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Enhancement of Developmental Competence of Immature Cattle Oocytes with Leukemia inhibitory Factor as a Culture Media Supplement

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ABSTRACT

Background: Leukemia Inhibitory Factor (LIF) plays an essential role in oocyte maturation and during early embryonic development. The present study aims to improve the *in vitro* cattle embryo production by supplementing culture media with LIF.

Methods: Fresh ovaries and oviducts were collected from an abattoir in 0.9% saline (30-35°C) supplemented with antibiotics. Total 542 cumulus-oocyte complexes were aspirated from ovaries and cultured in maturation media in 5% CO_2 incubator at 38.5°C with maximum humidity after 5-6 times washing. After 24 h matured oocytes were co-incubated with *in vitro* capacitated sperms in FBO medium. After 15-18 h cumulus cells were stripped off and presumptive zygotes were cultured in mCR2aa medium. After 40 to 42 h, cleavage was observed and embryos were cultured for 7-9 days. Culture media used to replace with fresh media after every 24 h. LIF was supplemented with 15, 30, 45 ng/ml.

Result: Supplementation of LIF in culture media increased maturation rate, cleavage rate significantly (P<0.05). LIF Supplementation @ 30 ng/ml during culture increased blastocyst development (in control group 7.0±1.3 and 7.1±1.2, 12.9±0.4, 10.2±1.5 in 15 ng/ml, 30 ng/ml and 45 ng/ml respectively) significantly.

Key words: Cattle oocyte, Cytokines, Embryo, IVF, Leukemia inhibitory factor, Maturation.

INTRODUCTION

In vitro Embryo Production (IVEP) has become the most emerging part in the field of reproductive biotechnology. IVEP is a part of assisted reproductive technology which has a great potential for speeding up the genetic improvement. IVEP includes *in vitro* maturation (IVM); *in vitro* fertilization (IVF) and *in vitro* culture (IVC). This technology allows the supply of embryos for sexing, cloning and nuclear transfer, which permits the preservation and rapid multiplication of genetically superior animals. It also helps for basic research on the mechanism of fertilization and early embryogenesis *etc*. (Hoshi, 2003). Therefore, it is very crucial to develop an effective culture system which can help to promote the early embryonic development from immature oocytes that leads to production of embryos beneficial to both research and commercial efforts. Within the follicular niche, there are several paracrine and autocrine factors are produced those are primarily responsible for oocyte maturation (Li *et al.* 2008; Gilchrist *et al.* 2008; Binelli *et al.* 2010). The addition of several paracrine and endocrine components to bovine cumulus oocyte complexes (COCs) during *in vitro* maturation (IVM) can enhance the success of *in vitro* embryo production and optimize the embryonic competence to produce healthy offspring after embryo transfer (Richter, 2008). Sometimes preimplantation embryos express receptors for many of these growth factors, indicating a functional role during preimplantation development (Zhang and Ealy, 2012). Leukemia inhibitory factor (LIF), a pleiotropic cytokine from the interleukin-6 family, promotes oocyte maturation and developmental competence in mice (De Matos *et al.* 2008), it's possibly acting directly on the oocyte as its receptor subunits found on the surface of immature and mature oocytes (Molyneaux *et al.* 2003). To improve the culture system comparison of media supplements for oocyte maturation, fertilization and embryo culture have yielded varying outcomes (Gasparrini *et al.* 2006, Borah and Biswas, 2020, Kumar *et al.* 2020, Umdor *et al.* 2021) and plays a predictable and significant role in the formation of *in vitro* embryos. According to Mo *et al.* 2014, LIF supplementation enhanced the nuclear and cytoplasmic maturation of intact cumulus oocyte complex (COCs), also observed in denuded oocytes also, indicating that LIF directly influences oocyte development. The present study was under taken with the

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objectives to improve the *in vitro* embryo development by supplementing culture media with LIF in cattle.

MATERIALS AND METHODS

Chemicals, cell culture media and supplements

Molecular biology grade chemicals and reagents were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and HIMEDIA Biosciences Pvt. Ltd., Mumbai, India unless otherwise stated. Leukemia Inhibitory Factor (from mouse recombinant, expressed in Escherichia coli) was from Sigma-Aldrich, St. Louis, MO, USA. All plasticwares, Petri dishes, Centrifuge tubes were purchased from Tarsons Products Pvt. Ltd. (Kolkata, India) and the 0.22 µm disposable syringe filters were used from Milipore Corp., Bedford, MA, USA.

Collection of ovaries

Fresh cattle oviducts, ovaries were obtained from a nearby abattoir (Kolkata, India) 3-4 h away from the laboratory. The ovaries were taken in the thermos flask containing warm (32-35°C) saline fortified with antibiotic (400 IU/ml penicillin). The collected ovaries were transported to the laboratory within 2-3 h of slaughter of the animal. After properly trimming of the adjacent tissues, ovaries were washed 3-4 times with warm saline (33-37°C) fortified with antibiotic (400 IU/ml penicillin) and used for oocyte aspirations.

Collection of oocytes

Immature oocytes were collected from visible surface follicles (> 3 mm diameter) of ovaries by aspiration method with a 19-gauge hypodermic needle attached to a 5 ml disposable plastic syringe containing aspiration medium (TCM-199+DPBS+0.3% BSA+50 ug/ml gentamicin sulphate. Searching of oocytes was carried out under a zoom-stereo microscope at 40X magnification and were shifted to 35 mm petri dish containing washing medium (TCM-199+10% FBS+0.81 mM Sodium Pyruvate+50 ug/ml gentamicin sulphate). The aspirated oocytes were graded on basis of cumulus layer.

In vitro maturation of oocytes

The usable quality oocytes were collected from the searching dish and washed 4-6 times with the washing medium followed by twice with maturation medium (TCM-199+10% FBS+5 ug/ml FSH-P+0.33 mM Sodium Pyruvate+50 ug/ml gentamicin sulphate). The supplementation of maturation media with three different concentrations of LIF (15 ng/ml, 30 ng/ml, 45 ng/ml) was done. For *in vitro* maturation, groups of 20-25 COCs were placed in 100 µl droplets of maturation medium overlaid with sterile mineral oil in 35 mm petri dishes and cultured for 24 h in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C. Oocyte maturation was determined 24hr after *in vitro* maturation. Oocytes are assessed by expansion of cumulus layer and polar body formation.

Sperm preparation and *in vitro* fertilization

The spermatozoa used for IVF throughout the study were from the same donor that had been tested for IVF earlier. Briefly, two straws of frozen-thawed cattle semen were suspended in 1.2 ml of working Brackett Oliphant (WBO) media each (Brackett *et al.* 1993) with 10 µg/ml heparin, 0.57 mM (Mili-molar) caffeine sodium benzoate and 1.23 mM sodium pyruvate and incubated for swim-up at 38.5°C for 15-18 minutes. After completion of incubation, capacitated sperms were taken by collecting supernatant (800 µl) from the eppendorf tubes and centrifuged at 2000 rpm for 5 min. After centrifugation, the supernatant was decanted and the pellet was dissolved in 1ml of WBO media for washing and again centrifuged at 2000 rpm for 5 min. Finally, the pellet was suspended in 1ml of Fertilization Brackett Oliphant (FBO) medium and centrifuged at 2000 rpm for 5min. *In vitro* matured oocytes droplets were inseminated with capacitated motile spermatozoa (1-2 million spermatozoa/ml) and placed in 5% CO_2 incubator at 38.5°C for 15-18 h with maximum humidity.

In vitro culture of presumptive zygotes

After 15-18h of sperm-oocyte co-incubation, the cumulus cells were stripped off from the oocytes by repeated gentle pipetting in washing medium. The oocytes were then washed 1-2 times with mCR2aa medium and cultured in 100 µl mCR2aa medium (cleavage media). After 48 h cleavage was checked and cleavage media was replaced by mCR2aa blastocyst medium (IVC media). The culture dish was kept in 5% CO_2 incubator with maximum humidity at 38.5°C for 8 days and media used to replace with fresh media every day.

Experimental design and statistical data analysis

IVM, IVF and IVC media were supplemented with three different concentrations of Leukemia Inhibitory Factor (T1-15 ng/ml, T2-30 ng/ml and T3-45 ng/ml). The control group was not supplemented with growth factors or any kind of growth promoting cytokine agent. Experimental data were analyzed using simple One Way ANOVA. Means were compared using Duncan Multiple Range Test (IBM R Statistical Package for the social sciences R (SPSS version 16.0). Mean difference among group analyzed by Tukey's Multiple Comparison test (GraphPad Prisam 9.4.0). Graphs were made by using GraphPad Prisam 9.4.0. and the values were expressed as means±standard error.

RESULTS AND DISCUSSION

Effect of LIF on the maturation of oocytes

LIF was supplemented with specific concentrations in maturation media and their varying effect on maturation was assessed by observing the cumulus cell expansion and polar body visualization. Comparison study was done between the treatment group and the control group. Total 542 immature oocytes were used for *in vitro* maturation study.

The maturation percentage were 83.60±1.17 in control group and 85.92±0.63, 89.58± 0.62 and 87.21±0.62 in T1, T2 and T3 respectively (Fig 1). LIF @ 30 ng/ml has significantly higher maturation rate as compared to T1 and control group ($p<0.01$), but not significantly different from T3. LIF in T3 have significantly higher maturation rate as compared to control group only. When concentration of LIF increased beyond @ 30 ng/ml showed no impact on oocyte maturation that means the impact was dose-dependent till (@30 ng/ml. These findings suggested that LIF has a biphasic effect on maturation of bovine oocyte.

Effect of different concentrations of LIF supplementation on the cleavage

These results demonstrated that there is a significant difference in cleavage rate between control and treatment groups. Fig 2 depicted that the mean percentage of cleavage rate were 72.00±0.612 in control group and 72.25 ±0.87, 78.12±0.87 and 76.73±0.06 in T1, T2 and T3 respectively. LIF @ 30 ng/ml shows significant difference with control group ($p<0.001$) as well as T1, whereas T3 also has significant difference with control group ($p<0.01$). There is no significant difference between T2 and T3 group. On the basis of these result, it could be concluded that Treatment 2 (*i.e.,* @ 30 ng/ml LIF) is the best concentration for cleavage rate after *in vitro* fertilization.

Effect of different concentrations of LIF on early embryonic development

The mean percentage of 2-cells stage, 4-cell stage, 8-cell stage, morula and blastocyst formation rates in case of control group and treatment groups (T1, T2 and T3) were

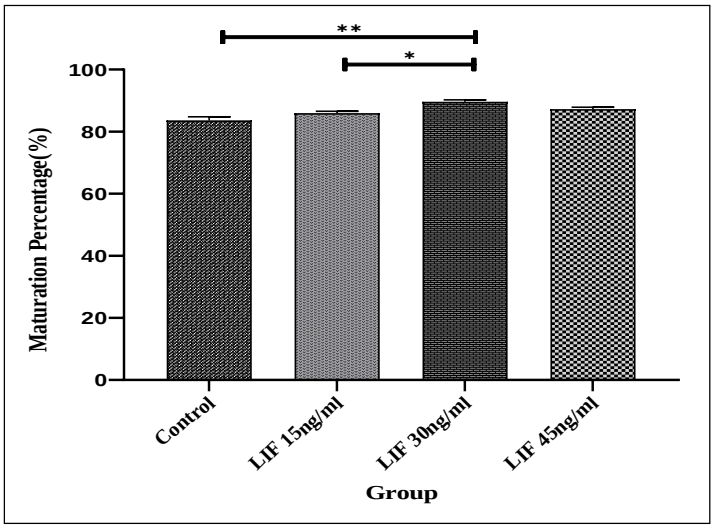

Fig 1: Effect of different concentrations of LIF supplementation on *in-vitro* Maturation. Data are expressed as mean±standard error of the mean from five independent experiments. Asterisks indicates **($p<0.01$). *($p<0.05$) level of significance.

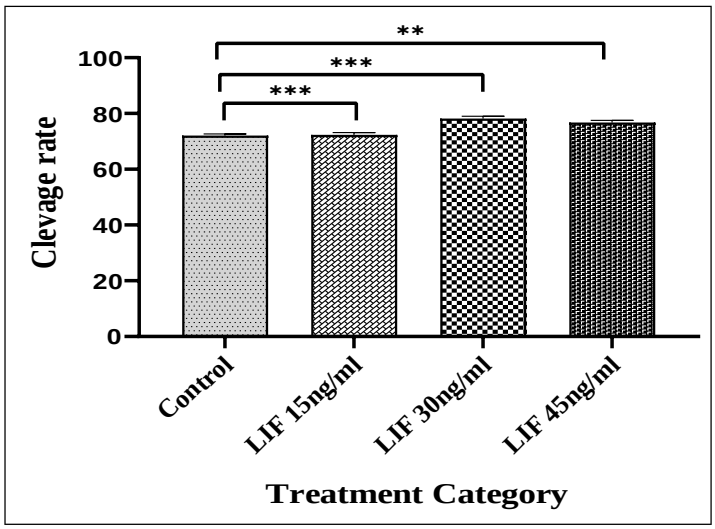

Fig 2: Effect of different concentrations of LIF supplementation on Cleavage. Data are expressed as mean percentage ± standard error of the mean from five independent experiments. Asterisks indicates ***($p<0.001$). **($p<0.01$) level of significance.

presented in table. The results demonstrated that there is a significant difference in 2-Cell Stage formation rate between treatment groups and control group. Treatment 2 is more significantly differ with the control group ($p<0.001$) as well as T1 group but not with T3. There are no significant differences among groups in 4-cell stage. The percentage of 4-cell stage formation rate was highest (*i.e.*, 83.91±0.67) in T2 as compared to other treatment groups as well as control group (Fig 3A). In 8-cell stage formation rate, there was no significant difference among groups. T2 has showed the best results (62.51±1.48) as compared to other treatment groups as well as control group (Fig 3B). Similarly, in morula formation rate there was no significant difference among treatment groups and control group. The highest percentages (30.27±2.20) of morula formation rate have been observed in T2 (Fig 3C). There was significant difference in blastocyst formation rate in T2 with control group ($p<0.05$) as well as T1 group ($p<0.05$), but not significantly differ with T3 group. The percentage of blastocyst formation was observed to be highest (12.96±0.49) in T2 as compared to other treatment groups as well as control group (Fig 3D). LIF have showed dosage-dependent effect when supplemented beyond 25 ng/ml. There was no detectable effect over control medium when LIF supplementation *used* @50 ng/ml and @100 ng/ml the percentage of metaphase II oocytes decreased significantly (Mo *et al.* 2014). LIF supplementation did not affect cleavage rate (86.46±2.33 in control group vs 84.01±2.90 in 25 ng/ml

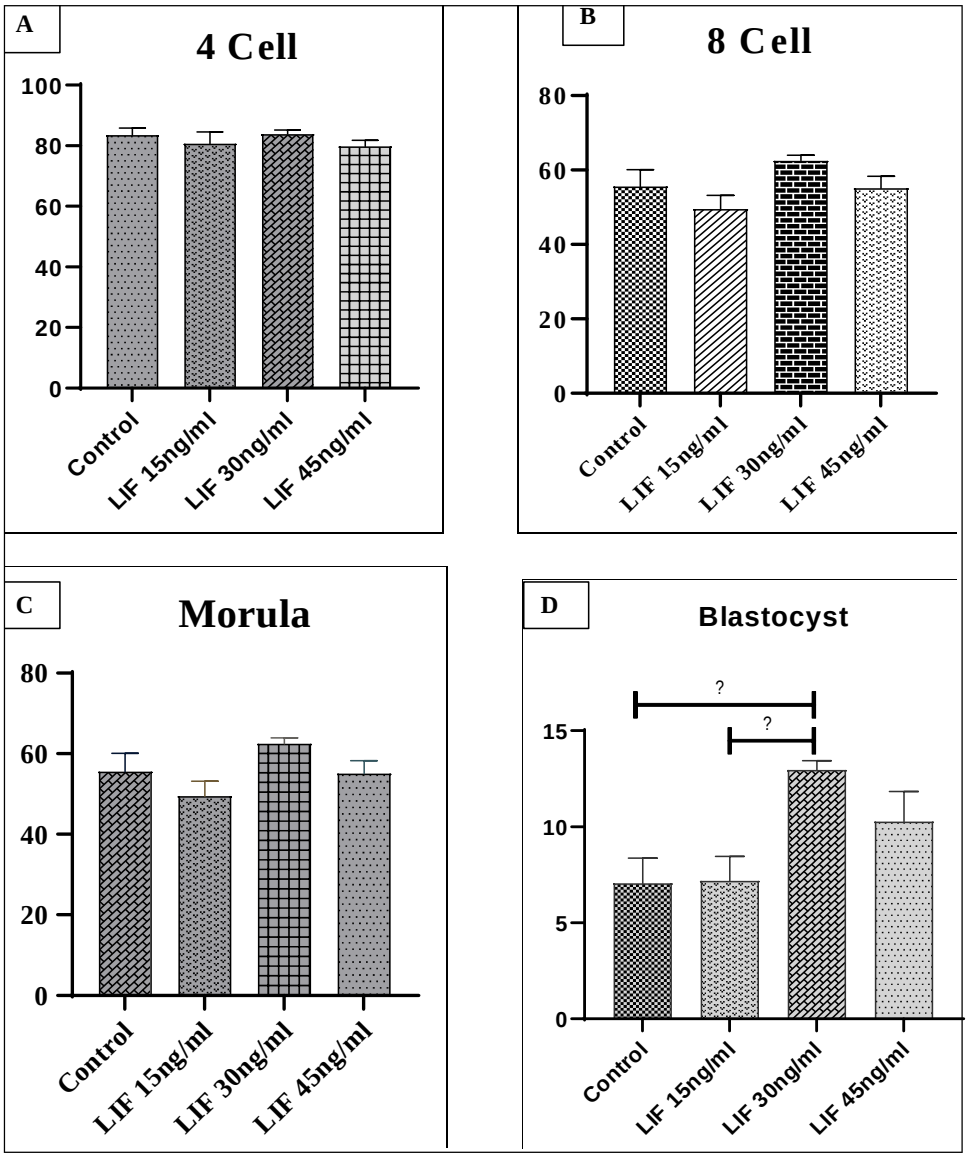

Fig 3: Effect of different concentrations of LIF supplementation on different stages of early embryonic development. A. 4 cells percentage B. 8 cells percentage C. Morula Percentage D. Blastocyst rate. Asterisks above the bars indicate statistically significant differences from the control group at $P<0.05$.

Table 1: Effect of different concentrations of leukemia inhibitory factor on early embryonic development.

Category	Total no of oocytesused	Matured Oocytes (IVM) Percentage (Mean±SE)	Cleavage % (Mean±SE)	4-cell % (Mean±SE)	8-cell % (Mean±SE)	Morula % (Mean±SE)	Blastocyst % (Mean ±SE)
Control	141	83.60^{A}±1.17	72.00^{A}±0.62	83.53±1.15	55.55±4.52	22.38±2.09	7.05^{A}±1.32
T1 (15 ng/ml LIF)	134	85.92^{AB}±0.63	72.25^{A}±0.87	80.68±1.95	49.51±3.67	24.27±2.42	7.18^{A}±1.28
T2 (30 ng/ml LIF)	134	89.58^{C} ± 0.62	78.12^{B}±0.87	83.91±0.67	62.51±1.48	30.27±2.20	12.96^{B} ± 0.49
T3 (45 ng/ml LIF)	133	87.21^{BC}±0.62	76.63^{B}±0.06	55.08±3.25	25.97±1.68	25.97±1.68	10.27^{AB}±1.57

Values with different superscript letters within the same column are statistically significant ($p<0.05$).

LIF supplemented group), blastocyst yield (26.00±2.55 and 24.86±2.48 in control and in 25 ng/ml LIF supplemented group respectively), but increased hatching rate (Vendrell *et al.* 2020).

CONCLUSION

In the present study @ 30 ng/ml LIF supplementation in IVM, IVF and IVC media resulted significantly higher maturation percentage, cleavage percentage and blastocyst formation as compared to control group, so this concentration of LIF could be used for improvement of in vitro embryo production.

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Conflict of interest: None.

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