JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

TEMPERATURE-DEPENDENT EFFECTS OF DENSITY GRADIENT CENTRIFUGATION ON MULTIPLE SPERM QUALITY PARAMETERS: A COMPARATIVE ANALYSIS AT 4°C, 20°C, AND 37°C

Michal Ďuračka[1], Filip Benko[2], Eva Tvrdá[2,3]*

Address(es): Ing. Michal Ďuračka, PhD.,
[1] Slovak University of Agriculture in Nitra, AgroBioTech Research Centre, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, +421 37 641 4918.
[2] Slovak University of Agriculture in Nitra, Institute of Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, +421 37 641 4918.
[3] Estonian University of Life Sciences, Institute of Animal Breeding and Biotechnology, F. R. Kreutzwaldi 1, 510 06 Tartu, Estonia.

*Corresponding author: michal.duracka@uniag.sk

https://doi.org/10.55251/jmbfs.12088

ARTICLE INFO

Received 21. 11. 2024
Revised 8. 5. 2025
Accepted 19. 5. 2025
Published 1. 8. 2025

Regular article

ABSTRACT

Percoll medium, a density gradient solution, is widely employed in reproductive biotechnologies for the efficient isolation of spermatozoa based on their specific density. This study examined the impact of temperature (4°C, 20°C, and 37°C) during and after Percoll separation on various sperm quality indicators. Semen samples from Holstein breeding bulls (n = 6) were layered onto density gradient solutions in 15 mL falcon tubes. Following centrifugation (15 min, 400 × g), sperm cells were washed to remove residual Percoll medium. Multiple parameters were assessed immediately post-separation and after 2 hours, including sperm motility, mitochondrial membrane potential, reactive oxygen species production, membrane and acrosome integrity, and DNA fragmentation. Statistical analysis employed two-way ANOVA with Tukey's multiple comparison test. The results showed a significant decrease in sperm motility after 2 h at 4°C, while mitochondrial membrane potential was least affected at 37°C. Membrane integrity remained stable at 37°C but was significantly disrupted at lower temperatures, particularly at 4°C (P<0.0001), with a corresponding increase in necrotic cells when compared to 37°C after 2h (P<0.0001). Although the lowest ROS production was recorded at 4°C in both times, the differences between temperature groups were not significant. DNA fragmentation increased significantly at higher temperatures (20°C and 37°C) compared to 4°C. In conclusion, while lower temperature (4°C) reduced ROS production and DNA fragmentation, other parameters such as motility, mitochondrial membrane potential, and membrane and acrosome integrity were best preserved at 37°C.

Keywords: density gradient separation, temperature, sperm quality parameters, ROS production, DNA fragmentation

INTRODUCTION

Percoll, a gradient medium for density gradient centrifugation (DGC), was for the first time introduced by Pertoft *et al.* in 1978 as colloidal silica particles firmly coated with polyvinylpyrrolidone with an average diameter of 17 nm. The medium is highly suitable for the separation of various cell types without significant consequences on cells and organelles in a density range of 1.00-1.20 g/mL (**Pertoft *et al.*, 1978**). This density range perfectly overlaps the density range of sperm cells (1.02-1.13 g/mL) originating from several species (**Lessley & Garner, 1983**). Afterwards, **Parrish *et al.* (1995**) established the gradient density centrifugation protocol on bovine spermatozoa for use in assisted reproductive technology techniques which is still used in its original version (**Arias *et al.*, 2017**) or with variations (**Ďuračka *et al.*, 2021**) till now. Besides the possibility of removing seminal plasma, diluents, impurities, other cells and bacteria (**Cojkic *et al.*, 2024**), the biggest advantage of density gradient separation lies in separating damaged spermatozoa from viable ones, which increases the overall quality of semen sample. Moreover, Percoll DGC effectively separated X and Y spermatozoa, establishing its potential as an available method for sperm sex-sorting (**Simbolon *et al.*, 2024**). After centrifugation, prooxidative agents such as leukocytes, cell debris, and morphologically abnormal spermatozoa are located separately from mature and motile spermatozoa at the bottom of a falcon tube (**Malvezzi *et al.*, 2014**). However, due to seminal plasma removal, sperm lack external antioxidant protection. Also, reactive oxygen species (ROS) are still produced due to shearing forces during centrifugation (**Aitken & Clarkson, 1988**). The detrimental effects of oxidative stress on sperm quality are well-studied and lead to impaired flagellar movement and membrane disruptions. Sperm DNA is highly susceptible to oxidative stress and its damage can significantly affect egg fertilization success. Even after fertilization, embryonal abnormalities affecting embryo development may occur (**Aitken *et al.*, 2022; González-Marín *et al.*, 2012; Sanocka & Kurpisz, 2004**). Previous studies demonstrated that the duration of centrifugation has a more significant impact on reactive oxygen species (ROS) production during the process than the centrifugal force applied (**Salam *et al.*, 2020; Shekarriz *et al.*, 2017**). **Aitken *et al.* (2010**) revealed that elevated ROS levels during DGC can induce oxidative damage to sperm DNA while not impacting sperm viability. Therefore, reducing the ROS production during the centrifugation is desirable. As shown by **Thijssen *et al.* (2014**), storage temperature is a key factor influencing spermatozoa quality after DGC. Highly fertile semen exhibits high oxidative phosphorylation activity, thus producing a large amount of ROS (**Gibb & Aitken, 2016**). We hypothesized that decreasing the storage temperature before DGC could reduce the metabolic rate, and thus the amount of ROS produced during and after the centrifugation. Therefore, this study focused on the effect of various temperatures during and after the DGC procedure on multiple spermatozoa quality parameters.

MATERIAL AND METHODS

Semen collection, study design and DGC

In this study, three different temperatures (37, 20, and 4°C) were applied during and after the DGC procedure. Percoll density gradient medium (Danaher, Washington, D.C., MD, USA) was diluted to 90% and 45% with Sp-TALP medium according to **Parrish *et al.* (1988**) and layered on the top of each other in a 15-mL falcon tube. Semen samples (n=6) from healthy Holstein breeding bulls (n=6) were collected during a regular collection and transported to our laboratory (approximately 20 min.; held in thermos pre-warmed to 37°C). Each sample was analysed in triplicate. After the transportation, part of the ejaculate was immediately layered onto the top of the pre-warmed percoll solution in a falcon tube. Another part was cooled to room temperature (20°C) for 15 min., respectively gradually cooled to refrigerator temperature for 30 min. (15 min. at 20°C and 15 min. at 4°C). After the equilibration, semen samples were layered onto the pre-equilibrated percoll solutions. During the whole procedure of DGC, the temperature was stable for each experimental group. After the first centrifugation (400 × g, 15 min.), the sperm pellet was washed in 3 mL BGM-3 medium (equilibrated to the corresponding temperature) and centrifugated at 400 × g for 5 min. The supernatant was removed and sperm was diluted to 50×10^6 sperm per mL of BGM-3 medium (equilibrated to the corresponding temperature) (**Duracka *et al.*, 2022; Parrish, 2014**). Before the sperm quality analyses, an aliquot of each sample was equilibrated to 37°C.

Computer-assisted semen analysis (CASA)

Total sperm motility (movement of spermatozoa ≥ 5 µm/s) was analyzed using the CASA system (HTM TOX IVOS II, Hamilton-Thorne Biosciences, Beverly, MA, USA) and Makler counting chamber (depth 10 µm; Sefi Medical Instruments, Haifa, Israel). Each sample (10 µL) was placed into the pre-warmed counting chamber and the movement of spermatozoa was objectively evaluated in 10 microscopic fields, while at least 300 cells were analyzed.

JC-1 analysis

Cathionic lipophilic JC-1 (Cayman Chemical, Ann Arbour, MI, USA) dye diluted to 5 mg/mL in Dulbecco's phosphate buffered saline solution (DPBS, Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate sperm mitochondrial membrane potential. One million cells were stained in each group for 30 min. at 37°C and the remaining dye was rewashed twice with 200 µL DPBS (600 × g, 5 min.) and diluted with 100 µL DPBS. The fluorimetric analyses were performed using the Glomax Multi$^+$ (Promega, USA) on a 96-well dark plate. The JC-1 polymers emit red fluorescence, while the monomeric form emits green fluorescence light (**Agnihotri *et al*., 2016**). The results are expressed as the rate of polymers to monomers.

Sperm membrane integrity assessment

Fluorescent staining was performed to evaluate sperm membrane intactness. Samples were incubated (37°C, 20 min.) with 10 µL carboxyfluorescein diacetate (CFDA; 0.75 mg/mL in dimethyl sulfoxide; DMSO) that emits a green fluorescence and with 10 µL 4′,6-diamidino-2-phenylindole (DAPI; 1 µM in DPBS) that emits a blue fluorescence. Moreover, necrotic cells were stained with 10 µL propidium iodide (PI; 2 mg/mL in DPBS) emitting a red fluorescence. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Afterwards, samples were centrifuged (600 × g, 5 min), washed with 200 µL DPBS twice, and transferred to a 96-well dark microplate. Fluorescence was measured using the Glomax Multi$^+$ (Promega, USA) (**Tvrdá *et al*., 2020**).

Sperm acrosome integrity assessment

The sperm acrosome integrity was evaluated using Peanut agglutinin (PNA-FITC conjugate) lectin from *Arachis hypogaea* which binds to the damaged acrosome. Each sample was incubated with 100 µL of 10 µM PNA in DPBS and 10 µL of 1 µM DAPI in DPBS for 30 min at 37°C. Afterwards, samples were transferred to a 96-well dark microplate and fluorescence was measured using the Glomax Multi$^+$ (**Benko *et al*., 2022**). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Global ROS production

Levels of ROS were evaluated with the chemiluminescent assay that utilizes luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) as a probe. Each sample was mixed with luminol (5 mM; 2.5 µL) in a 96-well microtitration plate and subjected to analysis on the Glomax Multi$^+$. Each measurement included negative (100 µL DPBS, 12.5 µL 30% H_2O_2, 2.5 µL) and positive control (100 µL DPBS, 2.5 µL) and blank (100 µL DPBS) (**Tvrdá *et al*., 2020**). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Results were expressed as relative light units (RLU)/s/10^6 spermatozoa.

Sperm chromatin structure assay

Sperm DNA damage assay was processed following the Januskauskas's *et al*. study (2001). In brief, sperm concentration was adjusted to 2 × 10^6 sperm/mL with TNE buffer, mixed with acid detergent and after 30 s at room temperature, samples were stained with acridine orange solution. The analysis of double-stranded DNA (green fluorescence) and single-stranded DNA (red fluorescence) was performed using the Glomax Multi$^+$ on a dark 96-well plate. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

Data obtained from the CASA system and the Glomax Multi$^+$ were evaluated using the GraphPad Prism program (version 8.4.3; GraphPad Software, San Diego, CA, USA). A two-way analysis of variance (ANOVA) was conducted, followed by Tukey's post hoc test, to assess differences in the observed data within and between measurement time points. The significance levels were set at *p<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. The graphs show mean values with error bars representing standard deviations.

RESULTS

Sperm motility evaluation

Our study utilizes six different semen samples collected from healthy Holstein breeding bulls that were subjected to DGC and incubated at different temperatures to observe the post-processing impact on sperm quality. Sperm motility did not show any significant differences amongst the groups immediately after the DGC procedure (0 h). Sperm motility decreased significantly after 2 hours of incubation at 4°C compared to both the initial measurement (0 h; Figure 1a) and to samples incubated at other temperatures for the same duration (Figure 1b).

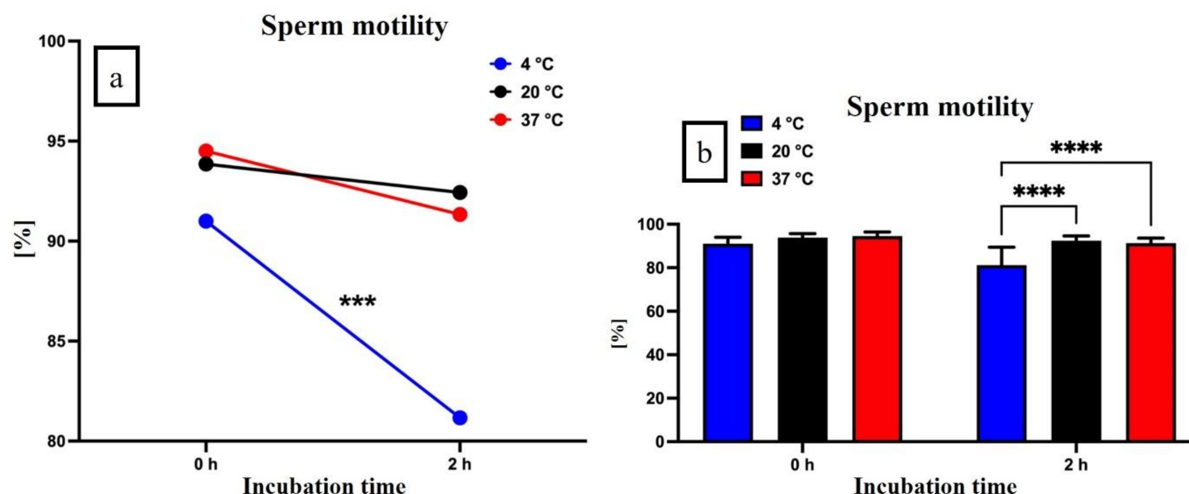

Figure 1a Development of sperm motility after DGC. **Figure 1b** Differences in sperm motility amongst the individual groups immediately after DGC and 2 h post-separation. The significance of differences between the observed groups/times is marked as *** P<0.001 or ****P<0.0001.

Mitochondrial membrane potential assessment

The green-to-red fluorescence ratio was significantly changed in each group after 2 h incubation compared to the initial ratios within the same groups. The most significant decrease in mitochondrial membrane potential (Figure 2a) was recorded at 20°C (P<0.0001). However, the lowest mitochondrial membrane potential was observed at 4°C. The highest mitochondrial membrane potential immediately after DGC was preserved at 37°C, which was significantly higher than at 4°C (P<0.05). After 2 h of incubation (Figure 2b), mitochondrial membrane potential in spermatozoa held at 37°C was significantly higher than sperm incubated at 20°C (P<0.05) and 4°C (P<0.01).

Sperm membrane integrity measurement

The eosin-nigrosin analysis showed that the highest percentage of sperm with intact membranes was preserved after DGC at 4°C while being significantly higher (P<0.05) than at 37°C at the same time (Figure 3b). As shown in Figure 3a, after 2 h, semen samples stored at 4°C recorded the most significant decrease (P<0.0001). On the other hand, sperm membrane integrity was not reduced when incubated for 2 h at 37°C when compared to the initial measurement, but a substantially higher percentage of sperm with intact membranes (P<0.05) was shown when compared to sperm held at 4°C.

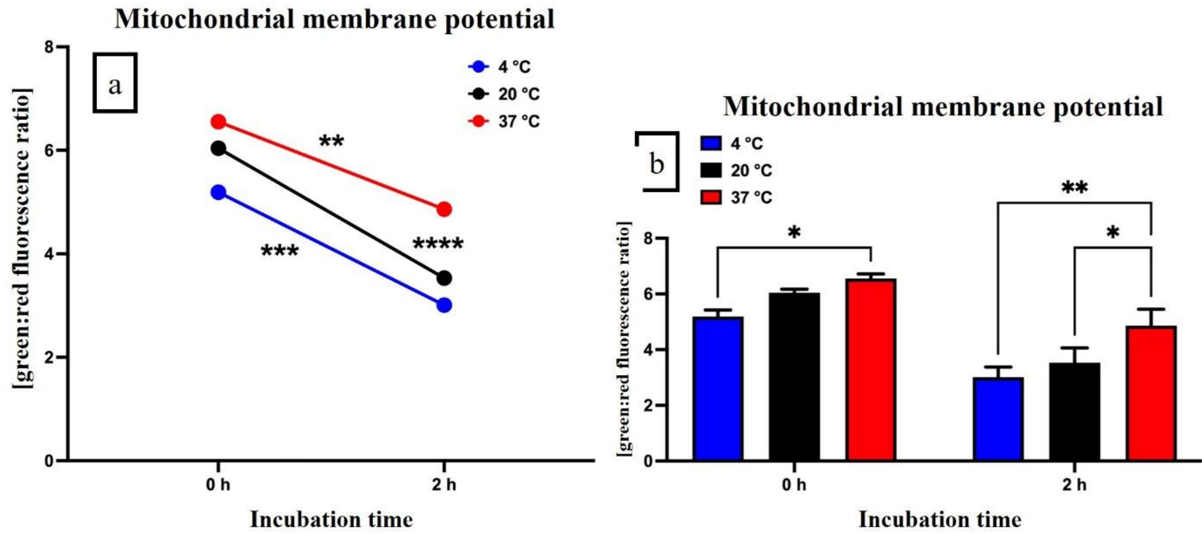

Figure 2a Development of sperm mitochondrial membrane potential after DGC. **Figure 2b** Differences in sperm mitochondrial membrane potential amongst the individual groups immediately after DGC and 2 h post-separation. The significance of differences between the observed groups/times is marked as * P<0.05, ** P<0.01, *** P<0.001 or ****P<0.0001.

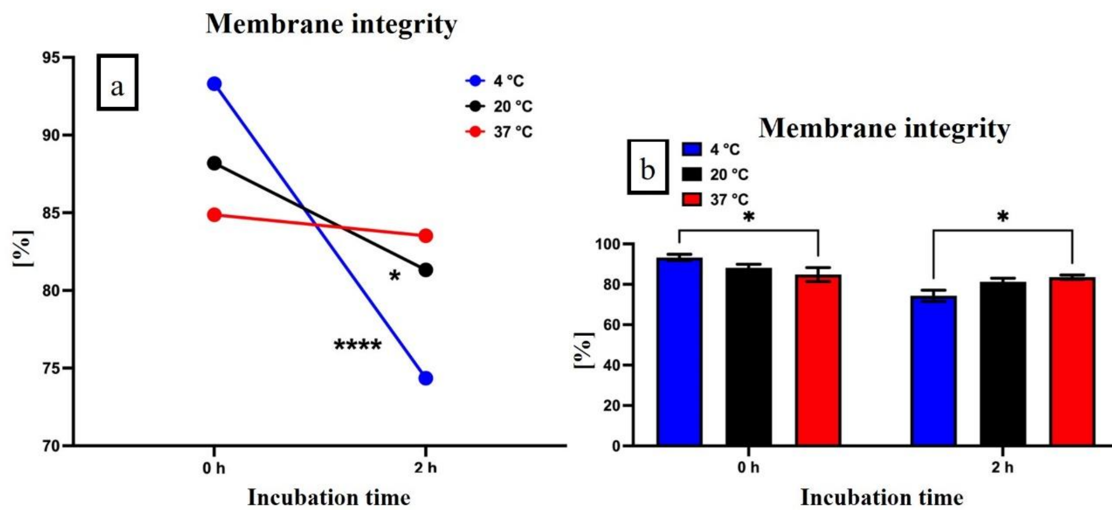

Figure 3a Development of sperm membrane damage after DGC. **Figure 3b** Differences in sperm membrane integrity amongst the individual groups immediately after DGC and 2 h post-separation. The significance of differences between the observed groups/times is marked as * P<0.05 or ****P<0.0001.

Representation of necrotic cells

The initial measurements did not show any significant differences in the representation of propidium-iodide-positive cells within and among the groups. After 2 h of incubation, a noticeable increase of necrotic cells was recorded at 4°C and 20°C when compared to the initial measurement within the groups (Figure 4a). The lowest proportion of necrotic cells after 2 h was observed at 37°C, which was significantly lower (P<0.01) than at 4°C (Figure 4b).

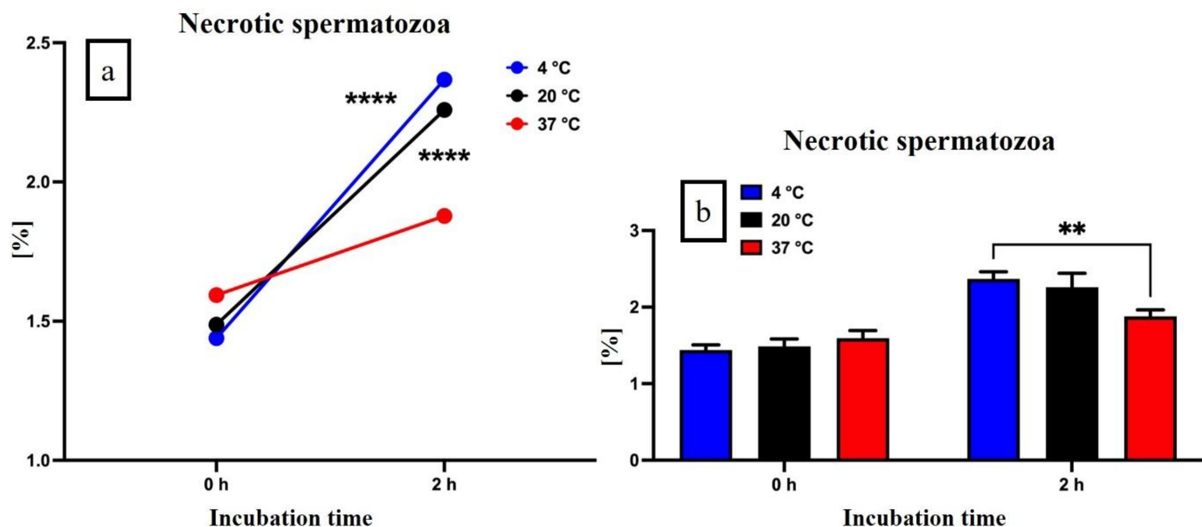

Figure 4a Development of necrotic cell counts after DGC. **Figure 4b** Differences in representation of necrotic cells amongst the individual groups immediately after DGC and 2 h post-separation. The significance of differences between the observed groups/times is marked as ** P<0.01 or ****P<0.0001.

ROS production analysis

The lowest production of ROS was recorded at 4°C in both times of analysis (Figure 5b). With the rising temperature, the ROS concentration increased, while the highest temperature was also characterized by the largest ROS production (Figure 5a). Each group recorded a similar significant (P<0.01) change after 2 h when compared to its 0 h measurement.

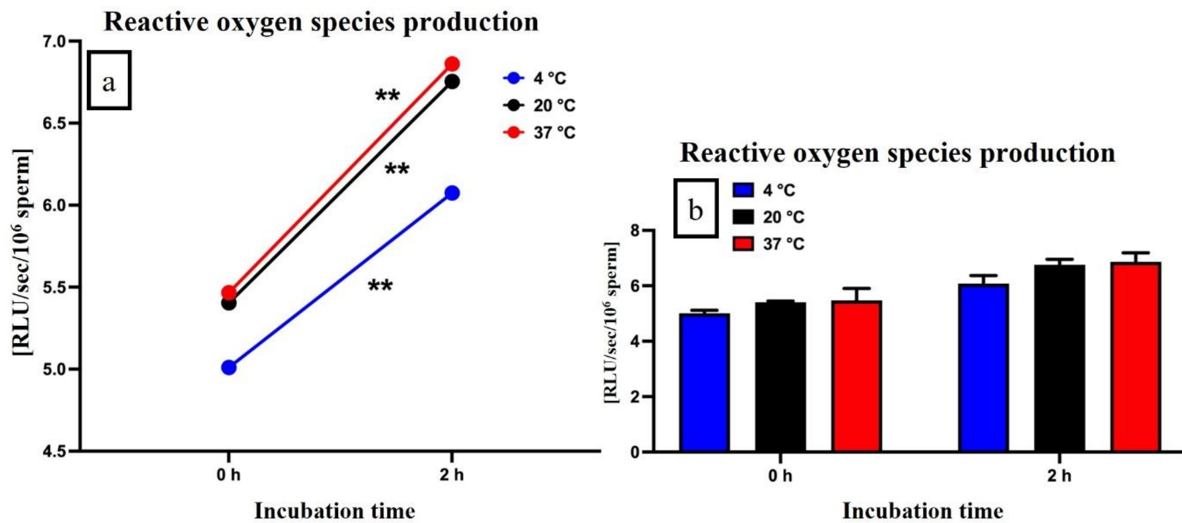

Figure 5a Development of the ROS production after DGC. **Figure 5b** Differences in the ROS production amongst the individual groups immediately after DGC and 2 h post-separation. The significance of differences between the observed groups/times is marked as ** P<0.01.

Sperm DNA fragmentation

The index of sperm DNA fragmentation showed significant deterioration (P<0.0001) in each monitored group after 2 h compared to the initial measurement (Figure 6a). However, the group held at 37°C showed a significantly higher representation of sperm with fragmented DNA at both times of analysis than those held at 4°C (P<0.0001). Additionally, the samples processed and incubated at 20°C performed higher sperm DNA damage at 0 h (P<0.01) and 2 h (P<0.0001) compared to 4°C (Figure 6b).

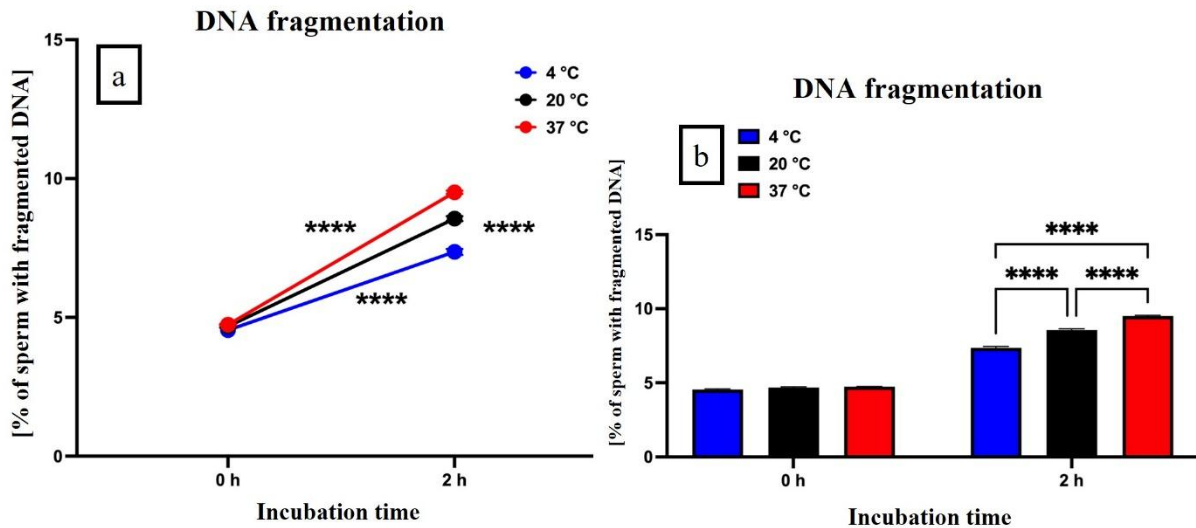

Figure 6a Development of the sperm DNA fragmentation after DGC. **Figure 6b** Differences in the sperm DNA fragmentation amongst the individual groups immediately after DGC and 2 h post-separation. The significance of differences between the observed groups/times is marked as ** P<0.01 or **** P<0.0001.

DISCUSSION

Numerous studies have examined how Percoll DGC affect sperm recovery rates and quality improvement across different animal species, improving their motility, membrane integrity, mitochondrial potential, acrosome and DNA integrity (**Arias *et al*., 2017; Camargo *et al*., 2023; Eberhardt *et al*., 2022; Oliveira *et al*., 2012; Parrish *et al*., 1995; Samardžija *et al*., 2006; Sepúlveda *et al*., 2018; Silva *et al*., 2020**). To date, it has not been investigated the impact of temperature during DGC on sperm quality. **Thijssen *et al*.** (**2014**) studied long-term *in vitro* sperm incubation (24 h) on human semen samples at testis temperature (35°C) and room temperature (23°C). Their results indicate that parameters like progressive motility, normal morphology, viability and acrosome integrity were better preserved at room temperature, while apoptotic cells were significantly lower compared to those incubated at 35°C. However, the impact of temperature during DGC has not been investigated so far. Studies mentioned above showed that DGC can effectively remove sources of ROS by removing immotile and damaged spermatozoa. Nevertheless, ROS formation during repeated centrifugation is not negligible. Consequently, optimizing centrifugation processes in assisted reproductive techniques is essential. Decreasing temperature during *in vitro* incubation can mitigate ROS formation by lowering metabolic rate (**Gibb & Aitken, 2016**). Therefore, our hypothesis posited that sustaining low temperatures before and during DGC could attenuate metabolic rate and ROS formation, thereby enhancing long-term sperm quality. Our experimental findings confirmed this hypothesis, demonstrating that reduced processing temperatures during DGC resulted in decreased ROS levels. Despite that sperm motility was not significantly affected immediately after DGC, mitochondrial membrane potential was affected at 4°C immediately after DGC and also at room temperature after 2 h post-DGC. **Sieme *et al*.** (**2015**) reported that cooling causes minor thermotropic non-cooperative phase transitions decreasing fluidity in membranes and domain formation. **Ortega-Ferrusola *et al*.** (**2008**) noted that centrifugation (600 × g, 10 min.) and cooling to 4°C did not affect most apoptotic markers except for mitochondrial membrane potential, showing increased sensitivity to a combination of mechanical and thermal stress. As previously reported by **Alamo *et al*.** (**2020**), mitochondrial membrane potential could serve as a predictive biomarker for sperm motility, which is particularly relevant for assisted reproductive techniques, as it allows for the early identification of sperm with diminished fertilizing potential. Distinct lipid phase transitions were observed during cooling and reheating, with different effects on the fluidity of sperm head or body regions (**Canvin & Buhr, 1989**). **Kim *et al*.** (**2012**) discussed that cooling down to 4°C was primarily responsible for the reduced sperm motility and destabilization of sperm plasma membrane in rats. Our study demonstrated that cooling bovine semen samples for the duration required for DGC may enhance the proportion of spermatozoa with

intact plasma membranes relative to 37°C. Preserving sperm in a cold environment following the DGC procedure may significantly increase the occurrence of sperm with membrane damage, as evidenced by the higher number of necrotic (propidium iodide-positive) cells. Research performed by **Aitken *et al*. (2010)** demonstrated that the majority of cells exhibiting positive TUNEL and 8OHdG markers were non-viable both before and following Percoll processing. While DGC effectively selected for motile and viable spermatozoa, thereby reducing the TUNEL-positive cell population, it simultaneously increased the proportion of viable cells with elevated 8OHdG levels in both patient and donor cohorts. Notably, this resulted in an increased frequency of viable TUNEL-positive spermatozoa exclusively in the patient population. These observations were primarily attributed to ROS generation during gradient centrifugation, rather than solely to the mechanical forces of centrifugation, although the latter can independently elevate ROS levels (**Aitken & Clarkson, 1988**). **Iwasaki and Gagnon (1992)** documented a four- to five-fold elevation in ROS levels in Percoll-processed spermatozoa compared to unprocessed samples. Temperature exposure has been demonstrated to significantly modulate DNA integrity in both unprocessed seminal specimens and processed spermatozoa. Thermal exposure at physiological temperature (37°C) induces accelerated deterioration of sperm DNA compared to sub-physiological thermal conditions. Specifically, when freshly liquefied seminal samples undergo one hour of incubation at 37°C, they exhibit significantly elevated levels of phosphatidylserine externalization and DNA fragmentation, as measured by TUNEL assay, relative to parallel specimens maintained at 34°C or 25°C **(Balasuriya *et al*., 2014; Matsuura *et al*., 2010)**. Our observations have revealed that fresh bovine semen subjected to 4°C before, during and following DGC had a significantly lower percentage of spermatozoa with damaged DNA than spermatozoa held at room temperature and physiological temperature. This temperature-dependent preservation of DNA integrity suggests the potential role of reduced thermal stress in maintaining genomic stability during sperm processing procedures. Therefore, we may hypothesize that the implementation of mid- or low-temperature protocols during sperm preparation for assisted reproductive technologies could enhance genomic integrity preservation. Specifically, if reduced processing temperature minimize DNA damage during and after DGC, the adaptation of current protocols might potentially improve reproductive outcomes.

CONCLUSION

In conclusion, this study provides evidence that temperature may significantly modulate sperm quality immediately after density gradient centrifugation as well as after this procedure in the long term. Our results demonstrated that maintenance of physiological temperature (37°C) before, during and after the density gradient centrifugation procedure helped preserve motion-related parameters such as sperm motility and mitochondrial membrane potential. Moreover, the sperm membrane was better preserved at 37°C, substantiated also by lower necrotic cell rate. Based on our results it cannot be fully understood if decreased ROS generation is responsible for lowering DNA damage under low temperatures, as there was no significant difference in ROS production between the individual groups. These findings have important implications for assisted reproductive technologies and their implementation could potentially enhance reproductive outcomes in specific cases when DNA stability is more prioritized than motion characteristics of spermatozoa. This pilot study warrants further research with a larger sample size and more in-depth investigation.

Acknowledgements: This research was supported by the Slovak Research and Development Agency of the Ministry of Education, research, development and Youth of the Slovak Republic (grant no. APVV-21-0095) and by the Scientific grant agency under grant no. VEGA 1/0067/24.

REFERENCES

Agnihotri, S. K., Agrawal, A. K., Hakim, B. A., Vishwakarma, A. L., Narender, T., Sachan, R., & Sachdev, M. (2016). Mitochondrial membrane potential (MMP) regulates sperm motility. *In Vitro Cellular & Developmental Biology - Animal*, *52*(9), 953–960. https://doi.org/10.1007/s11626-016-0061-x

Aitken, R. J., & Clarkson, J. S. (1988). Significance of Reactive Oxygen Species and Antioxidants in Defining the Efficacy of Sperm Preparation Techniques. *Journal of Andrology*, *9*(6), 367–376. https://doi.org/10.1002/j.1939-4640.1988.tb01067.x

Aitken, R. J., De Iuliis, G. N., Finnie, J. M., Hedges, A., & McLachlan, R. I. (2010). Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: Development of diagnostic criteria. *Human Reproduction*, *25*(10), 2415–2426. https://doi.org/10.1093/humrep/deq214

Aitken, R. J., Drevet, J. R., Moazamian, A., & Gharagozloo, P. (2022). Male Infertility and Oxidative Stress: A Focus on the Underlying Mechanisms. *Antioxidants*, *11*(2), 306. https://doi.org/10.3390/antiox11020306

Alamo, A., Luca, C. D., Mongioì, L. M., Barbagallo, F., Cannarella, R., Vignera, S. L., Calogero, A. E., & Condorelli, R. A. (2020). Mitochondrial Membrane Potential Predicts 4-Hour Sperm Motility. *Biomedicines*, *8*(7), 196. https://doi.org/10.3390/biomedicines8070196

Arias, M. E., Andara, K., Briones, E., & Felmer, R. (2017). Bovine sperm separation by Swim-up and density gradients (Percoll and BoviPure): Effect on sperm quality, function and gene expression. *Reproductive Biology*, *17*(2), 126–132. https://doi.org/10.1016/j.repbio.2017.03.002

Balasuriya, A., Serhal, P., Doshi, A., & Harper, J. C. (2014). Processes involved in assisted reproduction technologies significantly increase sperm DNA fragmentation and phosphatidylserine translocation. *Andrologia*, *46*(2), 86–97. https://doi.org/10.1111/and.12052

Benko, F., Mohammadi-Sangcheshmeh, A., Ďuračka, M., Lukáč, N., & Tvrdá, E. (2022). In vitro versus cryo-induced capacitation of bovine spermatozoa, part 1: Structural, functional, and oxidative similarities and differences. *PLoS ONE*, *17*(10), e0276683. https://doi.org/10.1371/journal.pone.0276683

Camargo, K. D., Pagoada, A. A., Hincapié, J. J., & Castillo, R. (2023). Efecto de diferentes tiempos de centrifugación utilizando el gradiente de Percoll 45-90% sobre la integridad de la membrana plasmática de espermatozoides bovinos. *Archivos de Zootecnia*, *72*(278), Article 278. https://doi.org/10.21071/az.v72i278.5713

Canvin, A. T., & Buhr, M. M. (1989). Effect of temperature on the fluidity of boar sperm membranes. *Journal of Reproduction and Fertility*, *85*(2), 533–540. https://doi.org/10.1530/jrf.0.0850533

Cojkic, A., Hansson, I., Johannisson, A., Axner, E., & Morrell, J. M. (2024). Single layer centrifugation as a method for bacterial reduction in bull semen for assisted reproduction. *Veterinary Research Communications*, *48*(1), 39–48. https://doi.org/10.1007/s11259-023-10178-y

Duracka, M., Liscinska, Z., Spanikova, S., Galovicova, L., Kacaniova, M., Lukac, N., & Tvrda, E. (2022). NARINGENIN, CURCUMIN AND ISOQUERCITRIN MITIGATE DAMAGES IN SPERMATOZOA CAUSED BY STAPHYLOCOCCUS AUREUS DURING BACTERIOSPERMIA IN VITRO. *Journal of Microbiology, Biotechnology and Food Sciences*, *12*(Special issue), Article Special issue. https://doi.org/10.55251/jmbfs.9489

Ďuračka, M., Husarčíková, K., Jančov, M., Galovičová, L., Kačániová, M., Lukáč, N., & Tvrdá, E. (2021). Staphylococcus-Induced Bacteriospermia In Vitro: Consequences on the Bovine Spermatozoa Quality, Extracellular Calcium and Magnesium Content. *Animals : an Open Access Journal from MDPI*, *11*(11), 3309. https://doi.org/10.3390/ani11113309

Eberhardt, M., Prochowska, S., Duszewska, A. M., Van Soom, A., Olech, W., & Niżański, W. (2022). The influence of Percoll® density gradient centrifugation before cryopreservation on the quality of frozen wisent (Bison bonasus) epididymal spermatozoa. *BMC Veterinary Research*, *18*(1), 305. https://doi.org/10.1186/s12917-022-03408-z

Gibb, Z., & Aitken, R. J. (2016). The Impact of Sperm Metabolism during In Vitro Storage: The Stallion as a Model. *BioMed Research International*, *2016*(1), 9380609. https://doi.org/10.1155/2016/9380609

González-Marín, C., Gosálvez, J., & Roy, R. (2012). Types, Causes, Detection and Repair of DNA Fragmentation in Animal and Human Sperm Cells. *International Journal of Molecular Sciences*, *13*(11), Article 11. https://doi.org/10.3390/ijms131114026

Iwasaki, A., & Gagnon, C. (1992). Formation of reactive oxygen species in spermatozoa of infertile patients*. *Fertility and Sterility*, *57*(2), 409–416. https://doi.org/10.1016/S0015-0282(16)54855-9

Januskauskas, A., Johannisson, A., & Rodriguez-Martinez, H. (2001). Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. *Theriogenology*, *55*(4), 947–961. https://doi.org/10.1016/S0093-691X(01)00456-3

Lessley, B. A., & Garner, D. L. (1983). Isolation of motile spermatozoa by density gradient centrifugation in Percoll®. *Gamete Research*, *7*(1), 49–61. https://doi.org/10.1002/mrd.1120070105

Malvezzi, H., Sharma, R., Agarwal, A., Abuzenadah, A. M., & Abu-Elmagd, M. (2014). Sperm quality after density gradient centrifugation with three commercially available media: A controlled trial. *Reproductive Biology and Endocrinology*, *12*(1), 121. https://doi.org/10.1186/1477-7827-12-121

Matsuura, R., Takeuchi, T., & Yoshida, A. (2010). Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. *Asian Journal of Andrology*, *12*(5), 753–759. https://doi.org/10.1038/aja.2010.46

Oliveira, L. Z., Arruda, R. P., Celeghini, E. C. C., de Andrade, A. F. C., Perini, A. P., Resende, M. V., Miguel, M. C. V., Lucio, A. C., & Hossepian de Lima, V. F. M. (2012). Effects of discontinuous Percoll gradient centrifugation on the quality of bovine spermatozoa evaluated with computer-assisted semen analysis and fluorescent probes association. *Andrologia*, *44*(1), 9–15. https://doi.org/10.1111/j.1439-0272.2010.01096.x

Ortega-Ferrusola, C., Sotillo-Galán, Y., Varela-Fernández, E., Gallardo-Bolaños, J. M., Muriel, A., González-Fernández, L., Tapia, J. A., & Peña, F. J. (2008). Detection of "Apoptosis-Like" Changes During the Cryopreservation Process in Equine Sperm. *Journal of Andrology*, *29*(2), 213–221. https://doi.org/10.2164/jandrol.107.003640

Parrish, J. J. (2014). Bovine In vitro fertilization: In vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology*, *81*(1), https://doi.org/10.1016/j.theriogenology.2013.08.005

Parrish, J. J., Krogenaes, A., & Susko-Parrish, J. L. (1995). Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. *Theriogenology*, *44*(6), 859–869. https://doi.org/10.1016/0093-691X(95)00271-9

Parrish, J. J., Susko-Parrish, J., Winer, M. A., & First, N. L. (1988). Capacitation of bovine sperm by heparin. *Biology of Reproduction*, *38*(5), 1171–1180. https://doi.org/10.1095/biolreprod38.5.1171

Pertoft, H., Laurent, T. C., Låås, T., & Kågedal, L. (1978). Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). *Analytical Biochemistry*, *88*(1), 271–282. https://doi.org/10.1016/0003-2697(78)90419-0

Salam, L., Rahim, A. I., & Al-Kawaz, U. (2020). *Which is matter in centrifugation based Reactive Oxygen Species (ROS) production? Force or time?* 6405–6408.

Samardžija, M., Dobranić, T., Karadjole, M., Getz, I., Vince, S., Gračner, D., Maćešić, N., & Filaković, I. (2006). The efficacy of gradient Percoll® on bull sperm separation for in vitro fertilization. *Vet. Arhiv*.

Sanocka, D., & Kurpisz, M. (2004). Reactive oxygen species and sperm cells. *Reproductive Biology and Endocrinology*, *2*(1), 12. https://doi.org/10.1186/1477-7827-2-12

Sepúlveda, B., Arias, M. E., Aguila, L., Zambrano, F., Sánchez, R., & Felmer, R. (2018). Gradient sperm selection for reproductive techniques in cattle: Is Isolate a suitable replacement for Percoll? *Andrologia*, *50*(3), e12921. https://doi.org/10.1111/and.12921

Shekarriz, M., DeWire, D. M., Thomas, Jr., Anthony J., & Agarwal, A. (2017). A Method of Human Semen Centrifugation to Minimize the Iatrogenic Sperm Injuries Caused by Reactive Oxygen Species. *European Urology*, *28*(1), 31–35. https://doi.org/10.1159/000475016

Sieme, H., Oldenhof, H., & Wolkers, W. F. (2015). Sperm Membrane Behaviour during Cooling and Cryopreservation. *Reproduction in Domestic Animals = Zuchthygiene*, *50 Suppl 3*, 20–26. https://doi.org/10.1111/rda.12594

Silva, E. C. B., Vieira, J. I. T., Nery, I. H. a. V., Silva, R. A. J. A., Lima, V. F. M. H., & Guerra, M. M. P. (2020). Sorting and cryopreservation of goat sperm with or without phenolic compounds. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *72*, 295–304. https://doi.org/10.1590/1678-4162-11354

Simbolon, C. N. A., Pramudhita, A. D., Rizqianti, Rr. A., Yekti, A. P. A., Isnaini, N., & Susilawati, T. (2024). Quality and Proportion of Spermatozoa in the Percoll Density Gradient Centrifugation Sexing Method at Different Gradients using AndroMed® Diluent. *BIO Web of Conferences*, *88*, 00042. https://doi.org/10.1051/bioconf/20248800042

Thijssen, A., Klerkx, E., Huyser, C., Bosmans, E., Campo, R., & Ombelet, W. (2014). Influence of temperature and sperm preparation on the quality of spermatozoa. *Reproductive BioMedicine Online*, *28*(4), 436–442. https://doi.org/10.1016/j.rbmo.2013.12.005

Tvrdá, E., Debacker, M., Ďuračka, M., Kováč, J., & Bučko, O. (2020). Quercetin and Naringenin Provide Functional and Antioxidant Protection to Stored Boar Semen. *Animals*, *10*(10), Article 10. https://doi.org/10.3390/ani10101930