed that the addition of IGF-I in serum free basic culture media had no positive effect on embryonic development of bovine embryos produced from oocytes matured and fertilized *in vitro* which conflicted with the results of studies reporting its positive effect [19, 20]. These controversial observations may be due to different culture conditions. However, stimulatory effects on the development of bovine embryos were observed within the range of 2 to 200 ng/ml when these amino acids were present in the culture medium indicating that the supplementation of amino acids is necessary for IGF-I to exert its beneficial effect [19]. The beneficial effect of IGF-I on embryonic development seen in this study was consistent with recent mouse studies reporting a stimulation of protein synthetic activity in embryonic cells, and an increase in cell numbers in the blastocysts formed in response to exogenous insulin and IGF-I [21, 22]. Similarly, IGF-I was reported to stimulate protein synthesis by pig embryonic discs [23].

The present findings related to *in vitro* fertilization and early embryonic development of cattle oocytes in serum (without IGF-I) and serum free culture media (with IGF-I) are presented in Table 5. To study *in vitro* fertilization performance, out of 91, 64 nos. *in vitro* matured oocytes and out of 105, 80 nos. *in vitro* matured oocytes were cleaved respectively and the mean fertilization rates (%) were found as 70.33 ± 3.21 and 76.19 ± 4.16 respectively in serum (without IGF-I) and serum free media (with IGF-I) and statistical analysis revealed no significant differences between serum and serum free media in respect of IVF percentages.

Table 5: Comparison of *in vitro* fertilization and early embryonic development of cattle oocytes in serum free (with IGF-I) and serum based culture media (without IGF-I)

Culture Media	Total COCS subjected to IVF (n)	IVF (%) (Mean±SE)	Cleavage (%)				
			4-CELL% (Mean±SE)	8-CELL% (Mean±SE)	16-CELL% (Mean±SE)	Morula % (Mean±SE)	Blastocyst % (Mean±SE)
Serum	91	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}
Serum Free + Igf-I	105	76.19±4.16^{ab}	57.14±4.83^{b}	45.71±4.86^{b}	37.14±4.72^{b}	27.62±4.36^{b}	4.76±2.08^{ab}

$^{a, b}$ Means bearing different superscripts in a column differ significantly (P<0.05)

The mean cleavage percentages at 4-cell, 8-cell, 16-cell, morula & blastocyst stages of embryos in serum (without IGF-I) and IGF-I supplemented serum free media were recorded as 47.25± 4.86 and 57.14 ± 4.83, 31.87± 4.99 and 45.71 ± 4.86, 20.88± 3.21 and 37.14 ± 4.72, 7.69± 4.32 and 27.62 ± 4.36 & 4.40± 2.09 and 4.76 ± 2.08 respectively but the mean cleavage percentages recorded at 4-cell, 8-cell, 16-cell and morula stages of embryos in IGF-I supplemented serum free culture media were found to be significantly higher (P<0.05) than in serum culture media (without IGF-I) and after that the differences were found to be statistically non-significant. These findings were in close agreement with the earlier reports [20, 4] where they concluded that IGF-I supplementation increased cleavage rate, but its effect on the rate of blastocyst production from original oocytes was not identified.

Similarly, the findings related to *in vitro* fertilization and early embryonic development of cattle oocytes in serum free culture media with or without IGF-I are also presented in Table 6. Following *in vitro* maturation and denudation, a total of 86 and 81 *in vitro* matured oocytes in serum free (without IGF-I) and serum free culture media (with IGF-I) were fertilized *in vitro* and out of 86, 48 and out of 81, 51 nos. *in vitro* matured oocytes were cleaved respectively and the mean fertilization rates (%) were recorded as 55.81 ± 3.42 and 62.96 ± 3.08 respectively in serum free (without IGF-I) and

serum free media (with IGF-I) and statistical analysis revealed no significant differences between serum free media with or without IGF-I in respect of IVF percentages.

Table 6: Comparison of *in vitro* fertilization and early embryonic development of cattle oocytes in serum free culture media with or without IGF-I

Culture media	COCs subjected to IVF (n)	IVF % (Mean±SE)	Cleavage %				
			4- Cell % (Mean±SE)	8- Cell % (Mean±SE)	16 Cell % (Mean±SE)	Morula% (Mean±SE)	Blastocyst % (Mean±SE)
SERUM FREE	86	55.81±3.42^{NS}	31.40±4.77^{a}	18.60±3.42^{a}	10.47±2.11^{a}	3.49±2.08^{a}	1.20±2.23^{NS}
SERUM FREE+IGF-I	81	62.96±3.08^{NS}	50.00±3.11^{b}	43.00±2.49^{b}	36.22±3.22^{b}	23.00±4.36^{b}	4.94±2.09^{NS}

$^{a, b}$Means bearing different superscripts in a column differ significantly (P<0.05), ^{NS}Non-significant

The mean cleavage percentages at 4-cell, 8-cell, 16-cell, morula & blastocyst stages of embryos in serum free (without IGF-I) and serum free media (with IGF-I) were recorded as 31.40± 4.77 and 50.00 ± 3.11, 18.60 ± 3.42 and 43.00 ± 2.49, 10.47 ± 2.11 and 36.22 ± 3.22, 3.49 ± 2.08 and 23.00 ± 4.36 & 1.20 ± 2.23 and 4.94 ± 2.09 respectively but the mean cleavage percentages recorded at 4-cell, 8-cell, 16-cell and morula stages of embryos were found to be significantly higher(P<0.05) in serum free media (with IGF-I) as compared to the values recorded in serum free culture media (without IGF-I) and after that differences were found to be statistically non-significant. Similar findings were also reported by earlier workers [10]. The present results showed that the addition of IGF-I in serum free basic culture media had no positive effect on embryonic development produced from bovine oocytes matured and fertilized *in vitro* which conflicted with results of studies reporting its positive effect and these controversial observations might be due to different culture conditions [20].

Conclusion

The addition of IGF-I in serum free maturation media had little beneficial effect than serum basic maturation media. Though the addition of IGF-I in serum and serum free culture media had beneficial effect on post fertilization period but the *in vitro* blastocyst production in IGF-I supplemented serum free culture media was comparable to serum free media without IGF-I.

Acknowledgement

The authors thankfully acknowledge the Head of the Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India for providing the infrastructural facilities to conduct the research work. Authors also wish to extend their gratitude to the in-charge of C.I.F., College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam for providing technical assistance during the research work.

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