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RESEARCH ARTICLE

Effects of Microchip Technology and Different Sperm Preparation Methods on In vitro Fertilization and Blastocyst Rates in Bovine Embryo Production

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Abstract

This study compared the effects of commonly used sperm preparation methods, Percoll Gradient, Swim-Up, and Brackett & Oliphant (BO), with the increasingly utilized microchip technique on in vitro fertilization and proportion of bovine blastocyst development. Abattoir-derived oocytes were aspirated from 2-8 mm follicles. Grade A and B oocytes were subjected to in vitro maturation in TCM-199 medium for 20-22 hours. The mature oocytes were randomly allocated into four groups, and fertilization was performed using four different sperm separation methods. Following fertilization, presumptive zygotes were cultured separately in CR1aa culture medium according to their experimental groups. The results showed that cleavage rates at 24-48 hours for the Microchip, Brackett & Oliphant (BO), Percoll Gradient, and Swim-Up methods were 57.7%, 35.5%, 23.4%, and 17.1%, respectively, with statistically significant differences among groups ($p < 0.05$). On day 7 of culture, blastocyst development rates were determined to be 41.02% for the Microchip method, 22.6% for BO washing, 14.3% for the Percoll Gradient, and 15.9% for the Swim-Up method, with statistically significant differences among the groups ($p < 0.05$). In conclusion, the microchip-based sperm preparation technique was found to be more effective than conventional methods in in vitro bovine embryo production, nearly doubling embryo yield and demonstrating the potential to reduce production costs.

Introduction

In farm animals, embryo production experiments, both in vivo and in vitro, which began with rabbits in the late nineteenth century, accelerated after the 1950s (Merton *et al*., 2003). The use of in vitro embryo technologies, including in humans, became widespread during the 1970s. The birth of the first baby conceived through In vitro fertilization (IVF) in 1978 marked a major milestone in the development of these technologies (Trounson & Conti, 1982). The fertilization stage is of critical importance in in vitro embryo production (Penariol, 2017; Samuel *et al*., 2018). IVF, one of the indispensable techniques of assisted reproductive technologies, remains a subject of intensive research (Knowlton *et al*., 2015; Yamanaka *et al*., 2016). Currently, a wide range of sperm separation methods is applied in different laboratories, particularly in farm animals. These methods are mainly classified as migration–sedimentation, gradient centrifugation, and filtration techniques (Merton *et al*., 2003; Kaymaz, 2015).

In vitro embryo production (IVEP) is a complex process in which the natural reproductive events are

mimicked under laboratory conditions to obtain embryos (Vajta *et al*., 2010; Ben *et al*., 2013; Penariol, 2017). This complexity directly influences the success of IVEP (Chen *et al*., 2013), and several stages of the process remain unclear (Hasler, 2001; Telfer *et al*., 2020). Procedures applied to both spermatozoa and oocytes for fertilization have a direct impact on the success of IVF. Proper sperm capacitation and oocyte maturation constitute the fundamental requirements for a successful fertilization process (Trounson & Conti, 1982; Demyda-Peyrás *et al*., 2015). Optimization of these stages significantly enhances embryo development capacity and, consequently, the overall efficiency of IVF (Harrison *et al*., 1993; Penariol, 2017; Nagata *et al*., 2018).

The motility of sperm is closely associated with fertilization success (Centola *et al*., 1998; Ben *et al*., 2013; Abraham *et al*., 2016; Penariol, 2017). The primary objective of sperm separation is to select spermatozoa with the highest progressive motility (Machaty *et al*., 2012; Chen *et al*., 2013; Demyda-Peyrás *et al*., 2015; Yamanaka *et al*., 2016). One of the commonly applied methods for this purpose is the swim-up technique, which is based on the upward migration of motile spermatozoa from the semen sample. However, this technique has notable limitations, particularly in samples with low sperm concentration or reduced motility, as it yields a relatively low number of motile spermatozoa (Zavos, 1992; Coscioni *et al*., 2001; Barceló-Fimbres *et al*., 2011). Alternative methods, including centrifugation-based techniques such as Percoll gradient (Samardzija *et al*., 2006) and Brackett & Oliphant (BO) (Brackett & Oliphant, 1975; Brackett, 1985), rely on sperm selection based on density differences. These approaches are effective in isolating motile spermatozoa (Ben *et al*., 2013; Arias *et al*., 2017; Penariol, 2017; Samuel *et al*., 2018; Nagata *et al*., 2018). However, the high centrifugation forces applied during these procedures may adversely affect sperm viability and induce various forms of cellular damage (Parrish *et al*., 2014; Yamanaka *et al*., 2016). During such processing, lipid peroxidation may occur, leading to disruption of membrane integrity, increased DNA fragmentation, and ultimately compromised fertilization outcomes (Van Kooij *et al*., 2004; Asghar *et al*., 2014; Knowlton *et al*., 2015; Li *et al*., 2016; Yamanaka *et al*., 2016). Moreover, Percoll has been reported to exert toxic effects on sperm cells (Schuster *et al*., 2003; Samardzija *et al*., 2006; Ben *et al*., 2013; ; Asghar *et al*., 2014;).

In recent years, non-toxic sperm separation microchip devices based on the principle of kinetic flow within a flat, slide-like microfluidic plane have been developed to avoid physical damage to spermatozoa (Phiphattanaphiphop *et al*., 2020; Telfer *et al*., 2020). These devices enable the selective isolation and enrichment of viable and motile spermatozoa (Barceló-Fimbres *et al*., 2011; Knowlton *et al*., 2015). By eliminating the need for centrifugation, which can cause substantial sperm damage, microchip systems provide a safer and more efficient selection process (Chen *et al*., 2013; Asghar *et al*., 2014; Anbari *et al*., 2021;). Microchip-based sperm selection devices are widely used in humans for applications such as in vivo insemination, IVEP, and intracytoplasmic sperm injection (ICSI) (Knowlton *et al*., 2015; Chinnasamy *et al*., 2016; Li *et al*., 2016; Phiphattanaphiphop *et al*., 2020). Moreover, recent advances in biotechnology have enabled bovine embryo production to be performed under laboratory conditions with improved efficiency and reduced costs (Parrish *et al*., 2014; Abraham *et al*., 2016; Penariol, 2017).

This study aimed to compare the effects of commonly used sperm separation methods, Percoll Gradient, Swim-Up, and Brackett & Oliphant (BO) washing with a microchip-based device that has recently been introduced for use in mammalian sperm selection, on fertilization success and blastocyst development in bovine in vitro embryo production.

Materials and Methods

All experiments were conducted with four replicates. Ovaries obtained from an abattoir located in the Ankara region were transported to the laboratory within a maximum of 2–3 hours in a thermally insulated container, maintained at 25–30 °C in 0.9% physiological saline solution supplemented with 100 mg/L kanamycin sulfate (Vetaş-KANOVET®, Istanbul, Türkiye). In the laboratory, oocytes were aspirated from follicles measuring 2-8 mm in diameter using an 18-gauge blunt-ended needle attached to a syringe. Cumulus–oocyte complexes (COCs) of grades A and B (Morphological Quality Classification), characterized by the presence of at least 3–4 compact layers of cumulus cells, were selected and subjected to in vitro maturation. Post-fertilization embryo development and quality assessments were performed in accordance with the standards established by the International Embryo Technology Society (IETS). Unless otherwise stated, all chemicals used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other consumables and pharmaceutical products are specified where relevant.

In Vitro Maturation (IVM)

Cumulus–oocyte complexes (COCs) were matured in TCM-199 medium (Medium 199, Gibco, USA) supplemented with 10% fetal calf serum (FCS), 5.5 mg/mL sodium pyruvate (Sigma-Aldrich, USA), 0.02 IU/mL follicle-stimulating hormone (FSH; Folltropin-V, BIONICHE, USA), and 50 µg/mL penicillin–streptomycin (Pen-Strep, Gibco, USA). Each drop was overlaid with mineral oil (Sigma-Aldrich, USA) and pre-equilibrated for at least 2–3 hours in a CO_2 incubator (Panasonic MCO-170M-PE, Japan) at 38.5 °C, 5% CO_2, and 98% relative humidity. A total of 20 appropriately selected

oocytes were placed into each 100 µL drop (5 µL medium per oocyte) and incubated for 22–24 hours to allow maturation.

Sperm Preparation for Fertilization and In Vitro Fertilization (IVF)

For fertilization, frozen semen straws (0.25 mL) obtained from four different commercially available Simmental bulls were used in this study. Sperm motility and kinematic parameters were evaluated using a computer-assisted sperm analysis system (CASA; IVOS, Hamilton Thorne, Beverly, MA, USA) (Table 1). To minimize the effects of individual bull fertility and breed-related variability, semen samples used for all experimental groups were pooled before fertilization (Barceló-Fimbres *et al*., 2011; Demyda-Peyrás *et al*., 2015).

The oocytes were randomly allocated into four groups and fertilized with spermatozoa obtained using four different sperm separation methods. Fertilization procedures were carried out in accordance with the method-specific protocols described in the relevant literature.

Microchip Method

In this study, a 3 mL sperm separation microchip specifically designed for bovine use (Fertile Bovine®, Germany) was employed. For the procedure, four semen straws (0.25 mL; 18 × 10^6 spermatozoa per straw) were thawed in a water bath at 37 °C for 30 seconds. Following the gentle mixing, a total of 1 mL of pooled semen (Table 1) was loaded into the microchip through the inlet port according to the manufacturer's instructions. The sperm-loaded microchips were incubated at 37 °C for 30 minutes. Following incubation, spermatozoa accumulated in the upper collection chamber, as specified in the protocol, were retrieved using a pipette. Sperm concentration was determined, and the suspension was diluted to a final concentration of 3 × 10^6 spermatozoa/mL. For fertilization, 5 µL fertilization drops were prepared and overlaid with mineral oil, then equilibrated for at least 2 hours in a CO_2 incubator (Panasonic MCO-170M-PE, Japan) at 38.5 °C under 5% CO_2 and 98% relative humidity. Subsequently, 95 µL of the prepared sperm suspension was added to each drop, and 18-20 oocytes were placed into each drop and incubated in the CO_2 incubator for 12-16 hours to promote fertilization (Li *et al*., 2016; Karcz *et al*., 2023).

Brackett & Oliphant (BO) Washing Method

A total of 1 mL of pooled semen (Table 1) was transferred into a 15 mL centrifuge tube, and 6 mL of the prepared BO solution was added. The tube was centrifuged at 400 × g for 5-10 minutes for sperm washing. The supernatant containing seminal plasma or cryoprotectants was carefully discarded. The washing step was repeated twice. Leftover sperm suspension was then diluted to a final concentration of 6 × 10^6 spermatozoa/mL. Fertilization droplets were prepared to provide 20,000-30,000 spermatozoa per oocyte by adding 95 µL of sperm suspension, and 18-20 oocytes were placed in 5 µL per droplet. Each fertilization droplet was overlaid with mineral oil and equilibrated for at least 2 hours in a CO_2 incubator at 38.5 °C with 5% CO_2 and 98% relative humidity. Fertilization was carried out for 6–8 hours under the same incubation conditions (Brackett, 1983; Kaymaz, 2015).

Table 1. CASA (Computer-assisted sperm analysis) parameters of sperm used for fertilization.

CASA Parameters	Samples				
	Bull 1	**Bull 2**	**Bull 3**	**Bull 4**	**Pooled**
CON (M/mL)	26.66	23.16	14.70	46.71	46.71
MOTIL (%)	50.30	50.10	67.60	58.60	58.60
P. MOTIL (%)	33.40	40.10	40	37.20	37.20
DAP (µm)	11.93	10.65	13.88	15.42	16.02
DSL (µm)	8.91	8.89	13	12.40	12.60
DCL (µm)	22	17.07	19.06	28.48	27.69
VAP (µm/sn)	29.11	25.97	29.43	34.62	37.81
VSL (µm/sn)	21.66	21.65	27.48	27.75	30.05
VCL (µm/sn)	52.84	41.87	40.13	62.94	64.22
STR (%)	38.22	41.86	63.97	47.25	61.19
LIN (%)	21.99	26.32	50.64	29.38	39.58
ALH (µm)	3.84	3.22	2.17	4.30	4.94
BCF (Hz)	8.42	9.16	14.33	9.88	13.72
WOB (%)	28.71	31.45	53.14	34.98	47.53

The abbreviations: CON (Concentration), MOTIL (Motility), P. MOTIL (Progressive Motility), DAP (Distance of Average Path), DSL (Distance Straight Line), DCL (Distance Curvilinear), VAP (Average Path Velocity), VSL (Straight-Line Velocity), VCL (Curvilinear Velocity), STR (Straightness), LIN (Linearity), ALH (Amplitude of Lateral Head Displacement), BCF (Beat Cross Frequency), WOB (Wobble).

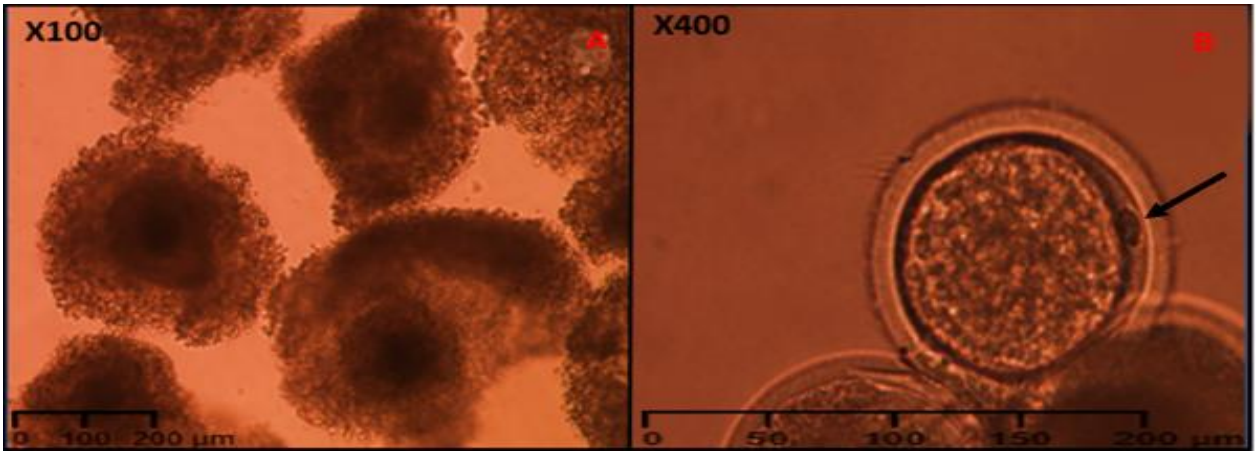

Figure 1. Post-maturation oocyte images: (A) Mature oocytes showing cumulus expansion (×100; Olympus SZH10); (B) Mature oocyte with visible first polar body (×200; Nikon Eclipse TE300).

Swim-Up Method

Thawed and pooled semen (1 mL; Table 1) was diluted 1:1 (v/v) with Tyrode's Albumin Lactate Pyruvate (TALP) solution and centrifuged at 300 × g for 5 minutes. After discarding the supernatant, the pellet was resuspended again at a 1:1 ratio with TALP solution to complete the washing process. The fertilization medium was supplemented with 0.2 mM sodium pyruvate, 6 mg/mL fatty acid–free bovine serum albumin (BSA; Sigma-Aldrich, USA), and 25 µg/mL gentamicin. Subsequently, 1 mL of washed semen was gently placed at the bottom of tubes containing 6 mL TALP (sp-TALP), positioned at a 60° angle, and incubated for 60 minutes. Fertilization drops (5 µL) were prepared, overlaid with mineral oil, and equilibrated for at least 2 hours in a CO_2 incubator at 38.5 °C with 5% CO_2 and 98% relative humidity. After incubation, the upper 1 mL fraction containing motile spermatozoa was collected and diluted with TL-HEPES to a final concentration of 1.25×10^6 spermatozoa/mL. Matured oocytes were washed in TL-HEPES, and fertilization droplets (95 µL) were prepared to contain 1.25×10^6 spermatozoa/mL. Subsequently, 15–20 oocytes (5 µL) were added to each droplet, and fertilization was carried out for 24 hours at 38.5 °C under 5% CO_2 and 98% relative humidity (Ben *et al*., 2013; Lucio *et al*., 2012).

Percoll Gradient Method

1 mL of pooled semen (Table 1) was mixed with 45% Percoll prepared in HSOF at a 1:1 ratio and carefully layered over 2 mL of 90% Percoll-SOF solution in a 15 mL conical centrifuge tube to prevent mixing the layers. Samples were centrifuged at 600 × g for 15 minutes. Supernatant was discarded, and 50 µL of HSOF was added to the sperm pellet. Following sperm counting, the suspension was diluted with 2% BSOF solution to achieve a final concentration of approximately 1×10^6 spermatozoa/mL. Fertilization drops (95 µL) containing 1.25×10^6 spermatozoa/mL were prepared, overlaid with mineral oil, and equilibrated for at least 2 hours in a CO_2 incubator at 38.5 °C with 5% CO_2 and 98% relative humidity. Subsequently, 15-20 oocytes (5 µL) were added to each drop. Fertilization was performed for 19-21 hours under the same incubation conditions (Samardzija *et al*., 2006; Penariol, 2017).

In Vitro Culture (IVC)

Following fertilization, cumulus cells were removed by gentle pipetting, and presumptive zygotes were transferred into CR1aa embryo culture medium. Culture was performed in 100 µL drops (15-20 embryos per drop) overlaid with mineral oil. Embryos were cultured for 7 days in a CO_2 incubator (Panasonic MCO-170M-PE, Japan) at 38.5 °C under 5% CO_2 and 98% relative humidity (Rosenkrans *et al*., 1993; Wan *et al*., 2009). The proportion of cleaved embryos was evaluated at 24-48 hours, morula formation on day 4 (96 hours), and blastocyst development on day 7 (156 hours). Cleavage and blastocyst stages were assessed visually using a stereo microscope (Olympus SZH10) and an inverted microscope (Nikon Eclipse TE300), and all observations were recorded.

Statistical Analysis

Statistical analyses of basic parameters and differences among experimental groups were performed using one-way ANOVA in IBM SPSS 24 software. Non-parametric data, including comparisons of embryo development rates, were analyzed using the chi-square test.

Results

The study consisted of four replicates. A total of 486 oocytes were aspirated across the four experimental trials. Of these, 345 were classified as grade A or B, corresponding to an A–B quality oocyte rate of 71.98%. The A–B quality oocytes were subjected to in vitro

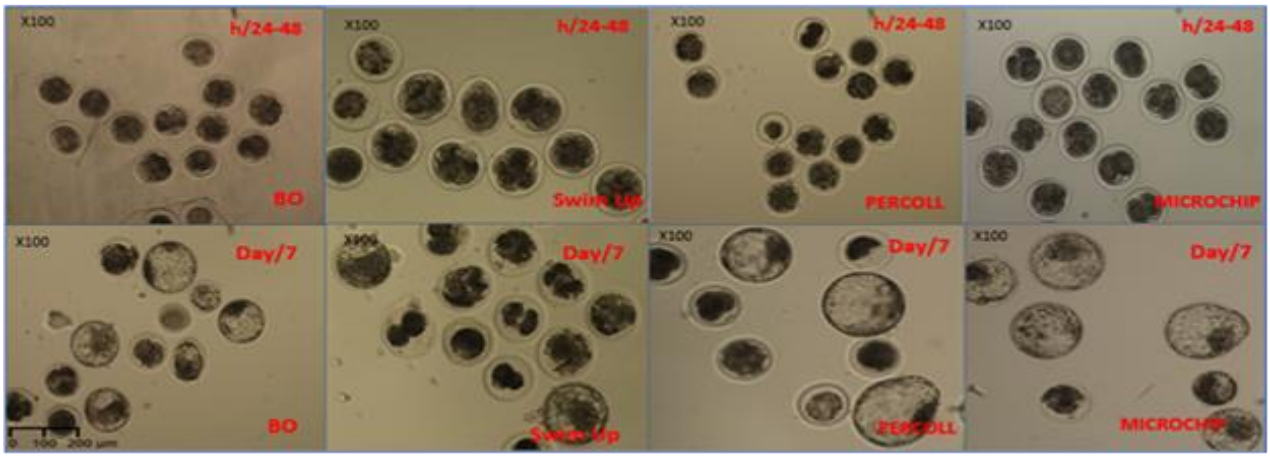

Figure 2. Representative culture medium images showing cleavage at 24–48 hours and blastocysts on day 7 following fertilization using the Microchip, Brackett & Oliphant (BO), Percoll Gradient, and Swim-Up methods (×100; inverted microscope, Nikon Eclipse TE300).

maturation without prior group allocation. A total of 299 oocytes completed maturation, yielding a maturation rate of 86.66%. Following maturation, the oocytes were randomly assigned to experimental groups (Figure 1) and used for fertilization.

In this study, cleavage for the Microchip, BO washing, Percoll Gradient, and Swim-Up methods was evaluated at 24–48 hours, while blastocyst development was assessed on day 7 (Figure 2).

According to the data obtained in this study, the proportion of cleavage at 24–48 hours, morula formation at 96 hours, and blastocyst development on day 7 were examined in detail for each method and evaluated using statistical analyses. These results, presented in Table 2, were used to compare the performance during embryo development.

Discussion

In this study, fertilization was performed using four of the most commonly applied sperm separation methods: Microchip, Percoll Gradient, Swim-Up, and Brackett & Oliphant (BO). The developmental competence of oocytes was evaluated based on the proportion of cleavage at 24–48 hours, morula formation at 96 hours, and blastocyst development on day 7.

Studies investigating the use of microchip-based sperm selection with frozen-thawed semen during the fertilization stage of IVEP in cattle are limited. Li *et al*. (2016) reported that the use of a different microchip device in bovine IVF with sex-sorted semen resulted in significantly higher cleavage compared to standard fertilization methods. The same study also demonstrated that microchip application increased the proportion of blastocyst formation in bovine IVEP, with an improvement of approximately 11-16% compared to the control group. In the present study, the group in which spermatozoa were selected using the microchip method exhibited nearly 20-24% higher blastocyst development compared to the other sperm separation techniques. This enhanced outcome may be attributed to differences in the microchip systems used, as well as advancements in microchip manufacturing technology over recent years, particularly the more efficient integration of nano-scale components, which has led to the development of more functional and effective devices.

Numerous techniques have been evaluated for preparing bovine sperm for fertilization (Lucio *et al*.,

Table 2. Developmental data obtained during fertilization and embryo culture according to experimental groups.

Method	Oocytes no	Cleavage (h 24-48)	Morulae (h 96)	Blastocyst (day 7)
Microchip	78	57.69 [a] (45/78)	48.72[a] (38/78)	41.02[a] 32/78
BO	62	35.48[b] (22/62)	25.81[b] (16/62)	22.60[b] (14/62)
Percoll-Gradient	77	23.38[b] (18/77)	19.48[b] (15/77)	14.30[b] (11/77)
Swim Up	82	17.07[b] (14/82)	14.63[b] (14/82)	15.90[b] (13/82)
P values		<0.001	<0.001	<0.001

BO, Brackett & Oliphant Method; h, hours; P, statistical difference.

2012; Penariol, 2017; Abraham *et al*., 2016; Yamanaka *et al*., 2016; Parrish *et al*., 2014; Prasad *et al*., 2018). Abraham *et al*. (2016) investigated the effects of different sperm preparation methods on the sperm fertilization ability. In the study, Bovicoll, Single-Layer Centrifugation Sperm Selection Technique (Mini-SLC), and the swim-up method were compared. It was reported that the cleavage proportions were 84.25 ± 5.29% and 80.5 ± 5.25% for the Mini-SLC and swim-up methods, respectively, while blastocyst formations were 15.6 ± 7.18% and 11.25 ± 8.5%, respectively. However, no statistically significant differences were observed among the groups in terms of cleavage or blastocyst development. The blastocyst development data reported were considerably lower than those observed in the microchip group in the present study and were more comparable to those of the swim-up method (Table 2). The separation principle of the Mini-SLC technique used by Abraham *et al*. (2016) is technically similar to the centrifugation-based BO washing and Percoll Gradient methods applied in the present study. In contrast, markedly higher blastocyst development was achieved using the microchip-based sperm selection technique. These findings support the hypothesis that the microchip method maynot induce damage to spermatozoa compared to conventional centrifugation-based techniques.

Percoll and swim-up methods were modified to identify an effective fertilization protocol (Lucio *et al*., 2012). In the study, cleavage ranged between 67-71%, and blastocyst development between 20-32%. These findings are consistent with the data obtained in the present study. Furthermore, the cleavage and blastocyst proportions reported by Parrish *et al*. (2014) and Prasad *et al*. (2018) using BO washing media, as well as those reported by Yamanaka *et al*. (2016) using the Percoll gradient method, agree with the results of the current study. In another study comparing Percoll, swim-up, and BO direct washing methods, cleavage proportions were reported as 70.1%, 72.6%, and 62.9%, respectively, while blastocyst formations were 34.6%, 26.9%, and 25.4%, respectively (Penariol, 2017). When compared with the findings of the present study, the blastocyst development reported in that study was lower than that achieved using the microchip method; they were relatively close to the results obtained with the Percoll method, and fertilization rates were similar for the same techniques. It is suggested that, unlike the present study, the use of an undefined medium during the Percoll gradient procedure may have contributed to the higher outcomes reported in that study.

Methods used to prepare bovine sperm for fertilization have also been evaluated across different local cattle and buffalo breeds. Rahman *et al*. (2020) conducted a study to determine an effective fertilization medium and sperm separation method to achieve optimal IVF in indigenous zebu cow oocytes. In their research, IVF was performed using sperm prepared with TCM-199, TALP, BO, Swim-Up, and Percoll Gradient methods, and cleavage proportions of 47.5%, 63.3%, 59.8%, 60.8%, and 62.3%, respectively, were reported. When compared with these findings, the results obtained in the present study, excluding the microchip group, were lower (Table 2). This discrepancy may be attributed to differences in the cattle breeds used in the respective studies. Ben *et al*. (2013) investigated the effects of different sperm preparation methods on yak sperm quality and in vitro embryo development by applying swim-up and Percoll gradient techniques to frozen–thawed semen samples from two bulls. For the first bull, cleavage proportions of 73.17 ± 10% and 62.14 ± 2.66% were reported for the swim-up and Percoll methods, respectively, while blastocyst proportions were 11.48 ± 1.90% and 8.44 ± 1.09%. For the second bull, cleavage rates of 75.83 ± 1.52% (swim-up) and 67.10 ± 2.20% (Percoll) were reported, with corresponding blastocyst proportions of 13.66 ± 1.63% and 12.08 ± 0.79%. Although the cleavages in that study were comparable to those observed with the microchip method in the present study, the blastocyst developments were markedly lower. In the present study, to eliminate bull-to-bull variation, semen from four different bulls was pooled before fertilization. Although direct comparison between yak and Simmental cattle used in the present study is limited due to interspecific physiological and biological differences, the embryo development data obtained in the current study were notably higher than those reported for yak cattle.

The use of microchip-based sperm selection in human in vitro fertilization studies has been shown to increase the proportions of cleaved embryos (Chinnasamy *et al*., 2016). In most of these studies, the proportion of high-quality blastocysts and the total number of embryos were reported to increase in a manner comparable to the findings of the present study. The data obtained in this study (Table 2) were higher than those reported in human IVF studies and markedly higher than outcomes observed in studies involving sperm from men with fertility disorders (; Asghar *et al*., 2014; Anbari *et al*., 2021; Ogata *et al*., 2021; Pujol *et al*., 2022; Mirsanei *et al*., 2022). This outcome is expected, as the bull semen used in the present study was commercially produced and derived from bulls with proven fertility. However, the more noteworthy observation is that the microchip-based results in this study were also higher than those reported for human sperm with normal fertility. This difference may be attributed to the fact that, in humans, fertilization often requires advanced intracytoplasmic assisted reproductive technologies such as intracytoplasmic sperm injection (ICSI), whereas in cattle, fertilization can be successfully achieved by co-incubating sperm and oocytes under appropriate in vitro conditions without the need for such invasive techniques. Additionally, for a more

comprehensive interpretation of these findings, it is important to consider the proportion of oocytes from females experiencing infertility due to various factors in human IVF studies.

In conclusion, the microchip method nearly doubled the number of embryos produced compared with conventional techniques. Microchip-based sperm preparation can be successfully applied in in vitro bovine embryo production as a more effective alternative to classical methods. By increasing embryo yield per production cycle, this approach has the potential to reduce the cost of in vitro embryo production by approximately 50%. Furthermore, it is suggested that this biotechnology be evaluated across a broader range of breeds and a larger number of mammalian species, and that quality-oriented studies focusing on DNA damage and acrosomal integrity be conducted.

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Ethical Statement

This study was approved by the Local Ethics Committee for Animal Experiments of the International Livestock Research and Training Center, Ministry of Agriculture and Forestry of the Republic of Türkiye, under decision number 188, dated 30.04.2021, with registration number 348.

Conflict of Interest

The authors declared there is no conflict of interest.

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