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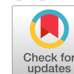

Development, Quality, and Production Parameters of *In Vitro* Embryo in Anatolian Water Buffaloes

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ABSTRACT

The aim of the study was to determine *in vitro* embryo development, embryo quality, and *in vitro* embryo production (IVEP) parameters by using Anatolian water buffaloes (AWB) oocytes. In this study, 184 ovaries of 92 AWB obtained from slaughterhouses were used. The tissue culture medium (TCM-199) was used for *in vitro* maturation (IVM), Brackett & Oliphant (BO) medium for *in vitro* fertilization (IVF), and Charles Rosencrans 1 amino acid (CR1aa) medium for *in vitro* culture (IVC). A total of 395 oocytes (2.15 per ovary) were obtained from the ovaries. Frozen AWB sperm was used for fertilization. The number of cleavages at the 24th hour was 93 out of 302 (30.79%), the number of morulae and compact morula at the 96th hour was 53 out of 302 (17.55%), the number of blastocysts at 7th day was 29 out of 302 (9.60%), and the number of hatched blastocysts were 12 out of 302 (3.97%) on the 8th and 9th days. In the quality assessment of 29 blastocysts recovered on day 7 in IVC, 7 of them (24.13%) were of the code 1 quality, 9 of them (31.03%) were of the code 2 quality, 8 of them (27.50%) were of the code 3 quality, and 5 of them (17.24%) were classified as the code 4 quality. This study provides the first data on *in vitro* embryo development, embryo quality classification, and embryo production in AWB. As a result, the potential of oocytes AWB for IVEP has been revealed, and a scientific background has been provided for future studies.

Keywords: Anatolian Water buffaloes; development; embryo; fertilization; oocyte

INTRODUCTION

The buffalo (*Bubalus bubalis*) is zoologically classified in the *Bubalina* group of the ruminant family. Through the archeological and agro-historical findings, it is known that buffaloes were brought to Anatolia and the Middle East in the first century AD (after Christ) by Arab traders from the Indus region of India under the name "Tarin" (Minervino *et al.*, 2020; Zhang *et al.*, 2020). Today, Turkey has a local breed-specific to the region called AWB (Özşensoy, 2020). These buffaloes are different from the Mediterranean buffaloes (MB), a river buffalo subgroup, with some differences in size and genetics (Özkan Ünal *et al.*, 2014; Turan *et al.*, 2021).

Embryo transfer (ET) technologies, one of the main assisted reproductive techniques of reproductive biotechnology, are widely used in cattle breeding as a valid technique for rapid reproduction of genetic superiority and can also be applied to buffalo species (Baruselli *et al.*, 2020; Duran *et al.*, 2017; Kumar *et al.*, 2020). With the development and widespread use of ET technologies, these technologies assist acceleration of genetic progress in buffalo breeding (Warriach *et al.*, 2015). Many researchers have emphasized the need to develop techniques for *in vitro* embryo production (IVEP) and cryopreservation in buffalo (Baruselli *et al.*, 2018; Gasparrini, 2002; Hasbi *et al.*, 2022; Nandi *et al.*, 2002; Palta & Chauhan, 1998; Singh *et al.*, 2015; Warriach *et al.*, 2015).

Media used for *in vitro* maturation (IVM) of buffalo oocytes are usually basic culture systems such as media prepared by adding additional pyruvate, lactate, and glucose to bicarbonate-buffered physiological salts, or complex media containing essential elements as well as amino acids, vitamins, and some other substances (Gasparrini, 2002; Hasler & Barfielt, 2021). After the IVM process, *in vitro* fertilization (IVF) is performed. Moreover, fresh semen, as well as frozen and thawed semen, can be used in the IVF process (Duran *et al.*, 2017; Rubessa *et al.*, 2019). Numerous methods are available for preparing sperm for IVF (Parrish, 2014; Romar *et al.*, 2016; Saini *et al.*, 2020; Volpes *et al.*, 2016). One of the most common mediums used for the processing of semen is the solution of Brackett & Oliphant (BO) (Akter *et al.*, 2023; Smetanina *et al.*, 2019). The BO washing medium base method has been reported to give better results in buffalo than fertilization by swim-up, Percoll, and TALP methods

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(Kumar *et al.,* 2020). The medium in which the single-celled zygote formed after the IVM and fertilized oocyte is maintained until the morula-blastocyst stage is called *in vitro* culture (IVC) (Duran *et al.,* 2017; Zhang *et al.,* 2020). The embryological developmental stage in buffalo varies depending on the day it is in culture, as in all mammals (Gasparrini *et al*., 2014). After days 6 and 7 of embryo culture in buffalo, blastocyst, expanded (extended) blastocyst, and hatched blastocyst can be obtained (Baruselli *et al.,* 2020; Kumar *et al.,* 2020).

Mammalian embryos are evaluated based on their morphology, shape, color, cytoplasmic density, homogeneity of cells, and determination of degenerative areas (Baruselli *et al.,* 2018; Hasbi *et al.,* 2022). In addition, it has been reported that the hatching blastocyst stage is a good indicator of embryo quality (Gasparrini, 2002; Kumar *et al.,* 2020). Embryo quality grades from 1 to 4 by the International Embryo Technology Society (IETS). For code 1 (1st quality), embryos are classified as excellent or good, transferable after freezing and thawing, and suitable for international trade. Code 2 (2nd quality) embryos are considered moderate quality and reasonable but with low post-freeze-thawing viability and are not commercially recommended. However, code 2 embryos can usually be transferred fresh. Embryos in code 3 (3rd quality) have a weak structure and poor quality, a low survival rate when frozen, and a low pregnancy rate when transferred fresh. Also, embryos in code 3 are not accepted as appropriate for trade. Embryos in code 4 (4th quality) are dead or degenerated embryos and UFO (unfertilized oocytes), and these embryos cannot be used (Duran *et al.,* 2017; Hasbi *et al.,* 2022).

This study aims to pioneer *in vitro* fertilization and embryo production studies on AWB. With this study, some *in vitro* embryo development parameters, information about the number of oocytes obtained per ovary, quality classification rates of oocytes, maturation rate of oocytes, fertilization rate of oocytes, blastocyst accession rates, and hatched blastocyst accession rates were determined for the first time in AWB. By revealing the AWB *in vitro* embryo production potential, infrastructure was explained for future studies.

MATERIALS AND METHODS

For this study, Ethics Committee Approval was granted by the decision dated 2785-29.11.2016, number 131, of the Ministry of Agriculture and Forestry, International Center for Livestock Research and Training, Local Ethics Committee for Animal Experimentation. Unless specifically noted, chemicals used are Sigma-Aldrich mediums and chemicals, 3050 Spruce Street, St. Louis, MO 63103, USA origin. Also, Gibco mediums and chemicals originate at 3175 Staley Rd., Grand Island, NY 14072, USA. Other substances and drugs were specifically mentioned in the section where they were used.

Material Supply

The AWB ovaries obtained from the slaughterhouses (Turkey, Aegean, and Central Anatolia regions) were used for the study, regardless of age, cycle times, yield characteristics, regional conditions, seasonal differences, and heat stress. In the study, 184 ovaries of 92 AWB slaughtered at different times in the abattoir were separated from surrounding viscera and tissues as soon as possible after slaughtering and treated with 100 mg/L kanamycin sulfate (Vetaş-KANOVET®, Istanbul, Turkey) at 15-21 °C. The ovaries were transferred within 3-4 hours in 0.9% saline to the IVF laboratory (Palta & Chauhan, 1998).

Oocyte Aspirations

The fatty and foreign tissues on the ovaries were removed and washed at least twice with 0.9% saline. Follicles 2-8 mm in diameter on the ovary were aspirated using 5-10 mL injectors with an 18-G needle attached. The Cumulus Oocyte Complex (COC) was aspirated into the injector with the fluid inside, without exploding the follicle, by entering the ovarian tissue 1-2 mm away from the follicles with the injector. Then, COCs were transferred into 90 mm diameter Petri dishes after being taken into conical bottom falcon tubes (Kaymaz, 2015; Palta & Chauhan, 1998). The Petri dishes were scanned by a stereomicroscope (Olympus SZH10), and the COCs were collected in a 35-mm Petri dish containing Dulbecco's Phosphate Buffer Solution (D-PBS). The oocytes, whose quality was graded according to the letter system, were classified as A (good quality), B (Middle quality), C (poor quality), and D (degenerate) (Totey *et al.,* 1992). This study determined the total number of oocytes and oocytes of all grades; the number and rate of mature oocytes and oocytes obtained for fertilization; the number of fertilized oocytes, and the rate of oocytes.

In Vitro Maturation

For maturation, M-199 (Gibco, Medium 199, Lot-1894670) containing 10% fetal calf serum (FCS), 5.5 mg/mL Na pyruvate, 0.02 IU/mL FSH, 10 ng/mL EGF, and 50 µg/mL penicillin-streptomycin (Gibco, Pen-Strep 15140) was used. Using the prepared medium, 100 µL drops that will contain 20 oocytes (5 µL per oocyte) were prepared. These drops were covered by oil. Then, the drops were incubated in a CO_2 incubator (Panasonic MCO-170M-PE) at 38.5 °C, 5% CO_2, and 98% relative humidity for 22-24 hours. Oocytes with cumulus expansion were considered as mature. In addition, oocytes with nuclear maturation were considered as mature with the detection of the first polar body, which is another maturation criterion (Palta & Chauhan, 1998).

In Vitro Fertilization

The BO medium based on the washing method has been used for the fertilization of oocytes. The solutions were prepared by the medium of BO for the fertilization of oocytes (Parrish, 2014). AWB bull semen prepared in 0.25 mL straws ($120X10^6$ /1 mL dose) was used for the study. For the removal, reconstitution and capacitation of cryoprotectants from frozen/thawed sperm, a modified BO solution was used. For this pur-

pose, Oocyte Washing Solution (OWS), Sperm Dilution Solution (SDS), and Sperm Washing Solution (SWS) were prepared from the solution. For the capacitation of spermatozoa, 5 IU heparin and 2 µM caffeine per mL were used in SWS. To achieve capacitation and adequate sperm density, 6 mL of SWS was added to the 0.25 mL straws (4 straws), which were dissolved in a 37 °C water bath and centrifuged at 1800 rpm for 5 minutes. After the separation of the supernatant, the same procedures were repeated again. Then, 100 µL drops containing $6X10^6$ spermatozoa in 1 mL were formed (SWS-OWS-SDS) by counting the spermatozoa on the Thoma strip. Petri dishes were kept equilibrated in a CO_2 incubator (Panasonic MCO-170M- PE) for 2 hr. 25.000-30.000 spermatozoa per oocyte in droplets, oocyte and spermatozoa are placed in droplets in 35 mm Petri dishes in a CO_2 incubator for fertilization for 16-18 hours; It was stored at 38.5 °C, 5% CO_2, and 98% relative humidity.

In Vitro Culture

Charles Rosencrans 1 amino acid (CR1aa) culture medium was utilized to provide oocyte development for 8-9 days after fertilization (Palta & Chauhan, 1998). After the fertilization step, the cumulus cells and remaining sperm cells were removed as quickly as possible by pipetting. Before the IVC, the potential zygotes were washed at least five times in different locations in a 35-mm Petri dish containing 2 mL of CR1aa culture medium. For the culture process, droplets containing 100 µL of CR1aa covered with oil were prepared in 35-mm Petri dishes. The prepared petri dishes were kept at equilibrium in a CO_2 incubator with 5% CO_2 and 98% relative humidity at 38.5 °C for at least 2 hours before culture. After that, embryo culture was performed with 20 potential embryos per droplet at 38.5 °C, 5%, and 98% relative humidity for ten days. In the culture, the 24th-48th hour cleavage number and rate; 96th hour (4th day) morula-compact morula numbers and rates; 7th-day blastocyst numbers and the rates and the number of hatched blastocysts on the 8th-10th day were determined. Embryo quality is graded using numerical codes from 1 to 4 established by the International Embryo Technology Society.

Evaluation of Results

Data obtained in the study were calculated using descriptive statistics. For this purpose, the arithmetic means of all data obtained from 6 different applications were used. The results of the various replications were calculated as percentage (%) frequencies. Minitab 19 software programs were used for statistical analyses.

RESULTS

In the study, 395 oocytes were obtained from 184 ovaries of AWB (n= 92) from the slaughterhouse. On average, 4.29 and 2.15 oocytes were collected per animal and ovary, respectively. In total, 334 oocytes were evaluated as A+B grade. However, 61 oocytes were C-grade and degenerated (Figure 1.1). Overall, the rate of A-B oocytes was 84.56%, and the rate of C and degenerated oocytes was 15.44%. It was found that the number of A-B oocytes was 1.81, while the number of C and degenerated oocytes was 0.33 on average per ovary (Table 1).

A total of 334 A-B grade oocytes were used for IVM. It was found that 329 of the 334 oocytes were mature by considering the cumulus expansions and cell morphology (Figure 1.2). The maturation rate was 98.51%. It was found that 5 of the oocytes were degenerated. The percentage of oocytes without maturation was 1.49% (Table 2).

After 24 hours of incubation, 329 mature oocytes were included in the fertilization process. After a fertilization period of about 16-18 hours, polar body existence in some oocytes (Figure 1.4) was detected. During the process, it was found that 5 out of 329 oocytes were not fertilized, and 21 of them degenerated. The fertilization process of 303 oocytes was completed and used in the IVC process (Table 3).

Figure 1.3 shows an image of one of the fertilization droplets. The fertilization process was accepted as successful in 93 oocytes with two or more cleavages by the microscopic examinations performed at the 24th hour. The cleavage rate of oocytes in the CR1aa culture in the first 24th hour was 30.79%. Also, the number and rate of oocytes without a cleavage were 209 out of 302 and 69.20%, respectively. Embryos detected at different times within the first 24 hours of the IVC period were provided (Figure 1.5 and Figure 1.6). In the microscopic examinations at the 96th hour (day 4) of IVC, the number of morulae was 53, while the ratio to the number of oocytes in culture was 17.55%. The number of blastocysts was 29. In addition, the rate of blastocysts was 9.60% on the 7th day of IVC. In the study, the number and the rate of oocytes that could not reach the blastocyst stage were 64 out of 93 and 68.82%, respectively. The number of hatching and hatched blastocysts was 12, and the rate was 3.97% in the microscopic examination performed on the 8th and 10th days after the embryos were cultured. As a result of the calculation based on the number of embryos that had cleavage, the rate of hatching and hatched blastocyst was 12.9% (Table 4).

The images of embryos at 8 and 10 days of the culture period are provided in Figure 1.6. The total number of blastocysts was 29 in the microscopic examinations on day 7. In addition, 7 out of 29 (24.13%) were in code 1 (excellent or good quality), 9 out of 29 (31.03%) were in code 2 (moderate quality), 8 of them (27.50%) were in code 3 (poor quality), while 5 out of 29 (17.24%) were in code 4 (degenerated embryos and UFO). The number of the obtained embryos with transferable quality (number of code 1 and code 2 quality embryos) was 16, and the rate of transferable embryos was 55.17%. Also, the number of embryos with code 3 and code 4 (average, poor quality, degenerated embryos and UFO) was 13, and the non-transferable embryo rate was 44.74% (Table 5). In the study, the number of embryos from blastocysts to hatched blastocysts was 12 out of 29 at 41.37%. The number of transferable embryos reaching the hatched blastocysts was 12 out of 16 at 75%.

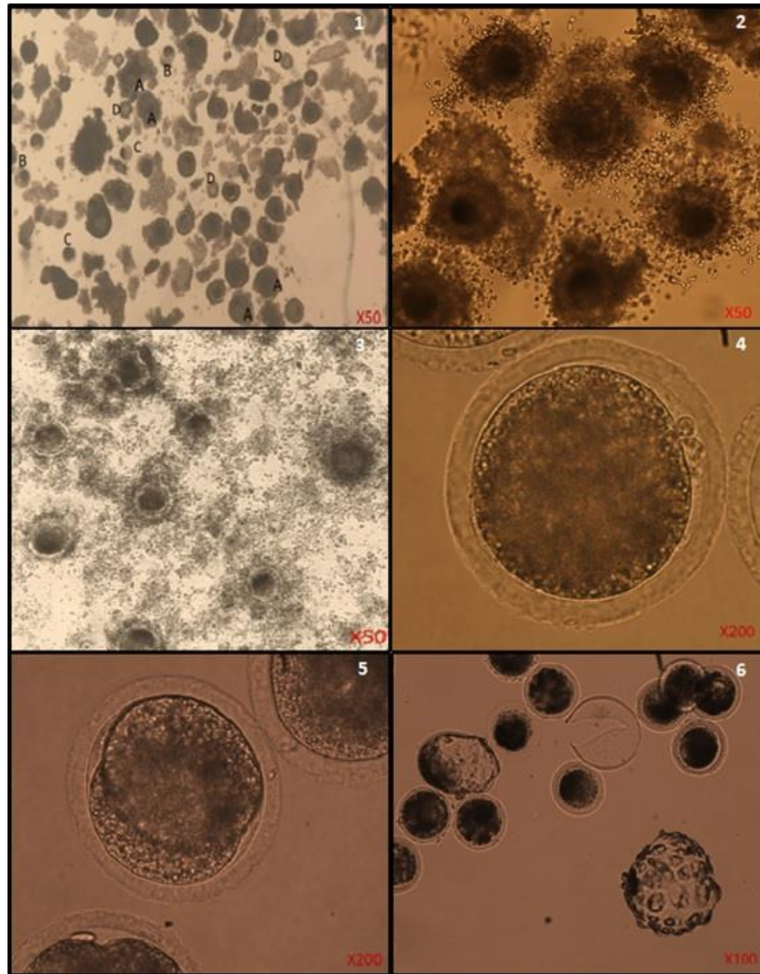

Figure 1. Anatolian water buffaloes (AWB) oocytes. (1) Oocytes quality classification, A-B excellent and good, C mediocre and poor, D “degenerate” quality oocytes (Stereomicroscope, Olympus SZH10), (2) Oocytes *in vitro* maturation, (3) Spermatozoa and oocytes in fertilization drops, (4) A fertilized oocyte and bipolar body, (5) Embryo detected in the 24th hour cleavage process *in vitro* culture, (6) 8th-10th *in vitro* culture day hatching and hatched blastocyst image (Nikon Eclipse TE300).

Table 1. The number of the Anatolian water buffaloes and other buffaloes breeds oocytes

Breeds	Data	Total (n)	Oocyte / Per Ovary	Oocyte (%)	References
AWB	Number of ovaries	184	-	-	The present study
	Total oocytes	395	2.15	-	
	A and B grade	334	1.81	84.86	
	C and D grade	61	0.33	15.44	
MIB*	Number of ovaries	1257	-	-	Gasparrini *et al.*, 2002; Di Francesco *et al.*, 2012; Gasparrini *et al.*, 2014
	Total oocytes	3771	3.0	-	
	A and B grade	2640	2.1	70	
	C and D grade	1131	0.9	30	
MB*	Number of ovaries	250	-	-	Ruhil & Purohit, 2016; Gasparrini *et al.*, 2002; Nandi *et al.*, 2002
	Total oocytes	915	3.66	-	
	A and B grade	378	1.51	41.3	
	C and D grade	537	2.14	58.7	
IB*	Number of ovaries	12263	-	-	Totey *et al.*, 1992; Madan *et al.*, 1994; Kumar *et al.*, 2020; Puri *et al.*, 2015
	Total oocytes	8995	0.73	-	
	A and B grade	5323	0.43	59	
	C and D grade	3672	0.31	41	

Note: *Data regarding buffaloes breeds are also given in other tables. AWB= Anatolian water buffaloes; MIB= Mediterranean Italian buffaloes; MB= Murrah buffaloes; IB= Indian buffaloes. A= excellent, B=good, C=poor and D= degenerate.

Table 2. *In vitro* maturation in Anatolian water buffaloes and other breeds of oocytes

Breeds	Total oocyte	Mature oocyte	Mature oocyte (%)	Non mature	Non mature (%)	References
AWB	334	329	98.51	5	1.49	0
MIB	750	696	92.8	54	7.2	1, 2, 3
MB	665	601	90.4	64	9.6	1, 4, 5
IB	136	117	86	19	14	6, 7, 8, 9

Note: AWB= Anatolian water buffaloes; MIB= Mediterranean Italian buffaloes; MB= Murrah buffaloes; IB= Indian buffaloes. References, 0= The Present study; 1= Gasparrini *et al*., 2002; 2= Di Francesco *et al*., 2012; 3= Gasparrini *et al*., 2014; 4= Ruhil & Purohit, 2016; 5= Nandi *et al*., 2002; 6= Totey *et al*., 1992; 7= Madan *et al*., 1994; 8= Kumar *et al*., 2020; 9= Puri *et al*., 2015.

Table 3. *In vitro* fertilization findings of oocytes in Anatolian water buffaloes and other buffaloes breeds

Breeds	Total oocyte fertilized	Fertilization completed	Non fertilization	Degenerated in fertilization	Referances
AWB	329	302 (91.79%)	5 (1.51%)	21 (6.38%)	0
MIB	277	261 (94.22%)	6 (2.16%)	10 (3.61%)	1, 2, 3
MB	490	265 (54.1%)	ND*	ND*	1, 4, 5
IB	136	128 (94.11%)	8 (5.89%)	8 (5.89%)	6, 7, 8, 9

Note: *ND= No Data. AWB= Anatolian water buffaloes; MIB= Mediterranean Italian buffaloes; MB= Murrah buffaloes; IB= Indian buffaloes. References, 0= The Present study; 1= Gasparrini *et al*., 2002; 2= Di Francesco *et al*., 2012; 3= Gasparrini *et al*., 2014; 4= Ruhil & Purohit, 2016; 5= Nandi *et al*., 2002; 6= Totey *et al*., 1992; 7= Madan *et al*., 1994; 8= Kumar *et al*., 2020; 9= Puri *et al*., 2015.

Table 4. The *in vitro* culture results of Anatolian water buffaloes and other buffaloes breeds

Breeds	Total oocyte	Cleavage 24th-48th h.	Morula 4th day	Blastocyst 7th day	Degenerate unfertilized	Hatched blastocyst	References
AWB	302	93 (30.79%)	53 (17.55%)	29 (9.60%)	209 (69.20%)	12 (3.97%)	0
MIB	277	146 (52.9%)	ND*	42 (14.9%)	131 (47.10%)	12 (4.33%)	1, 2, 3
MB	601	236 (39.3%)	85 (14.14%)	31 (5.15%)	365 (60.73%)	4 (0.57%)	1, 4, 5
IB	536	63 (11.75%)	26 (4.85%)	14 (2.61%)	473 (88.24%)	78 (14.63%)	6, 7, 8, 9

Note: *ND= No Data. AWB= Anatolian water buffaloes; MIB= Mediterranean Italian buffaloes; MB= Murrah buffaloes; IB= Indian buffaloes. References, 0= The Present study; 1= Gasparrini *et al*., 2002; 2= Di Francesco *et al*., 2012; 3= Gasparrini *et al*., 2014; 4= Ruhil & Purohit, 2016; 5= Nandi *et al*., 2002; 6= Totey *et al*., 1992; 7= Madan *et al*., 1994; 8= Kumar *et al*., 2020; 9= Puri *et al*., 2015.

Table 5. The quality of embryos at day 7th of *in vitro* culture of Anatolian water buffaloes and other buffaloes breeds

Breeds	Total embryos	Transferable embryo	Excellent or good quality (Code 1)	Fair quality (Code 2)	Poor quality (Code 3)	Dead or degenerating (Code 4)	Hatched blastocyte	Ref.
AWB	29	16 (55.17%)	7 (24.13%)	9 (31.03%)	8 (27.58%)	5 (17.24%)	12 (41.37%)	0
MIB	41	31 (75.60%)	31 (75.60%)	ND*	10 (24.40)	ND*	ND*	1, 2, 3
MB	100**	44 (44%)	44 (44%)	ND*	34 (34%)	22 (22%)	14 (14%)	1, 4, 5
IB	48	14 (28.16%)	13 (27.08%)	1 (2.08)	ND*	9 (18.75%)	4 (28.57%)	6, 7, 8, 9

Note: *ND= No Data. **The number is given as an example. Ref. = References; AWB= Anatolian water buffaloes; MIB= Mediterranean Italian buffaloes; MB= Murrah buffaloes; IB= Indian buffaloes. References, 0= The Present study; 1= Gasparrini *et al*., 2002; 2= Di Francesco *et al*., 2012; 3= Gasparrini *et al*., 2014; 4= Ruhil & Purohit, 2016; 5= Nandi *et al*., 2002; 6= Totey *et al*., 1992; 7= Madan *et al*., 1994; 8= Kumar *et al*., 2020; 9= Puri *et al*., 2015.

DISCUSSION

In the current study, IVF and IVEP data of AWB were investigated. Since there is no IVF and embryo production data in AWB in another study, data in this study were discussed with the data of other buffalo breeds. Oocyte aspiration data from the ovary of the present study was 2.15 (Table 1), which was lower than those reported an average of 3.0 oocytes per ovary in Mediterranean Italian buffaloes (MIB) (Gasparrini, 2002; Di Francesco *et al.,* 2012), and 3.66 oocytes per ovary in Murrah buffaloes (MB) (Ruhil & Purohit, 2016). It is thought that this high average may be due to the buffalo breed used in the study. The data in the present study (Table 1) showed that the number of quality oocytes (1.81) is higher than those reported by Totey *et al.* (1992) and Madan *et al.* (1994) in which the number of quality oocytes per ovary was found 0.45 and 0.47, respectively, in MB. Moreover, the number of quality oocytes in the present study is greater than that Puri *et al.* (2015) reported, in which an average of 1.15 quality oocytes per ovary was found in Indian buffaloes (IB). The quality of oocyte data in the present study (Figure 1.1) was similar to the average rate of 1.76 quality oocytes per ovary obtained in a study in Nili-Ravi buffaloes (Gasparrini, 2002). Some researchers reported that the rate of quality oocytes in buffaloes is generally 1.5 per ovary (Kumar *et al.,* 2020; Nandi *et al.,* 2002) (Table 1). It is thought

that the low oocyte number in the AWB may be due to the genetically low primordial follicle reserve in those buffaloes, the low number of antral follicles during the estrus cycle in buffaloes, and the high incidence of follicular atresia (Yılmaz *et al.,* 2014).

In this study, high maturation rates (98.51%) were obtained (Table 2). These results were similar to the 92.8% maturation rate in MIB (Gasparrini *et al.,* 2014). The data of this study were higher than 88 % maturation rate in Indian Kundi buffaloes (Puri *et al.,* 2015), 81.7 % in buffaloes from the New Delhi region (Totey *et al.,* 1992), 85% in MB (Nandi *et al.,* 2002), and 76.8% in MB (Madan *et al.,* 1994). The high rate of IVM in this study may be due to the use of the protocols literature data in the trials, with the best results according to the media used, maturation times, the standard in the maturation evaluation criteria, and breed characteristics.

Many researchers have reported that the cleavage rate in the first 24 hours can be considered as the fertilization rate (Beck-Fruchter *et al.,* 2014; Kumar *et al.,* 2020; Saini *et al.,* 2020) in scientific reports. It was also reported that the IVF rate is lower in buffaloes (45%-50%) compared to cattle (70%) (Sales *et al.,* 2015) (Table 3). At the end of 24 hours, two or more cleavages were observed in 93 (30.79%) of 302 fertilized oocytes cultured in this study. The cleavage rate at 24 hours found in the present study (30.79%) is lower than those reported by Sales *et al.* (2015) (45%-50%); Madan *et al.* (1994) (40%); Chauhan *et al.* (1997) (51%-56%); Di Francesco *et al.* (2012) (52.7%); Gasparrini *et al.* (2014) (52.7%-53.5%), and Konrad *et al.* (2017) (64%) (Table 4). In this study, the cleavage rate at the 24th hour (30.79%) was similar to those reported by Totey *et al.* (1992) (29.8%), Baruselli *et al.* (2018) (28.9%), and Nandi *et al.* (2002) (27%). In some studies, it is thought that different methods for obtaining the oocytes, the variety in buffaloes breeds, and differences in fertilization methods may lead to high cleavage rates (Table 4).

One of the critical periods after the first cleavage in embryo culture is the 96th hour. Morula, compact morula, and early blastocyst rates are vital production stages in this period (Konrad *et al.,* 2017; Totey *et al.,* 1992). Many researchers studying human IVF technology recommend transferring embryos at 72 or 120 hours. They reported that reducing *in vitro* residence time of these embryos increases their success (Petersen *et al.,* 2016; Yadid *et al.,* 2022). In this study, the 96th-hour data of IVC was 17.55% (Table 4). The rate of 17% was also reported in the study in MB (Konrad *et al.,* 2017), and for IB was close to 17% (Palta & Chauhan, 1998). However, the results found in the present experiment (17.55%) (Table 4) were lower than the 22% reported by Totey *et al.* (1992). It is thought that the differences in these data are the data in the studies carried out to test different media and chemicals. It can be claimed that the differences in those data also affect the use of various oocyte retrieval techniques.

In this study, the cleavage rate at the 24th hour (30.79%) was similar to those reported by Totey *et al.* (1992) (29.8%), Baruselli *et al.* (2018) (28.9%), and Nandi *et al.* (2002) (27%) (Table 4). In some studies, it is thought that different methods for obtaining the oocytes, the variety in buffaloes breeds, and differences in fertilization methods may lead to high cleavage rates.

In this study, on the 7th day of IVC, the number of blastocysts was 29 out of 302, and the rate was 9.60% (Table 4). This rate was higher than the blastocysts rate (8.1%) performed in MB, as well as in MB with a 6.7% blastocysts (Madan *et al.,* 1994). The blastocyst rate obtained in this study is similar to (9%) obtained from IB breeds (Palta & Chauhan, 1998), in MB (9.57%) (Shang *et al.,* 2007), and to MB (11.5%) (Gasparrini *et al.,* 2014). The 7th-day blastocyst rate of this study (9.6%) (Table 4) was lower than the 7th-day blastocyst rate (12.73%) reported in the MIB (Di Francesco *et al.,* 2012). Moreover, the 7th-day blastocyst rate of this study is lower than the rate (16.1%) reported in IB (Kumar *et al.,* 2020) and the rate (14.9%) reported in MIB (Gasparrini, 2002). The differences in those results are due to the use of different buffalo breeds and the utilization of various culture mediums. Considering the studies, generally, it can be observed that similar results were obtained in the buffaloes that are genetically similar to the AWB, such as the MB.

The number of hatched blastocysts in this study was 12 out of 302, and the rate was 3.97% (Table 4). This rate was considerably higher than the 0.57% rate of reaching hatched blastocyst from the total number of oocytes reported by Madan *et al.* (1994). In addition, this rate was similar to the hatched blastocyst rate of 4% reported by Palta & Chauhan (1998). However, it was considerably lower than the rate of total blastocysts reaching hatched blastocysts (14.63%) in studies reported by Rubessa *et al.* (2019) and Kumar *et al.* (2020). Various factors such as differences in mediums, breed, age of buffaloes, season, climate, and geographical region play a significant role in those results (Duran *et al.,* 2017).

A study reported 44% excellent - good quality blastocysts rate, 34% poor quality blastocysts rate, and 14% degenerated blastocysts rate (Nandi *et al.,* 2002). Accordingly, the embryo quality rates obtained in our study are very similar to the embryo quality data of that study (Table 5). Moreover, the excellent - good quality blastocyst rate obtained in the study is higher than the rate (11.3%) in a study reported by Gasparrini (2002). Achieving the hatched blastocyst is accepted as an indicator of embryo quality. According to a study by Gasparrini (2002), prolonging the culture period does not increase this rate. In this study, the number of hatched blastocysts was 12 out of 29, and the hatched blastocyst rate was 41.37% in the microscopic examinations performed on the 8th and 9th days of embryo culture. In addition, 12 embryos out of 16 transferable embryos reached the hatched blastocyst. Thus, the hatched blastocyst rate was 75%. Many factors affect hatched blastocyst access and embryo quality (Duran *et al.,* 2017; Kumar *et al.* 2020) (Table 5).

CONCLUSION

The present study is the first in which oocyte and various embryo data obtained from the ovary in AWB were determined by the assisted reproduction

technique in this breed. This study is the first pioneering study for AWB. The results may be slightly low but require improvement in future studies. Considering the literature studies, it was revealed that these data were genetically approximate to the Mediterranean buffaloes close to AWB and showed differences compared to other buffalo breeds.

CONFLICT OF INTEREST

This article is summarized from a part of Mehmet Ali Yılmaz's Ph.D. thesis. The authors declared that there was no conflict of interest in the publication of this paper.

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