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Effect of Insulin-Like Growth Factor-I (IGF-1) on oocyte competence and embryo development of buffaloes (Bubalus Bubalis)

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Abstract : The aims of this work were to study the effects of insulin-like growth factor-I (IGF-1) on: 1) In-vitro maturation (IVM) rate in buffalo oocytes, assessment of maturation was done by cumulus expansion for cytoplasmic maturation rate and presence of 1st polar body (MII) for nuclear maturation rate, 2) In-vitro embryo developmental competence of buffaloes, assessment of developmental competence of buffalo embryos was done by detection of cleavage rate and transferable embryo (morula and blastocyst) rate. Ovaries were collected from EL-Warak slaughter house. Good and excellent oocytes were cultured in TCM-199 vs. TCM-199 + 100 ng/ml IGF supplemented with 10% fetal calf serum (FCS) + 10 µg/ml follicle stimulating hormone (FSH) + 50 µg/ml gentamicin. COCs were matured for 22 h in incubator at 38.5°C in 5% CO_2 and humidified atmosphere. Matured oocytes were fertilized with frozen thawed semen (washed by fertilization TALP) and incubated for 18 hours, then in vitro cultured by SOF D=0 for 7 days. The experimental data were analyzed using paired t-test, P< 0.05 was considered to be statistically significant. The TCM-199 +IGF group showed no significant difference in cumulus expansion rate GIII, GII, GI or G0 (42.77±3.41, 20.11 ± 4.52, 12.78 ± 2.36 and 24.78 ± 2.57%, respectively) when compared with TCM-199 group (42.38 ±1.73, 20.07 ± 2.79, 9.53 ± 1.96 and 28.02 ± 3.57%, respectively). The maturation rate of buffalo oocytes with polar body (MII) was significantly higher in the TCM-199+IGF group (range 81.21±1.64 - 86.56±2.06%) when compared with those matured in TCM-199 (range 70.18±0.92 - 73.48±0.46%). The cleavage rate was significantly higher (P<0.05) in TCM-199+IGF (90.61±2.38%) when compared with TCM-199 (76.50±2.41%). The transferable embryo (morula and blastocyst) rate significantly increased (P<0.05) in IGF-I group (28.12±1.68 & 20.83±1.95%, respectively) when compared with TCM-199 group (20.20±2.85 & 12.85±2.51%, respectively). In conclusion addition of IGF-I to the in vitro maturation medium TCM-199 improved in vitro maturation and transferable embryo rates in buffaloes.

Keyword: Buffalo, in vitro embryo production, IGF-1.

Introduction

Buffalos (*Bubalus bubalis*) are multi-purpose animals with great importance in agriculture as milk and meat producers. Many countries depend heavily on buffalos' production for meat and milk, in addition to their value for labour and ability to resist environmental temperature, climate, stress and diseases. However, inherent reproductive problems, namely weak, silent oestrus signs[1], lower number of follicles compared to bovine [2], reduced response to ovarian stimulation[2,3,4], seasonal anoestrus[5], long post-partum anoestrus period [6], delayed age of puberty and low conception rates which collectively leads to less reproductive efficiency and limit the productivity of this species[7].

Some commercial applications of in vitro embryo production technology have included efforts to upgrade the productive and genetic performance of animals; to overcome infertility of valuable high yielding animals; to produce transgenic and cloned animals and to provide a source of sexed embryos. At the molecular level, the technique is used to elucidate events related to maturation, fertilization of oocytes and development of embryos, these events are difficult to study under natural conditions in living animals[8,9,10]Only few laboratories have been able to produce live buffalo calves from in vitro-derived buffalo embryos.[11,12] However, one major problem that has limited the application of this technology was the very low blastocyst yields around 10 %[7, 13] to 20% [14.15] of the oocytes subjected to IVM, when compared with the ~30 to 40% observed in cattle.[16]

Competent oocytes are the ones that are able to resume meiosis, complete the cytoplasmic and nuclear maturation process, have the ability to be fertilized, develop to normal transferable blastocyst, induce pregnancy after transfer to a recipient, sustain development of the fetus to full term, and result in the delivery of a healthy offspring [17, 18, 19.]

Improvements in understanding of the nutritional requirements of oocytes and paracrine, autocrine and endocrine regulation of meiotic maturation have led to improvements in in vitro oocyte maturation protocols (culture conditions and media components), which allow 90% of oocytes progressing to the metaphase II (MII) stage with a resulting 80% cleavage rate after fertilization in most farm animal species, including cattle (92.2%) [20,] and buffalo (80.4%) [21]. In vitro embryo production in buffalo was improved by using portable incubator lead to production of 42% transferable embryo [22] and addition of epidermal growth factor (EGF) in maturation medium and development of buffalo embryo in cultured buffalo oviduct epithelial cells (BOEC) giving 37% morula and 23% blastocyst [23]. Addition of growth factors as IGF1 to maturation medium is documented to improving the maturation rate by many authors[24,25,26.] Moreover, the global gene expression data generated in this study suggest differential expression of many genes associated with IGF-I system and its signaling in the preovulatory follicle[27]. IGF1 has a role in regulation of cell proliferation, survival and steroidogenesis in GCs. E2 production appeared to be regulated by intrafollicular concentrations of IGF-I and IGF-II 28 and concentrations of these may in turn be modulated by IGF binding proteins. IGF-1 has been shown to prevent apoptosis in early mammalian embryos and to act as a survival factor [28,29].

The aim of this work was to improve the in vitro embryo production in buffaloes with addition of IGF-I to the in vitro maturation medium.

Material and method

The present study was conducted in the Department of Animal Reproduction and Artificial Insemination, Veterinary Research Division, National Research Center, Cairo.

Oocytes Collection and In vitro Maturation:

Buffalo ovaries were collected from El-Warak slaughterhouse at Cairo, transported to the laboratory in a thermos containing normal saline solution (NSS, 0.9% NaCl + 100 IU penicillin and 100 µg/ml streptomycin). At the laboratory, ovaries were washed at least 3 times in pre-warmed saline solution (37°C) and then kept in water bath at 37°C until oocytes aspiration. Cumulus oocytes complexes (COCs) were aspirated from follicles 2-8 mm in diameter using an 18-gauge needle attached to a 10 ml sterile syringe containing 2 ml aspiration and

washing medium (phosphate buffered saline; PBS) + 6 mg/ ml bovine serum albumin F-V + 50 μg/ml gentamicin. After aspiration, follicular content was transferred to 15 ml Falcon tube and allowed to settle for 10 to 15 min in water bath at 37°C. COCs were evaluated under stereo microscope (x 90) and washed 3 times in oocyte aspiration medium. Excellent and good oocytes were transferred to in vitro maturation medium TCM-199 vs.TCM-199+100 ng/ml IGF supplemented with 10% fetal calf serum (FCS) + 10 μg/ml follicle stimulating hormone (FSH) + 50 μg/ml gentamicin. COCs were matured for 22 h in CO_2 incubator at 38.5°C in 5% CO in humidified atmosphere. The assessment of in vitro maturation in buffalo oocytes was done in 6 replicates. After 22 h of incubation, the cytoplasmic maturation of oocytes was assessed based on the degree of cumulus expansion to:

Grade 0 (G0): with no expansion.

Grade 1 (GI): with slight expansion in the outer layer of cumulus-cells.

Grade 2 (GII): with moderate expansion.

Grade 3 (GIII): with full expansion

The presence of first polar body in the perivetteline space (M II) was the criterion for nuclear maturation of the oocytes. Detection of polar body was done under inverted microscope in magnification 200X. The oocytes were fixed in 4% paraformaldehyde for staining with propidium iodide and image using confocal microscope (Zeiss LSM 710) in magnification 400X.

Cytoplasmic maturation rate was calculated as follows:

Expansion rate = No. of expansion grade's oocytes X 100 / Total no. of oocytes

Nuclear maturation rate (MII) was calculated as follows:

MII rate = No. of mature oocytes with 1^{st} Polar body X 100/ Total no. of oocytes

In vitro Embryo Production:

In vitro maturation of buffalo oocytes (TCM-199 vs. TCM-199+IGF-1 groups) was done in 8 replicates. Matured oocytes with full cumulus expansion and presence of 1st polar body were washed in fertilization medium (Fert-TALP supplement with 6 mg/l BSA). Frozen-semen was thawed in water bath at 37°C for 30 seconds. Motile spermatozoa were layered on the top of two layers of Percoll density gradient (90% and 45%) and centrifuged for 30 minutes at 2000 rpm. The supernatant and Percoll were removed and sperm pellet was suspended with 5 ml sperm-TALP medium containing 10 μg/ml heparin and 4 mg/ml BSA, then centrifuged again for 10 minutes at 1800 rpm. The supernatant was removed and the sperm pellet was re-suspended in fertilization- TALP medium supplemented with10 μM/ml hypotaurin, 20 μM pencillamine (PHE) + 1μg/ml heparin and 6 mg/ml BSA. Sperm concentration was adjusted to 1×10^6 sperm/ml and then allocated into 4-well culture plate. The sperm-oocytes were co-incubated for 18 h at 38.5°C under 5% CO2 in humidified air. The presumptive zygotes were washed at least 3 times then cultured in culture medium (IVC, modified synthetic oviduct fluid, mSOFaa medium) supplemented with 5 mg/ml BSA, 5 μg/ml insulin and 50 μg /ml gentamycin and incubated at 38.5°C under 5% CO2 in humidified air. Cleavage rate and embryo development to the morula and blastocyst stages were checked on Days 2, 5 and 7. Culture medium was changed every 48 h.

Statistical analysis

Data were expressed as mean ± standard error (SE). The significant of differences was tested by paired t-test and analysis of variance (ANOVA) followed by hoc test. Statistical analyses were performed using SPSS.

Results

Experiment I: Effect of addition of insulin growth factor (IGF) to the in vitro maturation medium on maturation rate of buffalo's oocytes

Number of ovaries used for this experiment was 179 for TCM199 (no=89) and for TCM-199+IGF (no=90).

1.1. Cytoplasmic Maturation:

The TCM-199 +IGF medium showed no significant difference in cumulus expansion rate (Table 1, fig1) of GIII, GII, GI or G0 (42.77±3.41, 20.11 ± 4.52, 12.78 ± 2.36 and 24.78 ± 2.57%, respectively) when compared with TCM-199 group (42.38 ±1.73, 20.07 ± 2.79, 9.53 ± 1.96 and 28.02 ± 3.57%, respectively).

Therefore, the supplement with IGF-I to the TCM medium has no effect on cumulus expansion rate.

Table (1): Effect of TCM-199 medium with or without IGF-I on cumulus expansion rate of in vitro matured buffalo oocytes

Media	No of Oocytes		GIII			GII			GI			G0		
	NO	Mean ± S.E.	No	Mean± S.E.	% (Mean± S.E)	No	NO	% (Mean ± S.E.)	No	Mean± S.E.	% (Mean± S.E %)	No	NO	% (Mean ± S.E.)
TCM	188	18.80 ± 2.96	77	7.70 ± 1.09[a]	42.38± 1.73 [a]	40	4.00 ± 0.84 [a]	20.07 ± 2.79 [a]	20	2.00 ± 0.56 [a]	9.53 ± 1.96 [a]	51	5.10 ± 0.78 [a]	28.02 ± 3.57 [a]
TCM+IGF	190	19.00 ± 1.39	82	8.20 ± 0.95 [a]	42.77± 3.41 [a]	36	3.60 ± 0.7 [a]	20.11 ± 4.52 [a]	23	2.30 ± 0.42 [a]	12.78 ± 2.36 [a]	49	4.90 ± 0.72 [a]	24.78 ± 2.57 [a]

a, b: Superscripts to be compared statistically within the same column. Values with different letters are significantly different (P<0.05).

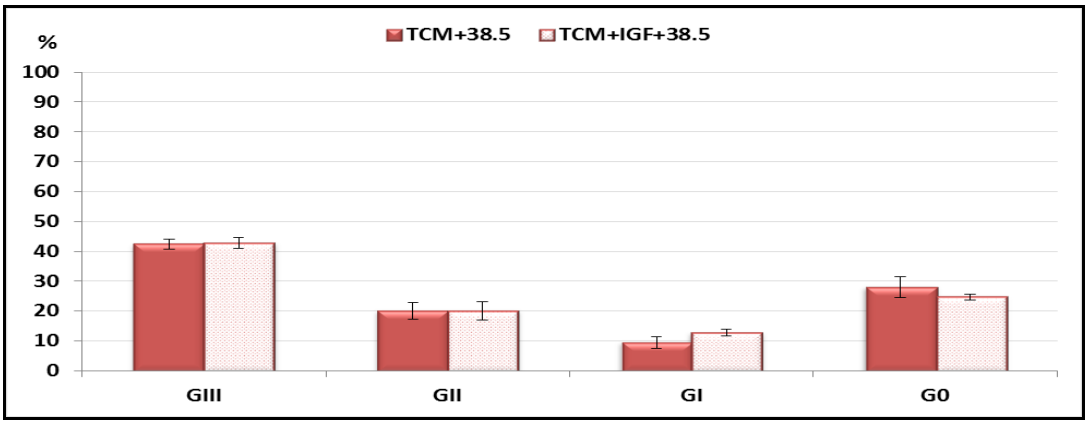

Fig (1): Effect of TCM-199 medium with or without IGF on cumulus expansion rate of in vitro matured buffalo oocytes

1.2. Nuclear Maturation (MII)

The matured buffalo oocytes with polar body (Table 2, fig 2, photo1) in TCM-199 and TCM-199+IGF media averaged 13.20 ± 2.10 and 15.60 ± 1.40, respectively. The respective matured oocytes without polar body were 3.20 ± 0.49 and 1.80 ± 0.33. The degenerated buffalo oocytes averaged 2.40 ± 0.52 and 1.60 ± 0.34 in TCM-199 and TCM-199+IGF, respectively.

The maturation rate of buffalo oocytes with polar body was significantly higher in the TCM-199+IGF maturation medium (81.21 ± 1.64%) when compared with those matured in TCM-199 (70.18 ± 0.92%). The buffalo oocytes without polar body matured in TCM-199 showing higher significant (P<0.05) difference (17.77 ± 1.47%) when compared with oocytes cultured in TCM-199 + IGF (9.23 ± 1.52%). The degenerated oocytes showed no significant difference between those matured in TCM-199 (12.05 ± 1.68%) and TCM-199+IGF-I (9.55 ± 2.31%).

Table (2): Effect of TCM-199 medium with or without IGF on nuclear maturation rate of in vitro matured buffalo oocytes

Media	No of Oocytes		1st pb			Without 1st pb			Degenerated		
	No	Mean ± S.E.	No	Mean ± S.E.	% (Mean± S.E)	No	Mean ± S.E.	% (Mean± S.E)	No	Mean ± S.E.	% (Mean± S.E)
Tcm	188	18.80 ± 2.96	132	13.20 ± 2.10^{a}	70.18 ± 0.92^{a}	32	3.20 ± 0.49^{a}	17.77 ± 1.47^{a}	24	2.40 ± 0.52^{a}	12.05 ± 1.68^{a}
TCM+IGF-I	190	19.00 ± 1.39	156	15.60 ± 1.40^{b}	81.21 ± 1.64^{b}	18	1.80 ± 0.33^{b}	9.23 ± 1.52^{b}	16	1.60 ± 0.34^{a}	9.55 ± 2.31^{a}

a-b: Superscripts to be compared statistically within the same column. Values with different letter are significantly different (P<0.05).

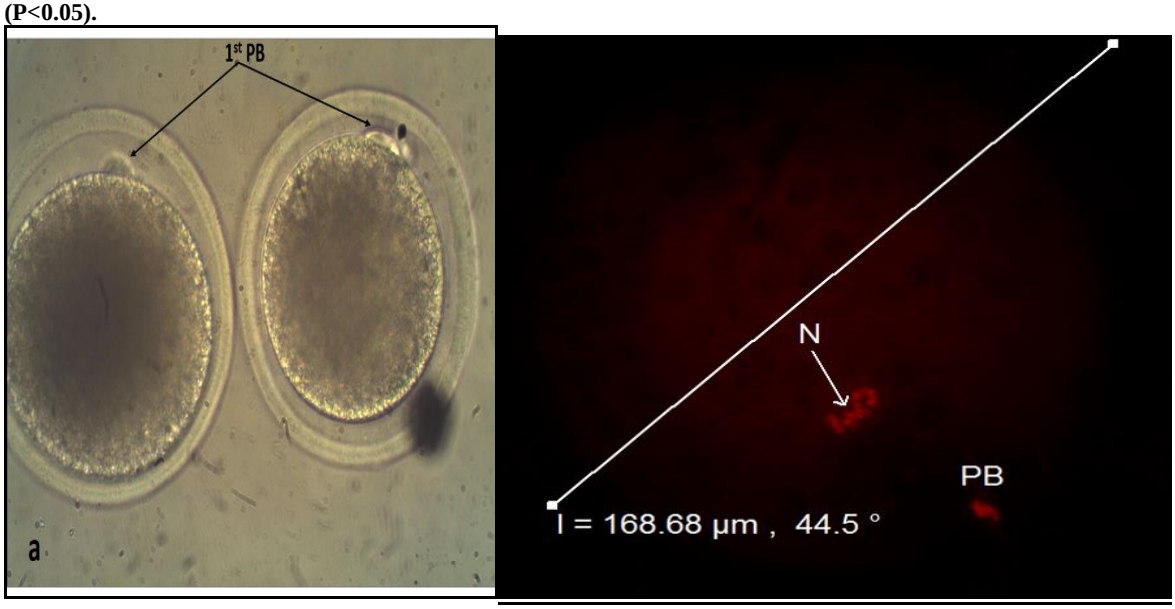

Photo 1, In vitro matured buffalo oocytes (nuclear maturation) with a-1st polar body (1st PB) inverted microscope 40X, b-oocyte stained with propidium iodide stain showed 1st polar body (PB) and nucleus with diameter 168.68 µm using confocal microscope Zeiss 710 magnification 400X laser wave length 561

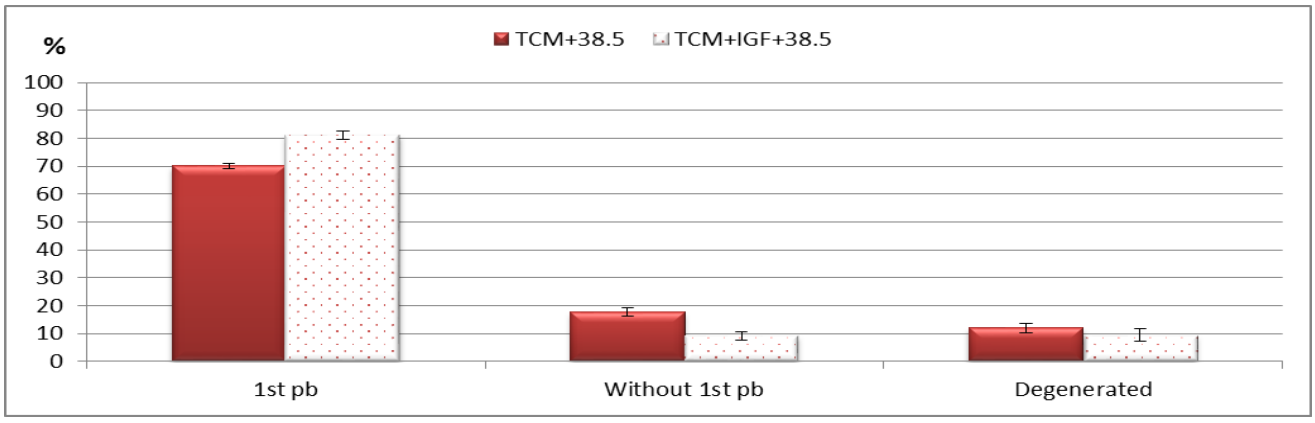

Fig. (2): Effect of TCM-199 medium with or without IGF-I on nuclear maturation rate of in vitro matured buffalo oocyte

Experiment II: Effect of IGF-I supplement to the in vitro maturation media on cleavage rate and embryo developmental competence in buffaloes

The mean number of maturation rate of in vitro matured buffalo oocytes (Table 3, fig 3) was 73.48±0.46 in TCM-199 and 86.56±2.06 in TCM-199+IGF-I. The cleavage rate was significantly higher (P<0.05) in TCM-199+IGF-I (90.61±2.38%) when compared with TCM-199 (76.50±2.41%). The developmental stages rate showed significant increase (P<0.05) in morula and blastocyst rates in IGF-I group (28.12±1.68 & 20.83±1.95%, respectively) when compared with TCM-199 group (20.20±2.85 & 12.85±2.51%, respectively).

Table (3): Effect of IGF supplement to the in vitro maturation media on cleavage rate and embryo developmental competence in the buffalo

Media	oocytes	No of matured Oocytes			Cleavage rate			morula			Blastocyst		
	No	No	Mean ± S.E.	% (Mean± S.E)	No	Mean ± S.E.	% (Mean± S.E)	No	Mean ±S.E.	% (Mean ± S.E)	No	Mean ±S.E.	% (Mean ± S.E)
Tcm	129	95	19.00± 2.47 [a]	73.48± 0.46 [a]	72	14.40± 1.63 [a]	76.50±2 .41 [a]	14	2.80± 0.37 [a]	20.20± 2.85 [a]	9	1.80±0 .37 [a]	12.85 ± 2.51 [a]
Tcm IGF	146	138	27.60± 0.68 [b]	86.56± 2.06[b]	125	25.00± 0.84 [b]	90.61± 2.38 [b]	35	7.00± 0.32 [b]	28.12± 1.68 [b]	26	5.20±0 .49 [b]	20.83 ± 1.95 [b]

a-b: Superscripts to be compared statistically within the same column. Values with different letter superscript different (P<0.05).

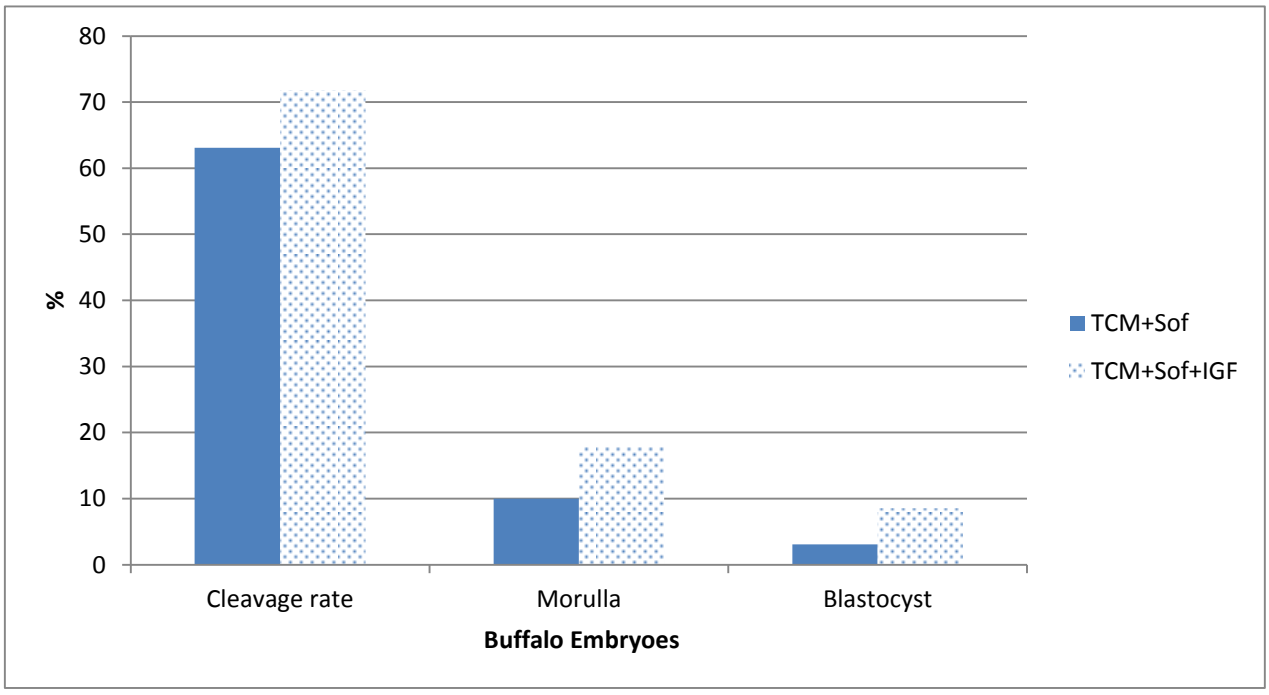

Fig. (3): Effect of IGF supplement to the in vitro maturation media on cleavage rate and embryo developmental competence in the buffalo

Discussion

1. Effect of addition of insulin growth factor (IGF) to in vitro maturation media on maturation rate of buffalos oocytes

1.1. Cytoplasmic Maturation

There was no significant difference between oocytes cultured in TCM group and TCM supplemented with IGF-I in cumulus expansion. This finding in buffaloes agrees with similar results that IGF does not affect cumulus expansion of in vitro matured oocytes in bovine[24,26,30.] This observation may be related to the mechanism of IGF action which does not act via cumulus cells or it interferes with the production of an expansion factor secreted by the oocyte[31.]

1.2. Nuclear maturation

In the present study, the nuclear maturation rate of TCM-199 buffalo oocytes ranged between 70 and 73%. A variety of media have been used for IVM and culture, but TCM-199 has been used for both maturation and culture more frequently than any other medium in buffaloes[32]. TCM-199 showed significantly better maturation rate (73.3%) than Ham´s 10 (61.6%)[33]. Moreover, TCM-199 medium exhibited higher maturation rate (77.6 ± 0.9%) than mSOF medium (40.9 ±0.7%)[34]. These differences in maturation rate may be attributed to the composition of the media[35]. TCM-199 contains both glutamine and glucose [36]. Presence of glucose is essential to generate ATP via glycolytic metabolism, while glutamine can feed into tricarboxylic acid cycle and serves as a potential energy source [37].

The finding of significant improvement in the nuclear maturation of buffalo oocytes cultured in TCM supplemented with IGF-1 as indicated by a marked increase in MII rate agrees with other authors[24,25,26]. Some authors did not find the beneficial effects of IGF-1 treatment during in vitro production of bovine embryos[38,39,40]. Hainaut et al.[41] postulated that maturation with IGF-1 is initiated upon activation of the membrane receptor for this growth factor and requires tyrosine dephosphorylation of p[34], the kinase component of maturation promoting factor (MPF). The kinetics of activation of MPF precede or occur simultaneously with GBVD in bovine oocytes[42,43] and MPF phosphorylates many of the proteins involved in nuclear membrane formation, chromatin condensation and microtubule reorganization[43]. According to Sakaguch[44 ,] the kinetics of activation of MPF can be assessed by H1 kinase activity. H1 kinase activity showed a rapid increases in correlation to IGF-1 supplement in media and the rapid meiotic progress of oocytes indicated by polar body extrusion. In vitro studies have shown that IGF1 synergizes with FSH to regulate the aromatase activity of granulosa cells [45].

Other in vitro effects of IGF1 include enhanced secretion of follistatin, inhibin-A, activin-A in granulosa cells[46,47,48], increased androstenedione production from theca cells[49] and protection from apoptosis in oocytes and granulosa cells [50,51,52] . In mouse embryos, the detrimental effects of oxidative stress induced by hydrogen peroxide could be alleviated by the addition of IGF-I to the culture medium[53]. Using TUNEL assay to determine DNA fragmentation, Wasielk et. al[54] reported that DNA fragmentation and apoptotic oocytes matured in media with IGF-I supplementation fell to zero which indicate that IGF-1 act as anti-apoptotic factor during oocyte maturation according to [54.] This action may be due to blocking of active caspase.

2. Effect of IGF supplement to the in vitro maturation media on cleavage rate and embryo developmental competence in the buffalo

Our results show that the supplementation of TCM-199 maturation medium with IGF-I significantly increased the cleavage rate than TCM-199 group (90.61 vs 76.5 respectively) and blastocyst rate (20.83 vs

12.85 respectively). These findings agree with Byrne et al.[55] and Block et al.[56] They revealed that The cleavge rate in our results was higher than that observed by Kandil et al. [57] (68.1 %), Manjunatha et al.[58], who reported that the cleavage rate in the good buffalo oocytes selected by brilliant crasyle blue was 71%. Gassparrini et al.[59], observed that the cleavage rate of buffalo oocytes matured in vitro in TCM-199 + cysteamine was 66 %. These difference may be due to the different maturation conditions. In the present work, addition of IGF-I (100 ng/ml) to the in vitro maturation medium increased the maturation rate and the subsequently the cleavage rate and the embryo development.

In our experiments, morula rate ranged between 20.20 and 28.12 and blastocyst rate between 12.85 and 20.83 in TCM-199 and TCM-199+IGF-1 maturation medium respectively. These result was similar to morula rate (22.6%) recorded by Gasprrini et al.[60], however, it was higher than blastocyst rate (18%) observed by Manjunatha et al.[61]. Gassparrini et al.[59] recorded 23.8% transferable rate of in vitro produced buffalo embryos. These differences may be due to the difference in culture condition. In bovine preimplantation embryos, IGF1 stimulates cell proliferation through the mitogen-activated protein kinase signaling cascade, while the anti-apoptotic actions are mediated via the Phosphatidylinositol 3-kinase (P13K)/AKT1 (also known as protein kinase B) pathway[62,63]. IGF-1 has been shown to prevent apoptosis in early mammalian embryos and to act as a survival factor[64]. Lu et al.[65], demonstrated that supplementing CM with 50 ng mL–1 IGF-1 could improve the developmental competence of buffalo embryos, increase the total cell number of blastocysts and decrease their apoptotic index, probably by down-regulating the mRNA level of pro-apoptotic bax gene and up-regulating the mRNA level of anti-apoptotic bcl-2 gene.

In conclusion, addition of IGF-I to the TCM-199 in vitro maturation medium improved in vitro maturation rate and transferable embryo rate (morula & blastocyst) in buffaloes.

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