The effect of swim up and Percoll gradient separation on ram sperm parameters, DNA integrity and embryo development

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ABSTRACT

Percoll gradient and swim-up are the most common methods used to obtain high quality intact sperm to be used for the *in vitro* fertilization procedures. In this study, we evaluated the effects of swim-up and percoll gradient methods on DNA integrity and other spermatological parameters. To determine DNA breakdown, toluidine blue staining was used. Motility, and progressive motility were analyzed via computer assisted semen analyzer (CASA) system and the others (morphology, viability, membrane and acrosome integrity) were evaluated via phase contrast and florescent microscopy. The membrane integrity was tested by hypo-osmotic swelling test. Also, the separated sperms were tested for *in vitro* embryos production. Our results showed no differences between the two methods of separation compared to the fresh samples in regard of sperm motility (*P>0.05*), however, in the separated sperms with both methods, progressive motility was higher than the fresh sperm and the highest results were obtained in Percoll method (*P*<0.05). In comparison to swim-up, Percoll gradient method maintained the highest normal sperm morphology rate and the lowest acrosome defects (*P*<0.05). In addition, the membrane integrity, intact acrosome and intact chromosome rates were also higher in the sperm samples separated by percoll gradient method (*P*<0.05). The blastocyst rate produced with sperm separated by Percoll gradient method was significantly higher than the swim-up group (*P*<0.001). Sperm samples obtained after Percoll gradient centrifugation could improve motility, morphological intactness, and DNA integrity. Accordingly, it was concluded that the Percoll gradient separation method is more useful then the swim-up for in vitro sheep embryo production. Also, Toluidine blue staining could be a suitable technique for determination of DNA integrity.

Keywords: Percoll gradient, Ram, Sperm DNA, Swim-up, Toluidine Blue

RÉSUMÉ

Effet du « swim up » et d'un gradient de Percoll sur divers paramètres du sperme de bélier, intégrité de l'ADN et développement embryonnaire.

Le gradient de Percoll et le « swim up » sont les méthodes les plus couramment utilisées pour obtenir des spermatozoïdes intacts de haute qualité à utiliser pour la fécondation in vitro. Dans cette étude, nous avons évalué les effets de ces techniques sur l'intégrité de l'ADN et d'autres paramètres spermatologiques. Pour déterminer la dégradation de l'ADN, on a utilisé la coloration au bleu de toluidine. Motilité, et motilité progressive ont été analysées à l'aide d'un système assisté par ordinateur (CASA). Les autres variables (morphologie, viabilité, intégrité de membrane et acrosome) ont été évalués en microscopie avec contraste de phase et fluorescence. L'intégrité de la membrane a été testée par test de gonflement hypo-osmotique. De plus, les spermatozoïdes séparés ont été testés pour la production d'embryons in vitro. Nos résultats n'ont montré aucune différence entre les deux méthodes de séparation par rapport aux échantillons frais en ce qui concerne la motilité des spermatozoïdes (P>0,05), cependant, dans les spermatozoïdes séparés par ces deux méthodes, la motilité progressive était plus élevé que celle du sperme frais. Les résultats les plus élevés ont été obtenus avec le gradient de Percoll (P<0,05). Par rapport à la technique dite de « swim up », le gradient de Percoll a aussi amélioré les critères morphologiques analysés (P<0,05). Le taux de blastocystes produit avec des spermatozoïdes séparés par gradient de Percoll a été significativement plus élevé que celui obtenu par technique de « swim up » (P<0,001). Il a été conclu que le gradient de Percoll était plus efficace que la technique dite de « swim up » dans la séparation des spermatozoïdes en vue de la production d'embryons d'ovins.

Mots-clés : bélier, sperme, conservation, fécondation in vitro

Introduction

Sperms are naturally subjected to maturation and morphological changes during the migration from site deposition to the site of fertilization (ampulla of the oviduct) of the female genital tract. If a sample obtained from the oviducts, it was observed that the sperm reaching this region had good morphology [18, 21, 31]. Simulation of the natural conditions is the main challenges of the *in vivo* assisted reproductive techniques (ART) to select high-quality sperm to be used for *in vitro* fertilization (IVF) of matured oocytes [1, 11, 28]. The sperm selection process includes the removal of seminal plasma components such as decapacitation factors, bacteria and other harmful agents. Also removing diluters and cryoprotectants of frozen semen [18, 21]. There are many methods used in the preparation of sperm for *in vitro* fertilization procedure. All these methods immerge in allowing the passage of sperm through different media to separate the highly motile ones from others [23]. Percoll gradient centrifugation and swim-up are the most used methods to obtain high-quality sperm with good progressive motility and intact structures [23, 26, 33].

To implement the swim-up method, it is necessary to have sufficient number of sperm with fast progressive motility. Swim-up is a method in which specimens of fresh or frozen-thawed semen are placed into the bottom of the centrifuge

tubes with sperm washing medium and the moving sperm is swim up to the top [26]. In Percoll gradient centrifugation method, sperm is placed on a suspension contains gradient colloidal silica particles followed by centrifugation, so that the sperms are moved to the bottom of the tube with centrifugal force. The sperm with the best motility and intact morphology move towards the bottom. As a result of the preparation, the immotile or abnormal sperm, the white blood cells and all other cellular excess in the semen are separated and only the motile and healthy sperm remain[18, 21]. Although, using gradient Percoll for sperm separation in *in vitro* embryo production is necessary to improve the quality of sperm[27], it has been demonstrated that centrifugation is a potentially sperm-damaging step during semen processing[9]. It has been reported [3]that centrifugation process could cause the production of reactive oxygen species (ROS) within the pellet, which brought irreversible damage to human spermatozoa and decreasing there *in vitro* fertilizing ability. Several studies have been conducted to compare the results of using swim-up and percoll gradient methods and to evaluate their influences on sperm quality. Most of these studies were applied on human and bovine spermatozoa and their findings were varied [16, 21, 33].

In order to obtain high-quality and quantity embryos, the chromosomes and DNA of both gametes should be intact [16, 18, 33]. Few previous reports has been conducted to assess the effectiveness of these methods on ram spermatozoa[15, 31].

Chromosome and DNA integrity could be assayed by TdT-mediated –dUTP nick end labeling (TUNEL), single gel electrophoresis assay, Toluidine Blue stain(TB), Sperm Chromatin Structure Assay (SCSA) and Chromatin Dispersion (SCD) assay[18]. TB stain has a strong binding and affinity for acidic tissue components resulted in darkly stains nuclear material of tissues rich with DNA contents [30]. Sperm exposed to centrifugation resulted in breakdown of nuclear material and make their chromosomes more stainable by TB. On the other hand, TUNEL is considered to be an expensive method with a lot of efforts to assay the quality of sperm.

Few previous reports has been conducted to assess the effectiveness of these methods on ram spermatozoa [15, 31], However, their effect has not been compared the intactness of DNA or the sperm cell membrane after sperm separation with Percoll or swim up methods. Therefore, the aim of this study was to compare the effectiveness of swim-up and Percoll gradient methods regarding DNA fragmentation and other sperm quality parameters. Sperm DNA fragmentation and chromosomal dispersion were evaluated with toluidine blue stain. Simultaneously, spermatozoa motility, progressive motility, morphology, viability, membrane and acrosome intactness were evaluated. In addition, to evaluate their effects on producing IVF embryos, swim-up and percoll gradient treated spermatozoa were used to fertilize *in vitro* matured oocytes.

Material and methods

RAMS AND SEMEN COLLECTION

Three Kivircik rams (3-4 years of age) with good quality semen characteristics (>80% motility and concentrations of at least 2.0x10^{9}spermatozoa/ml) were selected for semen collection and further analysis. The rams were clinically examined, and they were free from any general or genital diseases. Animals were maintained at the Department of Reproduction and Artificial Insemination of Faculty of Veterinary Medicine/ Istanbul University (Istanbul, Turkey). Semen were collected from rams by an artificial vagina twice a week. The semen from were pooled to eliminate variability among the evaluated samples. The pooled semen sample was immersed in a water bath at 28°C (15-30 minutes) until it could be assessed for total and progressive motility as well as sperm concentration. This study was replicated ten times for each group (Fresh, Swim-up and Percoll). The experimental procedures were approved by Istanbul University Ethics Committee on Animal Research (2017-303114).

SEMEN PROCESSING AND SEPARATION

Swim-up

For swim up, 200 µl of semen was covered with 5 ml of Bovine Serum Albumin supplemented Hepes buffered Synthetic Oviduct Fluid (BSA-HSOF) in 15 ml conical tube at 38.5 ºC for up to 30 min. the 3 ml of the medium was gently taken off from the top of the suspension and were then added into 15 m1 new conical tube. The tubes were then centrifuged at 600×g for 5 min at room temperature. The final pellet was re-suspended with 200 µl BSA-HSOF.

Percoll gradient

Spermatozoa were obtained by centrifugation, at 600x g, on a Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient (2 ml of 45% Percoll over 2 ml of 90% Percoll) for 15 min at room temperature. Spermatozoa collected at the bottom of the 90% fraction were washed in Bicarbonate supplemented Synthetic Oviduct Fluid (BSOF) medium and pelleted by centrifugation at 300x g for 5 min at room temperature.

Sperm motility and morphology analysis

Spermatological analyzes of fresh semen and semen separated by Percoll or swim-up methods were evaluated by computer assisted semen analyzer (CASA 12.3 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA). Before evaluation; of the samples, the semen analyzer was adjusted to ram sperm analysis protocol [25]. 600-800 spermatozoa were evaluated for each sample and 10 different filed were evaluated (magnification) at each time. Two independent sperm analysis procedures were performed for each sample

and the results were recorded. Semen samples were diluted 1:35 with TRIS buffered extender before estimating their total and progressive motility.

For morphological analysis; Sperm samples were fixed in 10% formaldehyde solution and 10 µ*l* of sperm samples were mixed with 100 µ*l* of fixative solution. One drop of the mixture was placed on a slide and covered with coverslip. Sperm morphological structures were examined under phase-contrast microscope with x100 immersion oil lens. The morphological analysis of acrosomes, head, mid-piece and tail abnormalities were evaluated as described previously [5]. A total of 200 cells were counted and percent values of normal and up normal sperm were recorded. Morphological analyzes were repeated twice.

HYPOOSMOTIC SWELLING TEST (HOST)

The functional integrity of the spermatozoon plasma membrane was assessed by hypoosmotic swelling test as described previously with slight modification [11]. Briefly; 20 µl of semen samples were added to 350 µl of sodium sulphate-fructose solution (50 mOsm) and incubated in at 41° C in a water bath for 45 min. To stop the reactions at the end of the incubation, the samples were made ready for examination by adding 50 µl Hancock solution. A total of 200 cells were examined at 400x magnification in a phase-contrast microscope, and the percent values of HOST-positive spermatozoa were recorded. Curled, spiral and shortened sperm tails were accepted as an indicator for intact plasma membrane of sperm. A total of 200 cells were counted and the percentages were recorded. The experiment was repeated twice, and the data were recorded.

SPERM MEMBRANE AND ACROSOME INTEGRITY

The membrane and acrosomal-integrity stains were performed on each replicant. Membrane integrity was assessed by using a combination of propidium iodide (PI) and Carboxyfluorescein Diacetate (CFDA) fluorescent stains. This staining protocol was modified from Nuri baspinar, et al. [6] and Yániz, J. L., et al.[32]. Samples from fresh, Percoll or swim-up separated sperm were diluted with normal saline (1:35). A 30 µl aliquot of the diluted spermatozoa was pipetted into an eppendorf tube and stained with 10 µl PI (0.5 mg/ml stock) plus 10 µl CFDA (0.46 mg/ml stock). The tubes were incubated at room temperature for 5 min after which 5 µl of Hancock solution was added to terminate sperm movement (Motility). Five µl of the stained suspension was placed on a slide under a coverslip. Two slides were prepared per sample to get a more precise estimate of the ratio of spermatozoa with intact or damaged membrane. Spermatozoa were assessed by fluorescent microscopy (Olympus BX 60) at an excitation wavelength of 485/20 nm excitation and 580-630 nm emission filters and a magnification of 400X. At least 200 spermatozoa per slide were evaluated to assess sperm membrane integrity. Sperm displaying red colorization was considered as damaged membrane, while displaying green colorization was considered to be intact membrane. Sperm displaying green-red colorization was considered moribund.

Acrosome integrity was assessed using Fluorescein Isothiocyanate (FITC)-labeled Lectins in combination with (PNA) peanut agglutinin (PNA–FITC) (L7381 FITC-PNA, Invitrogen). A 30 µl aliquot of the diluted spermatozoa was pipetted into 1.5 ml eppendorf tube and stained with 10 µl of PNA-FITC (100 µg/ml stock). The tube was incubated at room temperature for 5 min after which 5 µl of Hancock solution was added to terminate sperms movement. Five µl of the stained suspension was placed on a slide under a coverslip. Two slides were prepared per sample to get a more precise estimate of the acrosome integrity. Spermatozoa were assessed by fluorescent microscopy (Olympus BX 60) at an excitation wavelength of 450-490 nm excitation and 515 nm emission filters and a magnification of 400X. At least 200 spermatozoa per slide were evaluated. Spermatozoa displaying bright green or patchy green fluorescence were considered as acrosome non-intact or damaged, whereas cells which did not stain green fluorescence in acrosome cap were regarded as acrosome intact. (Figure 1)

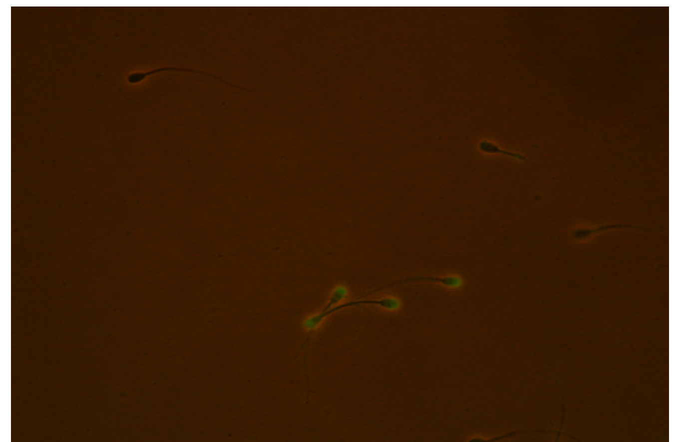

Figure. 1: Evaluation of acrosome integrity by PNA-FITC staining
The green color represents degenerated acrosome, not stained ones represent intact acrosome

SPERM DNA INTEGRITY

Toluidine blue (TB) staining method was performed as described previously by Dennis E. Marchesi et., al study [18] with slight modification. In brief; working solution of toluidine blue was prepared in 1:1 ratio with McIlvain buffer (pH 3.6) and Toluidine blue stock solution. Stock solution of toluidine blue was consisted 1 g TB powder, 10 ml of 1% acetic acid, 4.19 ml absolute alcohol, and 86 ml distilled water for 100 ml (pH 4.5) [30]. Air-dried slides were fixed in methanol for 30 min at 4°C. Slides were hydrolyzed in 0.1 N HCL solution for 5 min at 4°C washed briefly and incubated for 5 min in distilled water at room temperature.

Working solution of toluidine blue was applied to slides for 30 min on hot plate. All slides were washed briefly in distilled water than dehydrated for 30 s in butanol. End of the staining slides were incubated 3x5 min in xylene and

mounted with entellan. As a positive control (Figure: 3), spermatozoa were incubated for 10 min in Dithiothreitol (DTT 5 mM) [4].

Swim up, Percoll and fresh sperm smear were examined under light microscopy as described previously. Ten independent experiment were performed, at least 200 sperm cells were counted on each slide. The Sperm color light blue (LB), blue (B) and dark blue (DB) stained cells were determined (figure 2 and 3) (Figure 2)[13].

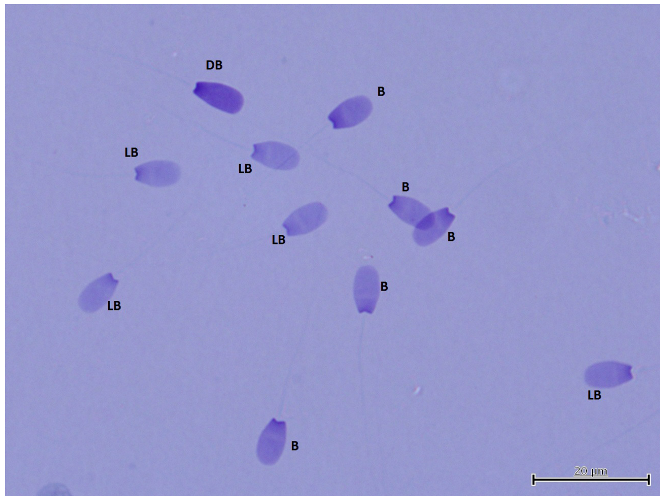

Figure.2: Staining sperm cells with Toluidine blue
Visual of light blue (LB), blue (B) and dark blue (DB) in Toluidine blue staining cells (20 µm)

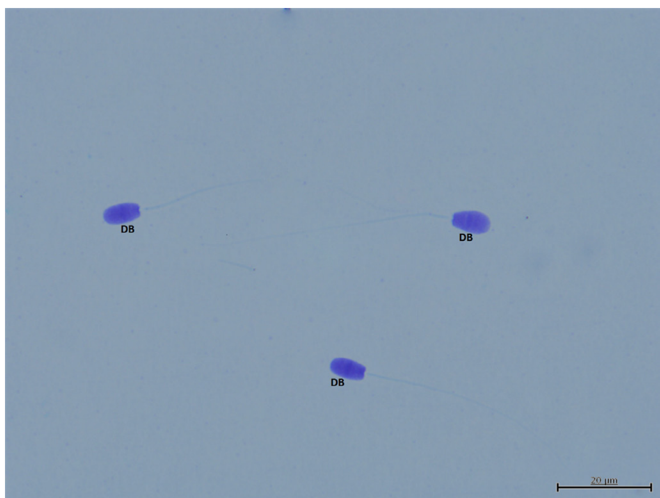

Figure.3: Positive control (20 µm)
DB: dark blue (dark stained)

IN VITRO MATURATION, FERTILIZATION AND CULTURE

Recovery and *in vitro* maturation of cumulus-oocyte complex (COCs):

Oocytes were obtained and matured *in vitro* as reported previously [7, 8]. Briefly, ewe ovaries obtained from a local slaughterhouse were brought to laboratory in Dulbecco's Phosphate Buffer Saline (D-PBS)(supplemented with penicillin G (250 IU/ml), streptomycin sulfate (125 µg/ml) and neomycin (125 µg/ml)) and incubated at 30-35 °C. COCs were recovered by slicing the ovaries with scalpel blade and washed three times with tissue culture medium TCM 199 (supplemented with hepes 10.5 mM, Na Hepes 10.5 mM, 0.33 mg/ml Na bicarbonate, 0.01 mg/ml heparin sodium salt, 62.5 IU/ml penicillin G, 0.05 mg/ml streptomycin sulfate and 3 mg/ml Bovine Serum Albumin (BSA)). After washing, a group groups of 35-40 COCs were transferred to 600 µl of TCM-199maturation medium (supplemented with 10% FCS, 1 mM Na-pyruvate, 10 µg/ml FSH and LH 10 µg/ml) under mineral oil and incubated in 4-well Petri dishes for 24 h at 38.5 °C in a humidified atmosphere of 5% CO_2.

***In vitro* fertilization of *in vitro* matured oocytes**

For IVF, matured COCs were washed twice in BSOF as described previously [7, 8]. Oocytes were then transferred into four-well plates containing 500 µl of BSOF medium supplemented with 2% estrus sheep serum per well (30 oocytes per well). For sperm preparation fresh semen from the same ram was prepared either by Percoll gradient centrifugation or by swim-up method as mention previously. The spermatozoa were counted on a haemocytometer and diluted in the appropriate volume of BSOF and added to each well, with a final concentration of $1X10^6$ spermatozoa/ml. The plates were then incubated for 21 h, in 5% CO_2 in humidified air, at 38.5°C. The COCs obtained from a given batch of ovaries constituted a replicate; a total of ten replicates were involved in this experiment.

***In vitro* culture of *in vitro* fertilized oocytes**

Embryo culture was carried out in Synthetic Oviduct Fluid (SOF) medium under paraffin oil in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N at 38.5°C. Twenty-one hours post in vitro fertilization; presumptive groups of 20–30 zygotes were denuded by repeated pipetting and washed in SOF before being transferred into 50 µl of culture droplets. Cleavage rate was assessed 48 h after placement in culture. The number of embryos that developed to the blastocyst stage was assessed on days 6 and 8 post insemination.

Statistical analysis

The results of ten independent experiments for sperm parameters were expressed as Mean +/- SD. Means were analyzed using a one-way analysis of variance, followed by Duncan's post hoc test to determine significant differences in all the parameters between all groups using the SPSS computer program (version 20; SPSS, IBM, Chicago, IL). Differences were considered statistically significant when the *P* values are less than 0.05.

The data obtained from the result of IVF, were analyzed using Mann-Whitney-U Test of using SPSS and *P*-values less than 0.05 were consider statistically significant.

Results

SPERM MOTILITY AND MORPHOLOGY

The effect of sperm preparation with swim-up and Percoll gradient method on sperm motility, progressive motility and morphology are presented in Table I. Overall sperm motility of the two separation methods was not significantly differ from the fresh samples, however progressive motility of sperms treated with the Percoll method was significantly higher (64.6%) than fresh untreated samples (42.4%) ($P<0.05$) with non-significant ($P>0.05$) higher rates than results obtained from swim-up separation method. The percent of total normal sperm morphology in untreated sample and sperms treated with Percoll were significantly higher than swim-up method ($P<0.05$). There was a significant increased tail defects with sperms obtained by two separation methods when compared to the control untreated raw semen. Acrosome defects were significantly lower in Percoll group in comparison to swim-up group (6.0% and 9.9% respectively). There were no significant differences related to the defect rates of head and mid-piece between all groups.

SPERM MEMBRANE, ACROSOME AND CHROMOSOME INTEGRITY

The result of Hypo-Osmotic Swelling Test (HOST), acrosome, membrane and chromosome integrity are shown in Table II. Our result showed a significant increased percent of membrane integrity tested by both HOST and CFDI/PI method, as well as the acrosome and chromosome integrity of sperm separated by Percoll method ($P<0.05$). Intact chromosome (light stained) rates were significantly higher in sperm treated by Percoll than swim-up and fresh groups ($P<0.05$).

IN VITRO FERTILIZATION

The capability of sperm separated by swim-up and Percoll gradient methods for *in vitro* fertilization are shown in Table III. Although the overall cleavage rates and degenerated oocyte rates fertilized with Percoll gradient separated sperm (76.1%) were not significantly different from those fertilized with swim-up separated sperm (79.2%), the rate of blastocyst (14.1%) obtained with the former method was significantly higher than of the later method ($P<0.001$). Morula rate

			Motility %			Sperm Morphology		
	M	PM	TNS	TDS	ADs	HD	MD	TD
Fresh	86.8±3.5	42.4±4.7^{b}	73.2±3.0^{a}	26.8±3.0^{b}	7.30±1.7^{ab}	8.20±1.9	2.20±0.6	9.10±2.7^{b}
Swim- Up	82.1±2.1	60.6±3.3^{a}	62.9±1.5^{b}	37.1±1.5^{a}	9.90±1.3^{a}	5.70±1.2	3.10±0.5	18.4±1.2^{a}
Percoll	86.2±1.9	64.1±3.4^{ab}	71.1±1.9^{a}	29.9±1.9^{b}	6.00±0.7^{b}	5.80±0.7	2.40±0.3	15.7±1.2^{a}

^{ab} values in a column with different superscript differ ($P<0.05$).
The results are obtained on ten independent experiments and expressed as mean ± SD.
Motility was analyzed by CASA whereas sperm morphology rates were measured by phase contrast microscopy following formaldehyde fixation.
M: motility; PM: progressive motility; TNS: total normal sperm; TDS: total defect sperm, AD: acrosome defects; HD: head defects; MD: mid-piece defects; TD: tail defects

Table I: Sperm parameters (Motility, Progressive Motility, and Morphology)

			Membrane integrity			DNA integrity		
	HOST +	**IA**	**IM**	**DM**	**M**	**LS**	**MS**	**DS**
Fresh	55.2±4.1^{b}	82.5±3.7^{a}	62.9±5.6^{b}	21.4±4.6^{b}	15.6±1.5^{b}	52.4±0.9^{b}	45.8±0.6	1.80±0.4^{a}
Swim Up	54.6±3.6^{b}	75.8±2.0^{b}	61.6±2.9^{b}	31.8±2.9^{a}	16.5±0.9^{b}	53.6±1.5^{b}	44.6±1.7	1.80±0.2^{a}
Percoll	66.7±2.8^{a}	87.2±1.6^{a}	75.8±1.9^{a}	13.4±1.5^{c}	10.7±0.6^{b}	59.4±0.9^{a}	47.8±2.6	0.20±0.1^{b}

^{abc} values in a column with different superscript differ ($P<0.05$).
The results are obtained on ten independent experiments and expressed as mean ± SD
Membrane integrity was analyzed by CFDI/PI whereas DNA integrity was measured after toluidine blue coloration. HOST+: Hypo-osmotic swelling test positive; IA: intact acrosome (analyzed by PNA/FICH); IM: intact membrane; DM: damaged membrane; M: moribund; LS: light-stained; MS: mid-stained; DS: dark-stained

Table II: HOST, Membrane, Acrosome and DNA Integrity

Protocol	Oocyte No.	Cleavage (%)	Degenerated (%)	Morulae (%)	Blastocyst (%)
Swim Up	1238	980 (79.2)	98 (7.9)	816 (65.9)^{a}	66 (5.3)^{a}
Percoll	1490	1134 (76.1)	84 (5.6)	840 (56.4)^{b}	210 (14.1)^{b}

^{ab} values in a column with different superscript differ ($P<0.001$).

Table III: cleavage, morula and blastocyst rates after in vitro fertilization by sperm separated by swim-up and Percoll gradient methods.

obtained by swim-up method was significantly higher than those obtained by Percoll gradient method (*P*<0.001).

Discussion

Many studies have been carried out to develop semen preparation methods to obtain sperms with good quality and superior parameters to achieve the highest *in vitro* and *in vivo* fertilization rates. Studies conducted on this subject generally focused on motility, morphology and viability of the sperm and compared them among methods. Sperm fertilization ability and blastocyst production rates are not only dependent on these parameterizations. Assessment of sperm cell membrane and acrosome stability as well as other sperm characteristics especially DNA integrity could provide valuable information regarding sperm fertilizing ability [2, 14]. Both swim-up and percoll gradient methods include centrifugation steps, which may cause harmful effects on spermatozoa DNA [18, 26]. While similar results can be achieved with Toluidine Blue Staining (TBS) using light microscopy and reasonable low cost materials [16, 18], techniques such as TdT-mediated-dUTP nick end labeling (TUNEL) and COMET or single gel electrophoresis have been used to evaluate DNA integrity [12, 20, 28]. These methods require very expensive equipment and kits as well as labor work.

The greatest acrosome intactness rate in the present study was achieved by Percoll gradient centrifugation method. There were no significant differences in acrosome integrity between Percoll and untreated semen whereas swim-up method significantly causes a negative effect on acrosome integrity.

Although, there have been many studies comparing spermatozoa separation methods in human and bovine, reports on other animals especially on small ruminant spermatozoa were limited. Swim-up procedures have been compared with percoll density gradients in separating poor-quality human sperm, the later have been suggested as the method of choice for separation of sperm for all human-assisted reproductive techniques before 1995 [22]. While percoll was not allowed for human semen processing by World Health Organization since 1999 [24], it is still the preferred spermatozoa separation method used to produce *in-vitro* derived embryos of domestic animals[10].

Sheep embryos have been produced in vitro using sperm prepared with swim-up and percoll gradient[19, 29] techniques from fresh semen. However, no direct comparison has been reported yet regarding the spermatozoa quality. Our study compared the quality of sperm separated from fresh-collected ram semen by swim-up or Percoll gradient techniques.

Percoll separation method yields significantly higher rates of viable sperm and maintain intact acrosome and cell membranes. At the same time there were a significant differences in morula and blastocyst formation rates after *in vitro* fertilization by spermatozoa separated by Percoll method. These findings indicate that spermatozoa DNA integrity was not significantly corrupted. We confirmed in the present study as there was a significant increase in DNA integrity of spermatozoa separated by Percoll gradient compared to those separated with swim-up method. This findinding is in agreement with previous studies that DNA intactness could affect IVF derived embryo rates [2, 14]. Even though, swim-up and percoll separation techniques maintain the total motility, progressive motility and morphological abnormalities of head, mid piece and tail, percoll method showed a significantly greater number of spermatozoa with intact membrane, DNA, intact acrosome and viability rates.

In contrast to our results, Marti et al., [19] showed that swim-up method yield higher viability rates than Percoll treated spermatozoa (67.4% versus 55.9%), our results showed a significant higher intact membrane rates of spermatozoa treated by percoll compared to swim up method (75.86% versus 61.60%). While we used a centrifugation time of 15 and 5 minutes at 600 and 300 × g respectively, the last researcher centrifuged semen for three times at 200, 1200 and 700 × g for 5, 15 and 5 minutes respectively. The different centrifugation velocity, duration and times differences could be the reason of their results were lower than ours.

Following Percoll density gradient centrifugation, we observed a significant increase in spermatozoa with intact DNA compared to fresh and swim-up treated spermatozoa. This finding was in contrast with Armand zini et al.,[33]who reported that density-gradient centrifugation couldn't select spermatozoa with higher DNA integrity. The difference in spermatozoa origin (human versus sheep) between the two experiments could be one of the reasons.

Our study shows that sperm processing with Percoll gradient centrifugation method significantly less harm DNA integrity based on Toluidine Blue staining compared to fresh un-treated or swim-up treated spermatozoa. This could be due to the stress caused by the relatively longer time period required compared to swim-up method [17]. DNA integrity is related to membrane and acrosome intactness as well as the viability rates. In addition, the IVF results showed that percoll treated spermatozoa resulted in significant higher morulae and blastocyst rates compared to swim-up treated spermatozoa. All these findings strongly suggest that Percoll sperm separation method could significantly reduce ram sperm defects during semen preparation for assisted reproductive techniques. The sperm separation method gives controversial results in different species. Previous work of Mehmood A. et al.,[21] found significantly increased rates of membrane integrity after separation of buffalo semen with swim-up method. This might be due to the species sperm structure differences or due to different semen source. In their study, they used a frozen/thawed semen compared to fresh semen in the present study. The effect of freezing/thawing process on spermatozoa membrane could be more

efficient to separate frozen semen with swim-up and fresh semen with Percoll method. On the other hand, Olivares et al.[23] suggested that Percoll method could be indicated when working to separate frozen sperm whereas swim-up when separating fresh goat semen. Olivares et al., [23] reported that mini-percoll density gradient could be the method of choice for goat frozen/thawed sperm selection which is in consistent with our results.

Percoll gradient can be used to improve the quality of spermatozoa; these are the spermatozoa that are needed for fertilization of invitro matured ewe oocytes in Assisted Reproduction Techniques. Sperm samples obtained after Percoll gradient centrifugation may be improved for motility, morphologically normal spermatozoa with intact DNA, acrosome and cell membrane. Accordingly, it was concluded that the Percoll gradient separation method is more useful than the swim-up for in vitro sheep embryo production. Also, Toluidine blue staining could be a suitable technique for determination of DNA integrity.

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