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The Effect of Follicular Fluid Supplementation on Embryo Development in Iraqi Buffalo

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Abstract
The study was carried out to evaluate the effect of supplementation of the buffalo follicular fluid (buFF) in culture medium (Ham's F-10) on the development of oocytes in vitro maturation (IVM) rate, oocytes in vitro fertilization (IVF) rates, and embryonic developmental rate. The experimental study extended from November 2019 to March 2020. Samples included (80) female ovary and (20) bull testes was a collection from Basrah center slaughterhouse after 20 minutes from slaughter. Harvested samples were transported by disinfected clean box at (37 ºC) in less than 1 hrs to the lab. Only A and B grads quality oocytes were selected and incubated in a prepared maturation medium. Spermatozoa were obtained from slicing caudal of epididymal. Matured oocytes and capacitated sperms were incubated fertilization medium. Fertilize oocytes were re-incubated in fertilization medium with changing 50% of the medium every day followed by an embryonic developmental assessment for 4 days. Results illustrated a significant increase in oocytes maturation rate in Ham F-10 media with a 20% buFF group compared control group. On the other hand, this study showed a significant increase in fertilization rate in Ham's F-10 medium with a 20% buFF group compared with the control group. Moreover, a high significant difference in embryonic development (2, 4,8,16 cells) in Ham's F-10 media with 20% buffalo follicular fluid group compared with the control group. Thus, It has been concluded that supplementation of 20% buFF to media could be enhanced in vitro oocyte maturation, fertilization, and in vitro embryonic development in buffalo.

Keywords: Buffalo, follicular fluid, Embryo development

Introduction

The zoological scale of buffalo in the class (Mammalia) order (Artiodactyla), Ruminantia (subordinate), family (Bovidae), sub-family (Bovinae), gender (Bubalus), species (Bubalus bubalis). Buffaloes are a very important resource in the agricultural economy in Iraq and some other countries such as India, China, and Pakistan Egypt, and Ext [1]. Riverine and Swamp buffalo are an irreplaceable milk producer, resistant to climate, parasite, stress, diseases, and the rustic and high ability for acclimation in climatic zones [2]. However, there are many problems in buffalos like delayed reproductive maturity, reproductive seasonality, calving interval long, silent estrus, low follicles, and poor super ovulatory response have been referred to low reproductive effecting of this species [3]. Therefore, the assisted reproductive technologies (ART) are very important to improve the genetic potential for this species [1].

Oocytes maturation is a very important step in the IVF process. Many studies were done to improve the in vitro maturation in mammal's oocytes [4]. Supplementation of in vitro maturation medium with hormones and different concentrations of serum plays an important role in embryo development [5]. Oocytes maturation consists of two steps: cytoplasm and nuclear maturation [6]. Bovine oocytes maturation is affected by several factors, such as time of oocytes collection, transportation temperature and follicular size [7], stage of oocyte development [8], the diameter of the oocyte [9], media composition [10], hormonal supplementation [11] serum addition like fetal calve serum (FCS) or bovine serum albumin (BSA) [12].

Buffalo follicular fluid is one of the alternatives of macromolecules that can be used as the supplementation in the maturation media because it is easy to get and cheap. Follicular fluid is a liquid semi-viscous and yellow in color, which dictates the follicle and surrounding oocytes [13]. Buffalo follicular fluid consists of many growth factors, follicle-stimulating hormone (FSH), luteinizing hormone (LH), several nutrients, and rich in hyaluronic acid [13]. Follicular fluid is a result of the transport of blood plasma components that cross the follicular barrier and secretory activity of granulosa and theca cells [14].

Analysis of the biochemical properties of the follicular fluid surrounding the eggs plays important role in determining the oocyte's quality and subsequent potential for fertilization and fetal development [15]. The analysis of the components of the follicular fluid provides information about metabolic changes in the blood serum, as the circulating biochemical environment may be reflected in the formation of the follicular fluid [16]. Several studies of the chemical components of follicular fluids have been grouped into the following categories hormones, growth factors in the TGF-beta family, other growth factors and interleukins, reactive oxygen species (ROS), proteins, amino acids, peptides, sugars, anti-apoptotic factors, and prostanoids [16]. Limited

studies were conducted to examine the effect of buffalo follicular fluid on in vitro fertilization in buffalo, so we designed this study to evaluate the effect of supplementation the buffalo follicular fluid on oocytes maturation, oocytes fertilization, and embryos production from buffalo oocytes.

Materials and methods

Media and reagents

All chemicals and media were obtained from Sigma Chemical Company (St. Louis, MO, USA) and Gibco (Invitrogen Corporation, Grand Island, NY, USA), respectively. All the experiments were carried out in the central laboratory, university of Basrah, college of veterinary medicine.

Recovery and *in vitro* maturation of buffalo oocytes

Immature buffalo cumulus oocytes complexes were aspirated from 0.8-1 mm follicles of slaughterhouse ovaries by using an 18-gauge needle connected with a syringe. The recovered oocytes were transferred into Ham's F-10 supplemented with 10% fetal calf serum (FCS). Oocytes with clear cytoplasm and with many layers of cumulus cells were selected for in vitro maturation [17] and incubated for 20 h in tissue culture medium at 38.5 °C, 5% CO2, and maximum humidity [18]. The oocytes divided into 2 groups, the first group (control group) was incubated inside in vitro maturation medium (Ham's F-10 plus 10% FCS) for 20 h at 38.5 °C, 5% CO2, and maximum humidity [18]. While the second group (treated group) was incubated inside in vitro maturation medium which supplemented with 20% buffalo follicular fluid for 20 h at 38.5 °C, 5% CO2, and maximum humidity. Oocyte maturation was recorded through the presence of cumulus cells expansion as well as the presence of the first polar body as described by [19].

Buffalo follicular fluid preparation

Buffalo follicular fluid (buFF) was collected by aspiration from pre-ovulatory follicles (1-2 cm in diameter) containing oocytes during the reproductive season. Follicular fluids (10–20 ml) for each sample were centrifuged twice at 1000 g for 15 min to removed cellular debris and transported to a sterilized

glass beaker for temperature inactivation at 50 ºC for 30 minutes in a water bath. The buffalo follicular fluid was sterilized by using syringe driven filter 0.22mm and then was kept in sterilization test tubes 1.5ml capacity at 20 oC for using within vitro maturation, in vitro fertilization, and in vitro culture medium development embryo [20].

Sperm maturation and Capacitation

Sperms was opined from slicing tail of epididymal of buffalo's bull testis. The sperm samples with 2ml of tissue culture media (TCM-199) with anti-fungal (Nystatin) and anti-bacterial (Penicillin) transported from petri dish to 10ml test tube , Samples incubated in (37Cº) for 4 hrs. .The descending protoplasmic droplet from tail of sperm was good signs for maturation [21]. The end step sperm capacitation was added 50 IU/ml heparin at the last 45minutes from time of sperm maturation [22].

In vitro fertilization and culture

Matured oocytes rinsed twice in Ham's F-10 medium before transmission to IVF Petri-dishes contain IVF medium (Ham's F-10) containing 20% buFF, 10% FCS, 100 IU/ml penicillin-streptomycin and100 IU/mL Nystatin [23]. While the control group was left without buFF. For in-vitro fertilization, approximately 1-2× 106 capacitated sperms were transferred for each matured oocyte inside the IVF medium [23]. Petri- dishes containing capacitated sperms plus matured oocytes were incubated at 38.5 °C, 5% CO2, and maximum humidity [24, 25]. One day after, the fertilization rate was recorded. In the following days, 50% of the medium was changed daily for 4 days and during this time the cleavage rate and embryo development was recorded according to [26].

Statistical Analysis

Chi-square test was used to compare maturation and fertilization rates among different groups [27]. All data were presented as means ± SEM and the differences were considered as significant at $P < 0.05$.

Results

Effect of buffalo follicular fluid on maturation of buffalo oocytes

The current study revealed that the oocyte maturation rate was increased significantly (P<0.05) in the treated group when supplemented with 20% buFF compared with the control group 77.78±4.06, 38.89±9.09 respectively (figure 1). Moreover, the percentage of non- matured oocytes in the treated group with 20% buFF was decreased significantly (P<0.05) in compared control group 22.22±4.06, 61.11±9.09 respectively (table 1).

Table 1. The effect of buffalo follicular fluid on In vitro maturation (IVM) of buffalo oocytes.

Groups	Ovaries No.	Cultured oocytes No.	Matured oocytes	Non matured oocytes
Control group	40	90	35 (38.89 ± 9.09) a	55 (61.11 ± 9.09) a
Treated group	40	90	70 (77.78 ± 4.06) b	20 (22.22 ± 4.06) b

Data are presented as number (percentage ± SEM). [ab] Different letters within each column indicate significant difference ($p < 0.05$)

The effect buffalo follicular fluid on *In vitro* fertilization (IVF) in buffalo

The present study showed that the fertilized oocytes were increased significantly (P<0.05) in the treated group with 20% buFF in comparison with the control group 84.29±2.64, 45.44 ±6.72 respectively (figure 2, 3 and Table 2). Non fertilized oocytes were increased significantly (P<0.05) in the control media group in comparison with a treated group that supplemented with 20% buFF 54.55±6.72, 15.70±2.64 respectively. While the percentage of embryos development

increased significantly (P< 0.05) in the treated group with 20% buFF 37.50±4.95 in compared with the control group15.32±3.63 (table 2).

Table 2. The effect of buffalo follicular fluid on IVF in buffalo oocytes.

Groups	Matured oocytes	Fertilized oocytes	Cleaved oocytes	Non fertilized oocytes
Control group	35 (38.89±9.09) a	17 (45.44 ±6.72) a	3 (15.32±3.63) a	18 (54.55±6.72) a
Treated group	70 (77.78±4.06) b	59 (84.29±2.64) b	22 (37.50±4.95) b	11 (15.70±2.64) b

Data are presented as number (percentage ± SEM). [ab] Different letters within each column indicate significant difference ($p < 0.05$)

The effect of buffalo follicular fluid on embryo

development in buffalo

The present study showed the embryonic development (2, 4, 8, and 16 cells) (figure 4, 5, 6, 7) significantly (P<0.05) higher in the treated group which supplemented with 20% buFF in compared with the control group (table 3).

Table 3. The effect of buffalo follicular fluid on embryos development in buffalo IVF.

Groups	Fertilized oocytes	2-cell embryo	4-cell embryo	8-cell embryo	8-cell embryo
Control group	17 (45.44±6.72) a	3 (15.32 ± 3.63) a	0 a	0 a	0 a
Treated group	59 (84.29±2.64) b	22 (37.50±4.95) b	19 (86.77±8.31) a	15 (78.05±6.09) a	12 (82.14±8.98) a

Data are presented as number (percentage ± SEM). [ab] Different letters within each column indicate significant difference ($p < 0.05$).

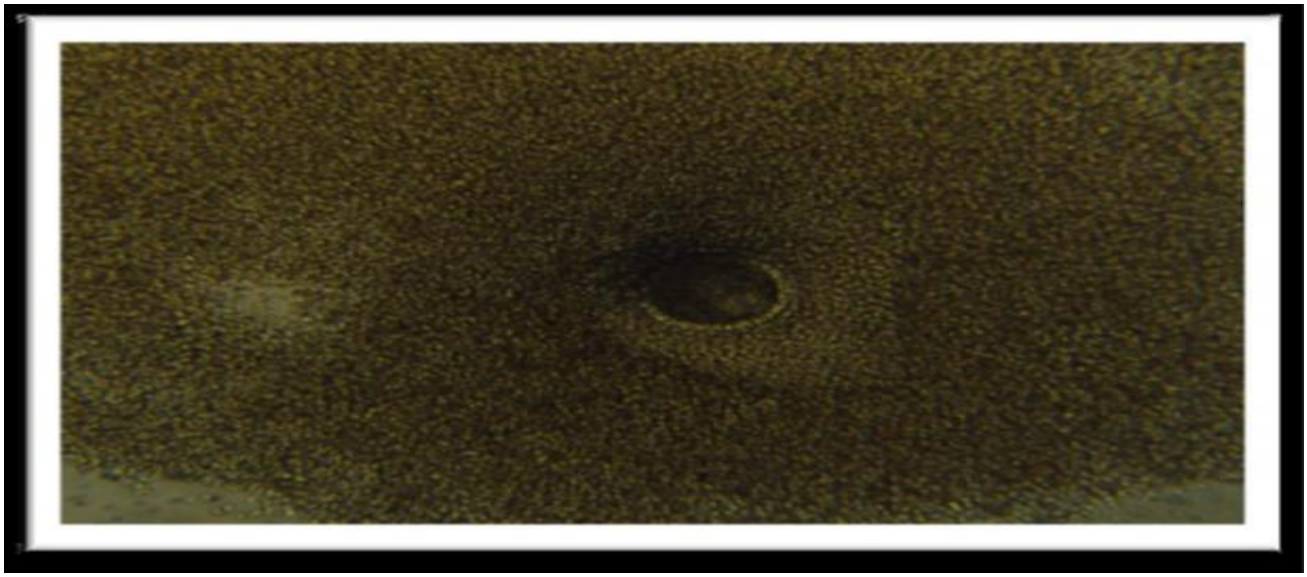

Figure 1. Matured Oocyte with cumulus cells expansions 10X

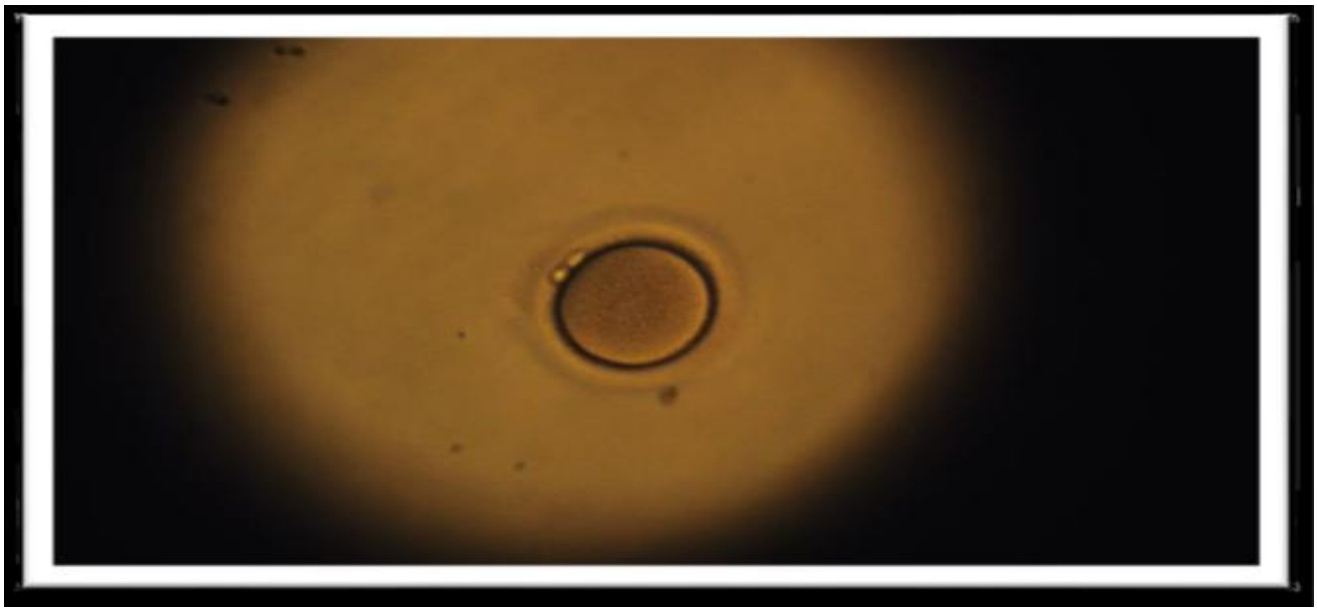

Figure 2. Fertilization Oocyte with two polar body - normal fertilization (10X)

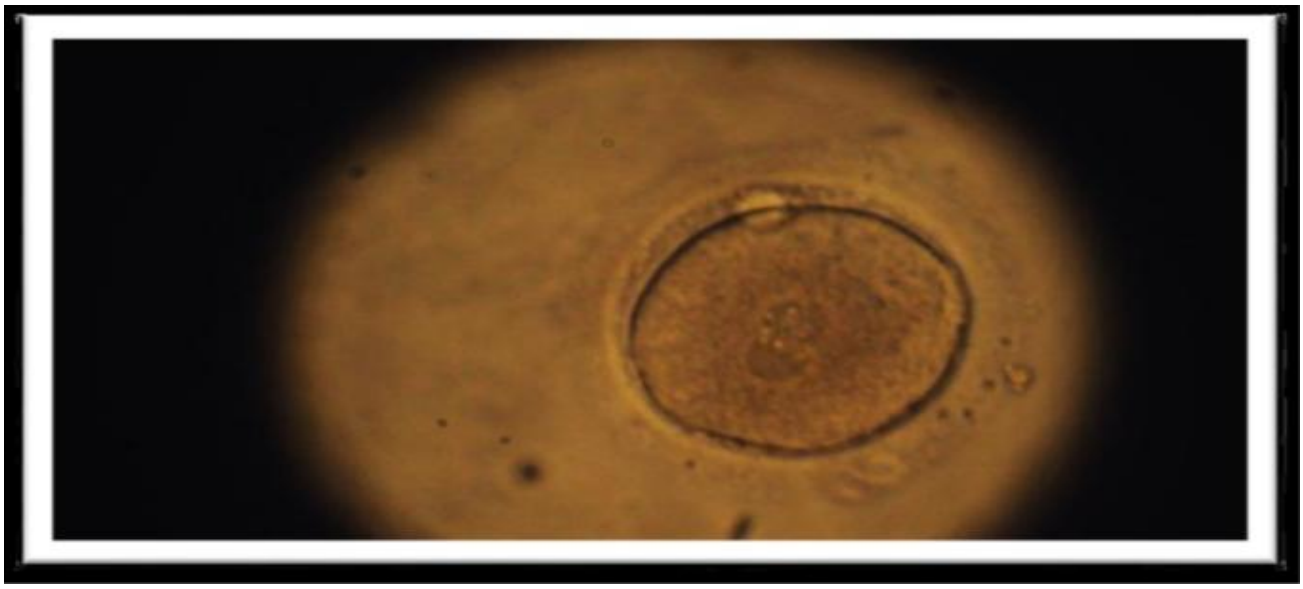

Figure 3. Fertilization Oocyte with two pronuclear - normal fertilization (10X)

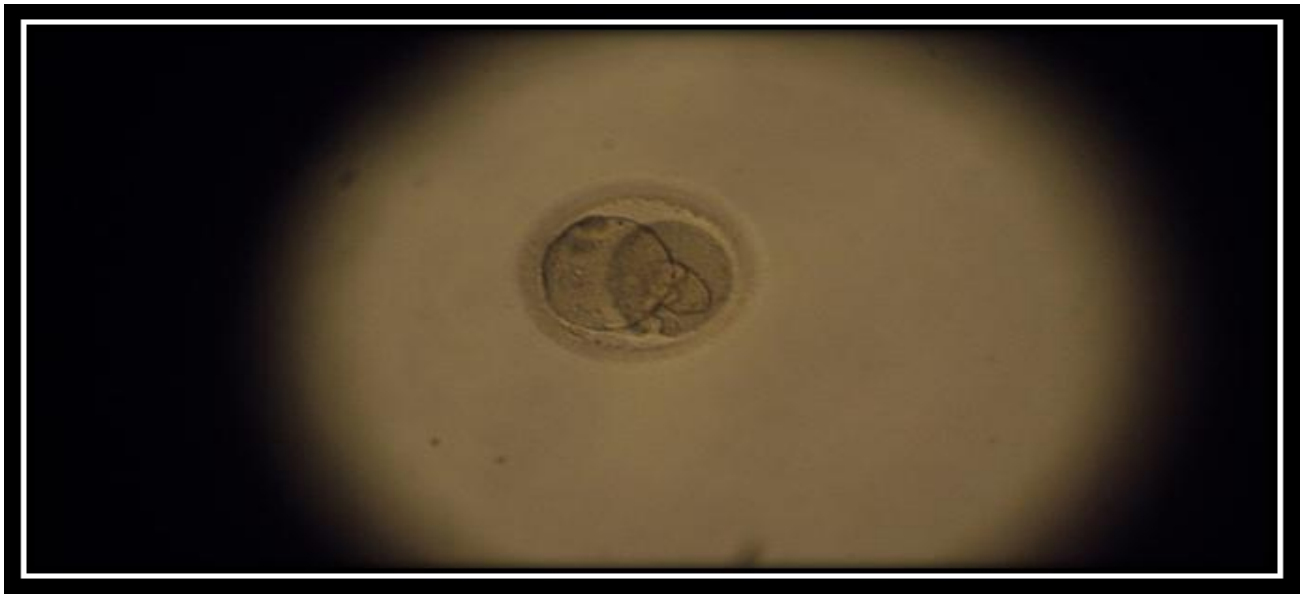

Figure 4. 2-cells embryo resulted from IVF in buffalo (10X)

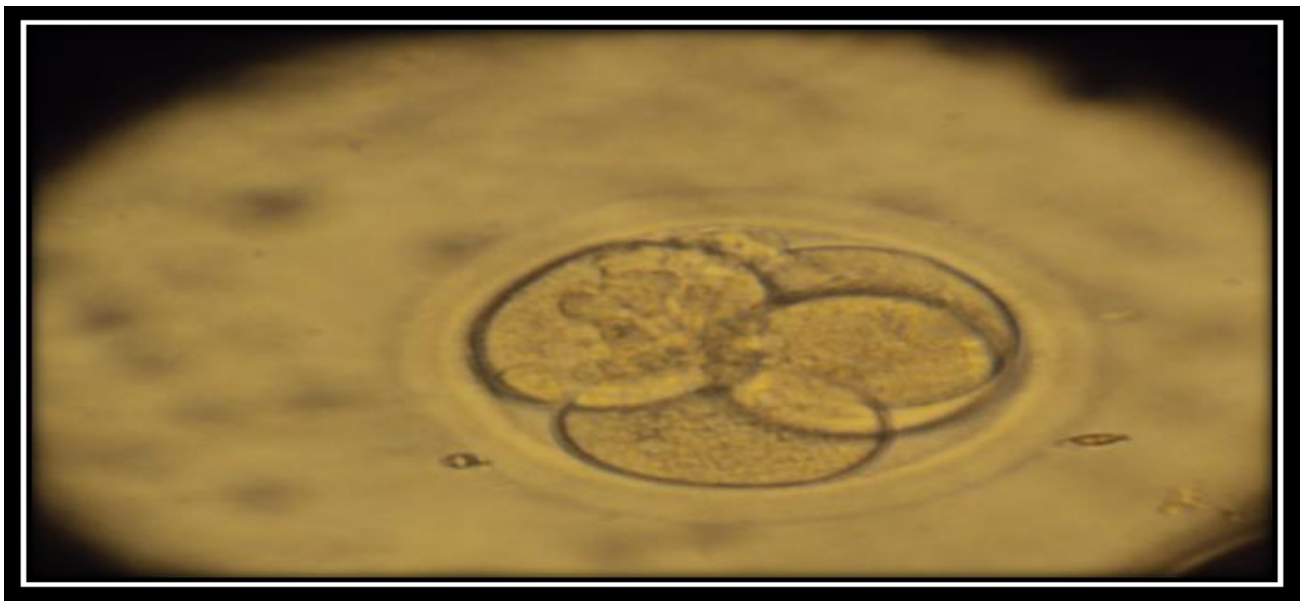

Figure 5. 4-cells embryo resulted from IVF in buffalo (10X)

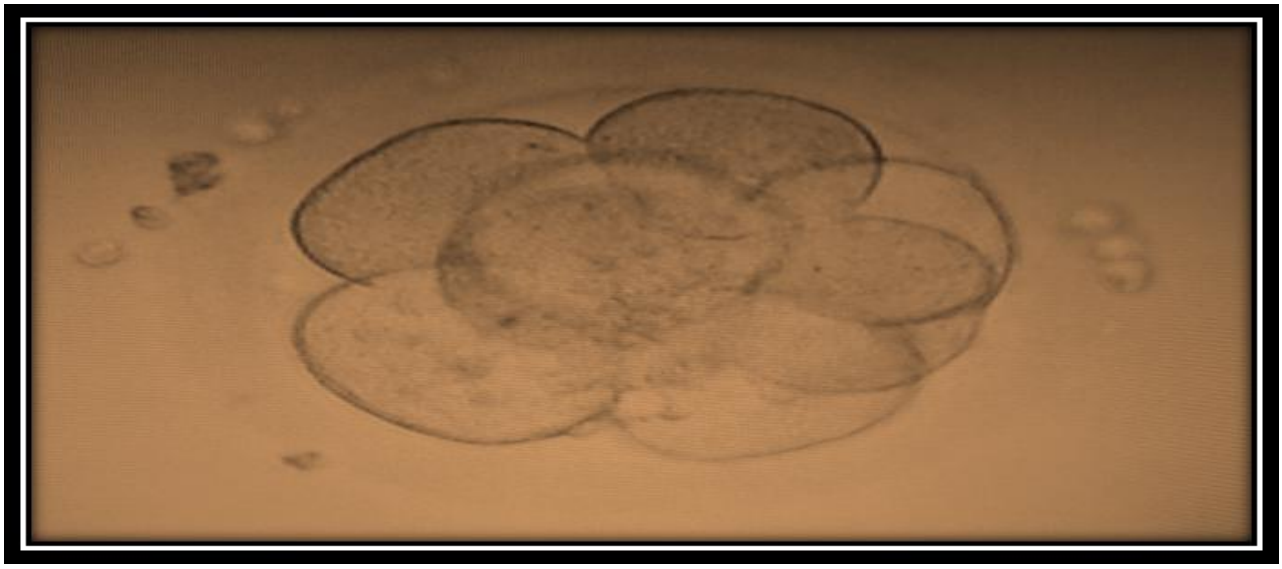

Figure 6. 8-cells embryo resulted from IVF in buffalo (10X)

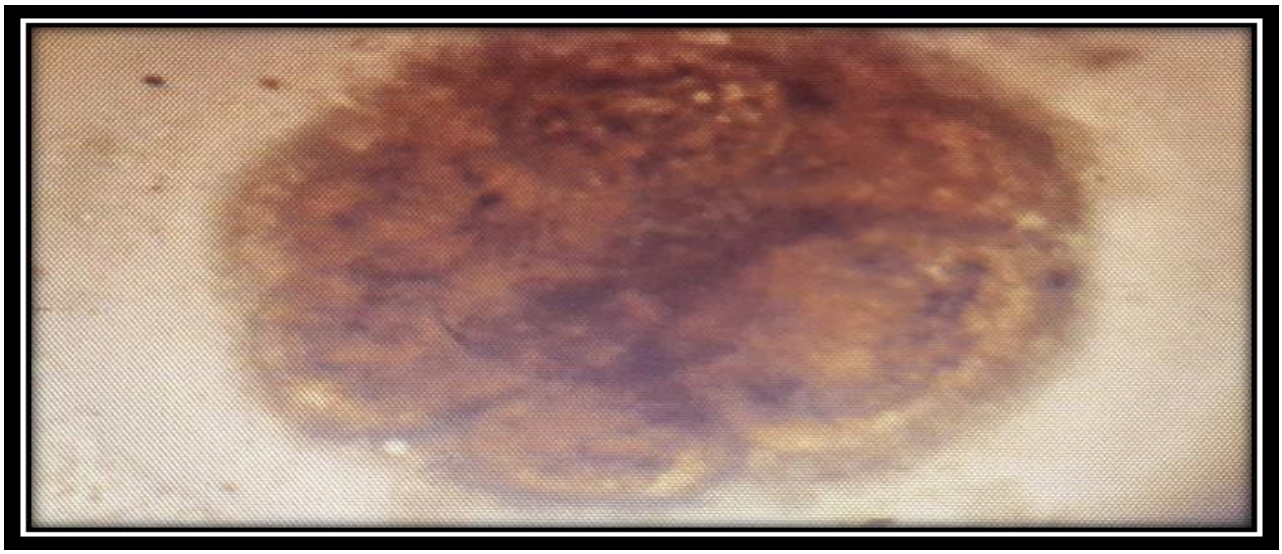

Figure 7. 16-cell embryo resulted from IVF in buffalo (10X)

Discussion

The current study showed that the maturation and fertilization rate of COCs with a buFF supplemented medium and improve the developmental rate of buffalo embryo by providing a positive action on the development of buffalo embryos from 2-cell to 16-cell. There is an important need to use the follicular fluid in the culture medium which contains various growth factors, hormones such as FSH, LH, moreover, it contains several nutrients and also rich with hyaluronic acid [13] that help in the process of development of embryos in buffalo IVF system. The study revealed that 20% of buffalo follicular fluid had a positive effect on oocytes maturation, fertilization, and subsequent embryo development in the treated group compared with the control group. This result is in agreement with previous studies which studied the effected of follicular fluid supplementation with IVM medium in other species.

In pigs, the use of a follicular fluid with a maturation medium enhanced oocytes maturation levels and promoted the formation rate of female and male nuclei [28]. The previous report have compared the effect of estrous cow serum (ECS) and follicular fluid in bovine IVF, it showed that presence of follicular fluid in IVM-IVF culture medium enhances the oocytes maturation rate, fertilization rate and improving the morula and blastocysts rate [29][30] Suggested the beneficial effect of follicular fluid on the maturation of buffalo oocyte due to the existence of insulin-like growth factor, growth factors, hormones (FSH, LH, and estradiol) and intra-ovarian peptides in more physiological properties in follicular fluid. On the other hand [31, 32] Suggested that the buffalo follicular fluid in culture medium enhances cytoplasmic and nuclear maturation. [33] Revealed that the fluid from normal follicle contains a balanced proportion of estradiol/progesterone (EPR) and insulin-like growth factor binding proteins (IGFBPs) enhance oocytes maturation and fertilization rates.

Another report mentioned the positive effect of using buffalo follicular fluid with IVF medium which promotes sperm penetration as well as improves cumulus cell expansion and enhancing ATP levels in oocytes [34,35] Fixed that the follicular fluid contains (electrolytes, hormones, amino acids, growth factors, and other components) which used as a natural material for blocking the meiosis [36]. Another study has documented that maturation medium supplemented with follicular fluid supplied an appropriate environment for oocyte development [37]. It also increases the degree of cumulus cell expansion and enhances embryonic development [38]. The addition of follicular fluid to the maturation medium had beneficial effects for the maturation and development ability of bovine oocytes [39]. [40] Suggested

that buffalo follicular fluid catalyze the synthesis protein cell cycle in embryos which in turn supported their development, the presence of enzymes like lactate dehydrogenase, ATPase, transaminase, and alkaline phosphatase, glycosaminoglycan's, proteins and steroids, gonadotropins antioxidants, might have promoted the maturation oocytes, cleavage rates and development embryo [41,42].

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