Effect of exercise intensity on sperm quality via testicular SOD, Bcl-2 expression, spermatogenic cells, and chromatin integrity

Efecto de la intensidad del ejercicio sobre SOD, Bcl-2 testicular, células espermatogénicas y calidad de cromatina espermática

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Abstract

Background: The effect of exercise on sperm quality and related mechanisms is still inconclusive.
Objective: This study aimed to analyzed the effect of different exercise intensities on the expression levels of testicular superoxide dismutase (SOD) and B cell lymphoma/leukemia gene-2 (Bcl-2), spermatogenic cell count, and sperm chromatin quality.
Methods: Experimental study with 40 young male mice (*Mus musculus,* Deutschland-Denken-Yoken strain, 23-35 grams) randomly allocated to four groups of 10 mice. The first group served as a control that did not swim, whereas mice in the low, moderate, and high-intensity groups swam three times per week with loads attached to their tails equivalent to three, six, and nine percent of their body weight, respectively, for four weeks. All groups were assessed for the testicular expressions of SOD and Bcl-2 with immunohistochemistry, spermatogenic cell count histologically, and sperm chromatin quality with aniline blue staining. Data were analyzed using a one-way analysis of variance with the Least Significant Difference and Games-Howell post-hoc test. The significance level used in this study is five percent.
Results: Higher expressions of SOD and Bcl-2 were observed between the control and intervention groups ($p<0.001$). The highest spermatogenic count, especially spermatocytes and spermatids, was found in the low-intensity group ($p<0.050$). No significant changes were observed among the groups regarding spermatogonia count and sperm chromatin quality.
Conclusion: Different exercise intensities have varying effects on the expressions of testicular SOD, Bcl-2, and spermatogenic cells. This study revealed that low-intensity exercise enhanced spermatogenic count by enhancing testicular SOD and Bcl-2.

Keywords

Bcl-2 genes; fertility; reproductive health; spermatogenesis; superoxide dismutase.

Resumen

Introducción: El efecto del ejercicio en la calidad del esperma y los mecanismos relacionados es aún inconcluso.
Objetivo: Analizar el efecto de diferentes intensidades de ejercicio sobre la expresión testicular de superóxido dismutasa (SOD) y del gen leucemia/linfoma-2 de células B (Bcl-2), el recuento de células espermatogénicas y la calidad de la cromatina espermática.
Metodología: Estudio experimental con 40 ratones machos jóvenes (*Mus musculus,* cepa Deutschland-Denken-Yoken, 23-35 gramos) asignados aleatoriamente a cuatro grupos (n=10). El grupo control no realizó ejercicio, mientras que los grupos de ejercicio de baja, moderada y alta intensidad nadaron tres veces por semana durante cuatro semanas, con cargas atadas a sus colas equivalentes al tres, seis y nueve por ciento de su peso corporal, respectivamente. Se evaluaron las expresiones testiculares de SOD y Bcl-2 con inmunohistoquímica, el recuento de células espermatogénicas histológicamente y la calidad de la cromatina espermática tinción con azul de anilina. Los datos se analizaron con ANOVA de un solo factor, prueba de Diferencia Mínima Significativa y Games-Howell, con un nivel de significancia del cinco por ciento.
Resultados: Se encontraron expresiones significativamente mayores de SOD y Bcl-2 en los grupos de intervención ($p<0.001$). El grupo de baja intensidad presentó el mayor recuento de espermatocitos y espermátides ($p<0.050$). No ubo diferencias significativas en espermatogonias ni en la calidad de la cromatina.
Conclusiones: Las distintas intensidades de ejercicio influyen de forma variable en la espermatogénesis y en los niveles testiculares de SOD y Bcl-2, siendo el ejercicio de baja intensidad el más beneficioso.

Palabras clave

Genes bcl-2; fertilidad; salud reproductiva; espermatogénesis; superóxido dismutasa.

Introduction

Sperm quality is decreasing worldwide. Auger et al. (2022) observed the trend of decreasing sperm quality among several populations in the last decades. Levine et al. (2023) calculated that the mean decline of sperm concentration among groups worldwide was 51% or 0.87 million/ml/year/man between 1973-2018, and this rate doubled after the year 2000. Many endogenous and exogenous factors influence sperm quality, including exercise (Agarwal et al., 2014).

Recent studies tried to explore the effect of exercise on sperm quality, but the results are still inconclusive (Giulioni et al., 2023). Abdullahi et al. (2019) reported that low and moderate exercise intensity increased sperm quality, while high-intensity exercise lowers sperm motility, morphology, and viability but has the highest sperm count. Another conflicting study found that the higher the exercise intensity, the worse the sperm quality and the related indicators (Manna et al., 2004). Conversely, Sun et al. (2019) found in healthy men that the higher the exercise intensity, the better the sperm motility.

The magnitude and permanence of the response of the body to exercise depend on the type, frequency, interval, and intensity of the exercise (Belladelli et al., 2023). Various exercise intensities affect antioxidant activity (Simioni et al., 2018) such as superoxide dismutase (SOD) (Ernawati et al., 2020; Riris et al., 2019; Syarifah et al., 2021), and the production of oxidants (Manna et al., 2004; Taruna et al., 2022) differently. Oxidants may activate apoptosis regulated by B cell lymphoma/leukemia gene-2 (Bcl-2) (Rojas et al., 2017). Effects on spermatogenesis can be evaluated by spermatogenic cell count (Agarwal et al., 2014; Susanti et al., 2020; Wulandari et al., 2019). Apoptosis causes sperm chromatin condensation that can be examined by the aniline blue method (Dutta et al., 2021). The impact of exercise intensity by load gradient on swimming on testicular SOD, Bcl-2, spermatogenic cell count, and sperm chromatin quality by aniline blue method is still unclear.

Thus, this study aimed to analyze the effects of different exercise intensities on the expressions of testicular SOD and Bcl-2, spermatogenic cell count, and sperm chromatin quality in mice. The use of load on swimming by animals to produce gradient exercise intensity has been used extensively in basic research (Albar et al., 2021; Dewi et al., 2024; Rahayu et al., 2021; Sari et al., 2023), but we have not found this method used in the study of male fertility. The findings on the optimum intensity to enhance sperm quality can be used to inform exercise recommendations. We hypothesize that different exercise intensities will have distinct effects on testicular SOD, Bcl-2, spermatogenic cell count, and sperm chromatin condensation, with lower testicular SOD and Bcl-2, and higher spermatogenic cell count and sperm chromatin quality in low- and moderate-intensity exercise compared to high-intensity exercise.

Method

Ethical clearance

The ethical clearance was approved and registered by the Health Research Ethics Committee of Medical Faculty Airlangga University Surabaya (No.113/EC/KEPK/FKUA/2023) according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

Experimental animals

Forty healthy male mice (Mus musculus, Deutschland-Denken-Yoken strain, 25-35 grams, six to eight weeks old) were acquired from Veterinary Farma (Surabaya, East Java, Indonesia). They were kept in polypropylene cages with wire mesh tops and wood chaff bases that were cleaned and changed daily, in a 25°C ventilated room with 50-60% humidity, and 12:12-hour light-dark cycle. They were acclimated for one week with ad libitum access to a standard pellet diet (Hi-Pro-Vite CP511, Charoen Pokphand, Jakarta, Indonesia) and water. The body weight of all mice was measured weekly (Riyono et al., 2022). More than 20% weight loss was considered a humane endpoint, and the indicated mice should be excluded and terminated (Talbot et al., 2020). Any mice that got sick, died, or could not swim continuously as expected would be excluded from the study.

Research design

This experimental study with a randomized posttest-only control group design was conducted at the Biochemical Animal Laboratory. The four groups of 10 young male mice were randomly allocated using the lottery method (Elfil & Negida, 2017), where rolled up paper written `control`, `low`, `moderate`, or `high` as many as 10 each were put into a jar and shaken. Each rolled paper was taken from the jar, as an uncategorized mouse was chosen, so the chosen mouse was assigned to the group category as written on the rolled paper. Power analysis was conducted using G*Power (Faul et al., 2007) to determine the sample size, with 80% statistical power, a 0.050 significance level, and a 0.750 Cohen's f effect size based on prior studies (Smalheiser et al., 2021), resulting in a minimum sample size of eight mice per group. Additionally, a 20% attrition rate was applied, and the number was adjusted to 9.6, which rounded up to 10 mice per group. After the acclimatization, the intervention was given for four weeks. Twenty-four hours after the last intervention session to avoid the acute effect of exercise (Manna et al., 2004), mice were sacrificed and dissected for the collection of testes and epididymis. The testes were assessed for testicular expression of SOD and Bcl-2 using immunohistochemistry, and spermatogenic cell count was determined histologically. The sperm assessed for chromatin quality were taken from the epididymides. The group allocation of the mice was only known by those who put the mice to swim, but was blinded to the assessors for the rest of the study, which included organ harvest, specimen processing, and outcome measurement.

Protocol of intervention

The control group did not swim but was exposed to shallow water. The low, moderate, and high-intensity groups swam with loads attached to their mid-tails (Poole et al., 2020) equivalent to three, six, and nine percent of their body weight, respectively, adjusted weekly (Rahayu et al., 2021). The water temperature was maintained at 23-25°C (Kregel et al., 2006). Mice in exercise groups were adapted to swimming in tubs without and with loading on different days for three minutes (Dewi et al., 2024). The tubs were round, 50 cm in diameter, 37.5 cm high, with a water level of 18 cm to prevent the mice from diving and jumping out (Dewi et al., 2024; Kregel et al., 2006). Swimming was performed in the afternoon to adjust to the nocturnal behavior of mice (Kregel et al., 2006), with an increasing duration for adaptation of five minutes in the first week one, 10 minutes in second week, and 15 minutes in the third and last weeks (Riyono et al., 2022). The intervention was given thrice weekly, interspersed with a rest day (Lutfi et al., 2021). Each exercise group was made to swim in the same tub at the same time to minimize the potential confounders. The water bubble system helped the mice to swim continuously (Evangelista et al., 2003; Kregel et al., 2006). The indicator that mice could not swim anymore and were nearly drowned was when they produced a big bubble, were subsequently discarded from the water, and were excluded from the study (Kregel et al., 2006). At the end of this study, none of the mice were excluded.

Testicular tissue processing

The testes were fixed for 24 hours in 10% neutral buffered formaldehyde. The testes were then sequentially dehydrated in ascending concentrations (70%, 80%, and 90%) of ethanol, paraffin-blocked, serially sliced five micrometers thick, and put on Poly-L-Lysine coated slides for immunohistochemistry and hematoxylin and eosin staining (Safrina et al., 2023).

Immunohistochemistry

Testicular SOD expression was assessed with immunohistochemistry staining according to manufacturer protocol, using primary antibody anti-SOD monoclonal antibody (MDB29064, Medikbio, Malang, East Java, Indonesia) diluted optimally 1:100 overnight at 4°C and secondary antibody Biotin-conjugated goat anti-mouse IgG (bs-0296G-Biotin, Bioss, Woburn, MA, USA) with 1:200 dilution for 15 minutes at room temperature. Then, it was followed by the application of Strept Avidin-Horseradish Peroxidase (SA-HRP) and Diaminobenzidine (DAB) (NM100H and DB801 L, Biocare, Concord, CA, USA) for subsequently 20 minutes and 2 minutes at room temperature. Meanwhile, testicular Bcl-2 was determined using anti-Bcl-2 monoclonal rabbit antibody (ab32124, Abcam, Shanghai, Jiangsu, China) diluted 1:100, incubated at 4°C for one night, and subsequently incubated in the prediluted secondary antibody Rabbit/Mouse Universal Streptavidin-HRP Kit (DAB) (CW2069S, CWBIO, Taizhou, Jiangsu, China) according to the outlined instructions. All samples were finally counterstained using Mayer's hematoxylin

(HMM125, ScyTek, Logan, UT, USA) for 30 seconds and mounted using Entellan (107960, Merck, Darmstadt, Hesse, Germany).

The immunohistochemistry-stained sections were examined sequentially under the light microscope (Olympus BX41, Evident Corporation, Shinjuku-ku, Tokyo, Japan). The data obtained was analyzed by Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). Immunohistochemistry analyses of the expressions of testicular SOD and Bcl-2 were assessed as the mean percentage of immunoreactive cells, which is the percentage of the number of cells with a brown color compared to all visible cells in 10 fields of view (Ernawati et al., 2020; Fan et al., 2017). Testicular SOD expression appeared in the form of a brownish color in the cytoplasm of Sertoli cells and germ cells (Ernawati et al., 2020). Meanwhile, testicular Bcl-2 expression was observed in brownish-stained cytoplasm and the nucleus of germ cells (Xi et al., 2017).

Spermatogenic cell count

The testicular sections with hematoxylin and eosin staining were examined sequentially by light microscope at 40x magnification. The number of spermatogonia, spermatocytes, and spermatids was counted in three fields of view of seminiferous tubules in each slide (Manna et al., 2004; Wulandari et al., 2019). The spermatogonia, spermatocytes, and spermatids count were summed for total spermatogenic cell count (Susanti et al., 2020).

Evaluation of sperm chromatin quality

Samples of sperm were taken from the minced epididymides in one milliliter of phosphate buffer saline (PBS, pH 7.4). The sperm were incubated at 36°C for 30 minutes to swim into the PBS. A drop of sperm suspension was placed on a slide for aniline blue staining (Hachemi et al., 2019). A light microscope was used to observe at least 200 sperm cells for each slide. The sperm chromatin quality was determined by the percentage of sperm with blue-stained heads divided by the total sperm count (Dutta et al., 2021).

Statistical analysis

The research data were expressed as mean ± standard deviation (SD) and analyzed using the Statistical Package for the Social Sciences version 25.0 (International Business Machines Corporation, Armonk, NY, USA) software. The Shapiro–Wilk test for normality data distribution resulted in $p>0.050$ for all data, indicating that all data were distributed similarly to the normal distribution standard and therefore subjected to one-way analysis of variance (ANOVA), which was considered statistically significant if $p<0.050$. The Levene test was performed to investigate the homogeneity of variances, revealing a homogenous distribution of all data other than Bcl-2 and Spermatid Count. The Least Significant Difference (LSD) post-hoc test was performed in SOD, Spermatocyte Count, and Total Spermatogenic Cell Count data with a significant difference and homogeneous distribution, while the Games-Howell post-hoc test was conducted in Bcl-2 and Spermatid Count data with a significant difference but did not show a homogeneous distribution. The effect size was calculated using the Eta-squared formula commonly employed in ANOVA models to show the degree of correlation between the variables with the result 0.014 and higher indicates a large effect size, while a smaller result greater than or equal to 0.060 shows a medium effect size and a small effect size for the smaller amount (Cohen, 1988).

Results

The mean ± standard deviation (SD), minimum, maximum, one-way ANOVA p-values, and effect size of all parameters are presented in Table 1.

Table 1. The Effects of Various Exercise Intensities on Testicular SOD, Bcl-2, Spermatogenic Count, and Sperm Chromatin Quality

Parameter Variable (Measurement Unit)	Study Group Mean ± SD (Minimum–Maximum)				One-Way ANOVA (p-value)	Effect size (Eta-squared)
	Control (n=10)	Low-Intensity Exercise (n=10)	Moderate-Intensity Exercise (n=10)	High-Intensity Exercise (n=10)		
Testicular SOD Expression (%)	25.4 ± 10.0 (12.5–40.4)	52.8 ± 14.7 (31.0–74.6)	39.7 ± 13.1 (22.0–56.8)	34.4 ± 11.4 (15.3–50.4)	< 0.001	0.414
Testicular Bcl-2 Expression (%)	17.0 ± 4.0 (12.2–24.4)	37.4 ± 3.7 (31.8–43.0)	27.7 ± 7.1 (16.9–39.7)	24.6 ± 1.4 (22.0–27.0)	< 0.001	0.743

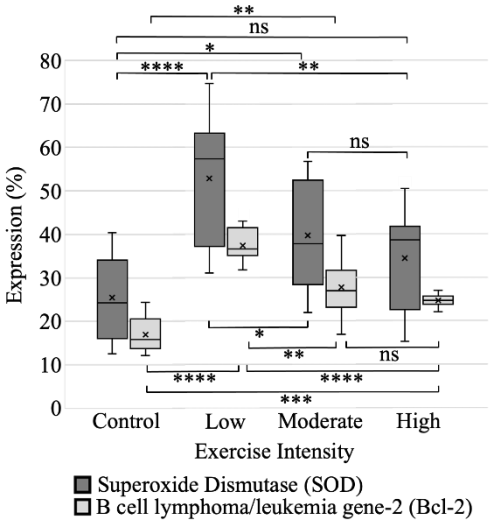
Spermatogonium Count (cells)	30.3 ± 2.6 (27–35)	33.6 ± 6.3 (25–46)	29.0 ± 5.0 (23–38)	28.6 ± 3.4 (22–34)	0.077	0.171
Spermatocyte Count (cells)	39.1 ± 3.0 (35–43)	45.8 ± 4.8 (39–54)	37.3 ± 4.1 (29–42)	38.9 ± 5.7 (30–46)	0.001	0.368
Spermatid Count (cells)	102.9 ± 15.0 (83–125)	104.9 ± 6.8 (94–117)	96.1 ± 8.0 (82–111)	87.2 ± 14.4 (65–111)	0.007	0.281
Total Spermatogenic Cell Count (cells)	172.4 ± 16.8 (146–196)	184.6 ± 13.2 (164–200)	162.2 ± 10.3 (151–182)	154.7 ± 21.4 (120–187)	0.001	0.355
Sperm Chromatin Quality (%)	53.9 ± 19.4 (24–80)	70.9 ± 12.0 (58–88)	62.6 ± 21.6 (32–88)	54.4 ± 22.9 (30–92)	0.182	0.125

SOD: superoxide dismutase; Bcl-2: B cell lymphoma/leukemia gene-2.

Effects of various exercise intensities on testicular SOD and Bcl-2

The study revealed a significant enhancement ($p<0.001$) with large effect size (0.414) in the expression of testicular SOD in groups subjected to low and moderate exercise intensities when contrasted with the control group. Additionally, a significant elevation ($p<0.001$) and large effect size (0.743) in testicular Bcl-2 expression were discerned across all groups engaging in varying exercise intensities compared to the control set. Intriguingly, the group partaking in low-intensity activities demonstrated superior augmentation in both testicular SOD and Bcl-2 expressions, surpassing those observed in the remainder of the studied groups (Figure 1).

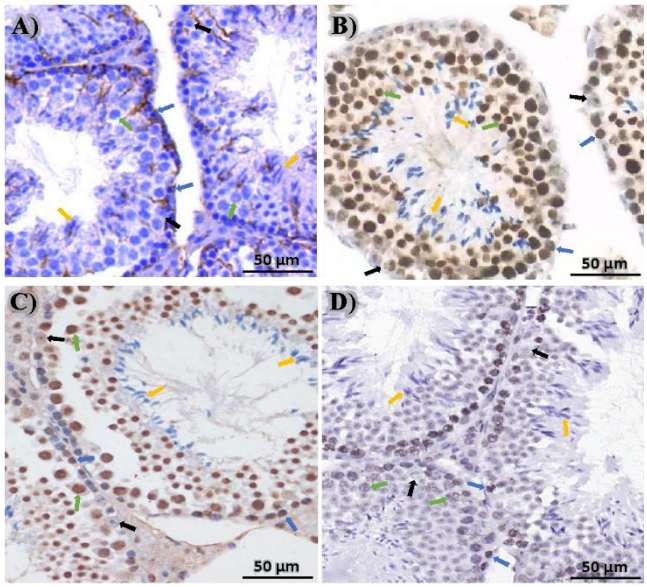
Figure 1. The effect of various exercise intensities on the testicular SOD and Bcl-2.



(ns) Non-significant with $p\geq0.050$. (*) Significant with $p<0.050$. (**) Significant with $p<0.010$. (***) Significant with $p<0.001$. (****) Significant with $p<0.0001$.

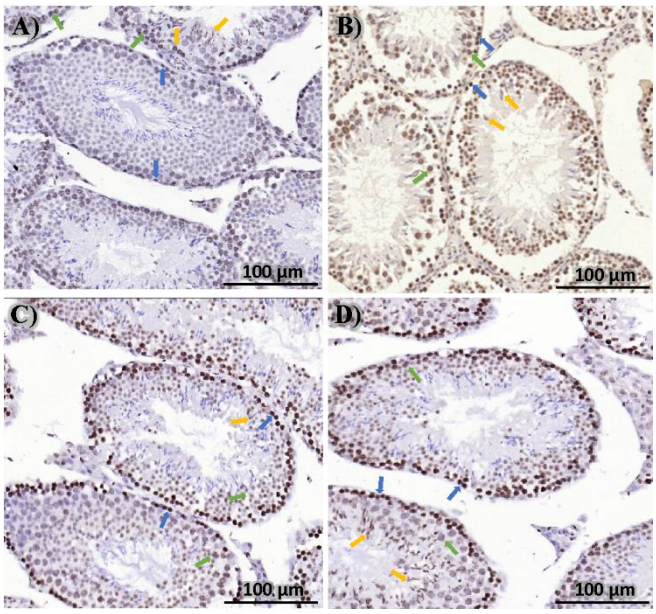
The control group exhibited immunoreactive patterns predominantly localized in the cytoplasm of spermatogonia, as illustrated in Figure 2, panel A. Contrastingly, the low-intensity cohort displayed pronounced immunoreactive responses in the cytoplasm of not only spermatogonia but also spermatocytes and Sertoli cells, captured in Figure 2, panel B. Meanwhile, the moderate and high-intensity groups presented moderate and faint immunoreactivity within the cytoplasm of spermatogonia, spermatocytes, spermatids, and Sertoli cells, as shown in Figure 2, panels C and D, respectively. Figure 3 delineates the immunoreactive intensity towards Bcl-2 protein within the nucleus and cytoplasm of germ cells, showcasing strong (panel B), faint (panel A), and moderate (panels C and E) responses corresponding to low, control, moderate, and high-intensity groups.

Figure 2. Expression of testicular SOD in immunohistochemistry with 400x magnification in control (A), low (B), moderate (C), and high-intensity group (D).



Blue arrow: spermatogonia; green arrow: spermatocytes; yellow arrow: spermatids; black arrow: Sertoli cells.

Figure 3. Expression of testicular Bcl-2in immunohistochemistry with 100x magnification in control (A), low (B), moderate (C), and high-intensity group (D).



Blue arrow: spermatogonia; green arrow: spermatocytes; yellow arrow: spermatids.

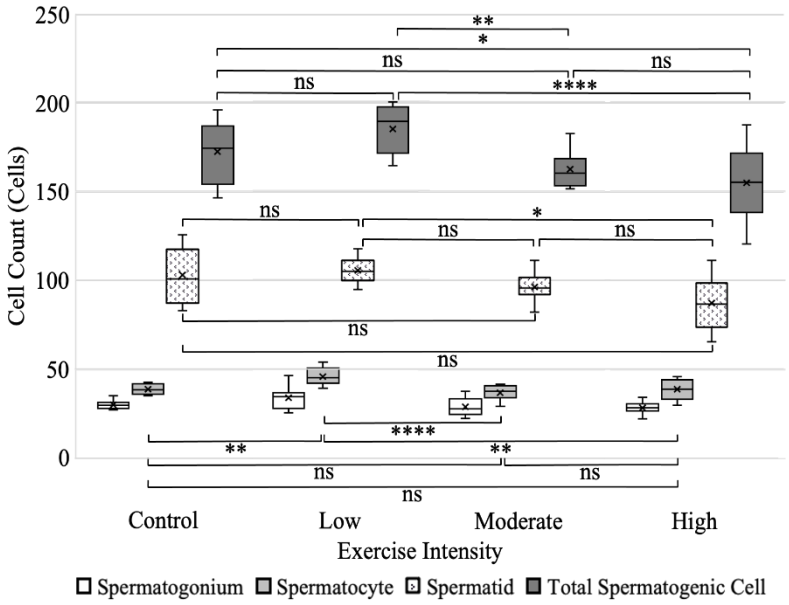
Effects of various exercise intensities on spermatogenic cell count

In this investigation, the low-intensity exercise regimen was associated with a notably significant ($p<0.002$) higher count of spermatocytes against the other groups involved in the study, with a large effect size (0.368). Conversely, the cohort subjected to high-intensity exercise registered a significantly reduced spermatid count when juxtaposed with the low-intensity group ($p<0.018$) with a large effect size (0.281). Additionally, both moderate and high-intensity exercise groups lagged in total spermatogenic cell count significantly ($p<0.003$) behind the low-intensity group with a large effect size (0.355).

Compounding this, the high-intensity group's total count of spermatogenic cells was markedly diminished ($p<0.018$) compared to the control group. Nonetheless, the count of spermatogonia did not exhibit any significant variance among the studied groups ($p=0.077$) despite the large effect size (0.171). The

detailed outcomes concerning the counts of spermatogonia, spermatocytes, spermatids, and total spermatogenic cells are visually represented in Figure 4.

Figure 4. The effect of various exercise intensities on spermatogonium, spermatocyte, spermatid, and total spermatogenic cell count.



No significant spermatogonium cell count differences among groups tested by one-way ANOVA. (ns) Non-significant with p≥0.050. (*) Significant with p<0.050. (**) Significant with p<0.010. (***) Significant with p<0.001. (****) Significant with p<0.0001.

Effects of various exercise intensities on sperm chromatin quality

No significant (p=0.182) differences in sperm chromatin quality were observed among the studied groups (Table 1), and the effect size was medium (0.125).

Discussion

The impact of exercise varies depending on the exercise protocol, potentially enhancing or inhibiting physiological processes such as antioxidant activity, apoptosis, and spermatogenesis (Belladelli et al., 2023; Giulioni et al., 2023; Maleki et al., 2022; Ibañez-Perez et al., 2019; Matos et al., 2019). This study found the different effects of various exercise intensities on the expression of testicular SOD and Bcl-2, spermatogenic cell count, but no differences in sperm chromatin quality. Given that numerous lifestyle and environmental factors such as smoking, alcohol, heat, radiation, psychological stress, nutrition, and clothing influence sperm quality (Agarwal et al., 2014; Giulioni et al., 2023; Schuppe & Köhn, 2022), an animal model was used to minimize confounding variables and isolate the impact of exercise intensity.

SOD is an important enzymatic antioxidant that protects cells from oxidant production (Ernawati et al., 2020). This study found that low and moderate-intensity exercise increased testicular SOD expression, aligning with previous studies (Daud et al., 2022; Yi et al., 2020) that observed peak SOD activity at moderate intensity and the lowest at high-intensity exercise. However, other studies (Manna et al., 2004; Samanta et al., 2006) found decreased SOD activity at any exercise intensity and that it got lower as the intensity of exercise increased. A meta-analysis conducted by Xu et al. (2022) found that athletes needed high-intensity exercise to increase SOD plasma levels but inactive individuals benefited from moderate as well as high-intensity.

Despite increasing oxidant production due to increased metabolism and oxygen consumption, exercise also increases antioxidant activity as a defense mechanism against oxidative stress (Simioni et al., 2018).

The imbalance between antioxidant and oxidant activity causes oxidative stress that disturbs spermatogenesis, including sperm maturation, and thus affects fertility (Agarwal et al., 2014). The majority of oxidants formed in sperm is superoxide which undergoes a dismutation reaction catalyzed by SOD to produce hydrogen peroxide which then is degraded into water by catalase and glutathione peroxidase (Agarwal et al., 2014). An imbalance between the activity of SOD against glutathione peroxidase and catalase causes hydrogen peroxide buildup that will inhibit SOD activity (Daud et al., 2022). This explains the phenomenon of decreasing SOD activity in increasing exercise intensity in this study.

Different results were also due to inconsistent definitions of exercise intensity in each study. For example, this study defined low-intensity exercise as 15 minutes of swimming three times weekly with a three percent body weight load, whereas Manna et al. (2004) classified it as an hour of swimming five days a week. The latter may have increased oxidant production that exceeded antioxidant capacity, thereby suppressing SOD activity. Additionally, heterogeneous study subjects may influence exercise intensity effect on SOD activity, whereas postulated that athletes have higher antioxidant protection against oxidants, while the sedentary and obese groups have lower protection than the control group (Xu et al., 2022). High-intensity exercise is relatively heavier in the sedentary and obese than in trained athletes.

The process of spermatogenesis encompasses the migration of the germ cells from the base of the seminal tubules to the lumen, causing epithelium restructuring, which produces ROS (Mruk et al., 2002). Appropriate levels of SOD can protect spermatogenic cells from ROS by preventing malondialdehyde production, conserve intracellular enzymes and other antioxidants (Celino et al., 2011). This is proven in the study of Ishii et al. (2005) that the spermatogenic cells of SOD1-knockout mice are more susceptible to ROS production and cell death.

Bcl-2 is an anti-apoptotic that reduces oxidant production to prevent cellular damage by oxidative stress (Asadi et al., 2021). Apoptosis is needed in physiological spermatogenesis to maintain sperm quality by removing abnormal germ cells, especially in sperm maturity (Asadi et al., 2021). However, increased apoptosis in oxidative stress states becomes pathological by damaging germ cells, interfering with spermatogenesis, and adversely affecting sperm production and male fertility (Asadi et al., 2021). Increased testicular Bcl-2 expression illustrates increased apoptosis in seminiferous tubules (Fan et al., 2017; Rojas et al., 2017).

This study found increased testicular Bcl-2 expression with low and moderate-intensity exercise, indicating the suppression of apoptosis in the seminiferous tubules. This is consistent with the research of Pahavani et al. (2020), where moderate and high-intensity exercise increases Bcl-2 expression, and the study of Malekloo et al. (2022) that found Bcl-2 is upregulated in low and moderate-intensity exercise but downregulated at high-intensity exercise. In contrast, Saberi et al. (2022) found decreased Bcl-2 expression at high-intensity exercise (80-85% maximal velocity) with increased apoptotic cell count. Similarly, Nasir et al. (2022) found that high-intensity exercise reduces Bcl-2 protein levels. Different protocols of exercise intensity in each study may also cause these conflicting results. Exercise intensity to a certain level may reach a minimum point to trigger Bcl-2 activity in preventing apoptosis, and along with the increasing intensity, reaching a point where apoptosis activity increases and cannot be suppressed by Bcl-2 activity, which subsequently initiating negative feedback mechanism to decrease Bcl-2 expression (Yao et al., 2018).

Spermatogenic cell count describes the ongoing spermatogenesis activity. Increasing spermatogenesis activity will increase spermatogenic cell count and vice versa (Susanti et al., 2020). In this study, the low-intensity exercise increased spermatogenic cell count, particularly the spermatocyte, suggesting improved spermatogenesis. On the other hand, higher-intensity exercise seemed to negatively impact spermatogenesis as observed in decreased spermatocyte, spermatid, and total spermatogenic count, likely due to increased oxidative stress and testicular apoptosis as seen in diminished SOD and Bcl-2 expression compared to the low-intensity group.

Meanwhile, increased spermatogenesis activity in the low-intensity group may be due to increased SOD and Bcl-2 activity along with the lower oxidant production in contrast to the higher-intensity exercise group. This mutual combination of activities may result in an increased number of good-quality germ cells, as a controlled apoptosis function avoids the excessive cellular damage caused by oxidative stress. The direct relation of the antioxidant activity to spermatogenic cell count was also observed in the study of Susanti et al. (2020). However, several other studies found decreased spermatogenic cell count in

exercise (Manna et al., 2004; Samanta et al., 2006). The conflicting findings can also be attributed to variations in exercise intensity protocols among studies. When the intensity exceeds the antioxidant defense capability, it triggers oxidative stress, leading to damage to lipids, proteins, and deoxyribonucleic acid (DNA), ultimately inhibiting spermatogenesis and reducing the spermatogenic cell count (Agarwal et al., 2014). Changes in antioxidant activity affect cytokine secretion, steroidogenesis, hormone production, and spermatogenesis (Dutta et al., 2022).

The current study found no significant differences in spermatogonia counts across groups. Similar findings have been reported in previous studies, where spermatogonia numbers remained unchanged despite decreased antioxidant levels and declines in spermatocyte and spermatid numbers (Manna et al., 2004; Samanta et al., 2006). Dutta et al. (2022) further observed biochemical alterations in maintained spermatogonia quantity, related to changes in antioxidant activity, suggesting that spermatogonia may still undergo molecular modifications while the cell count remains stable. These findings indicate that although the quantity of spermatogonia was not affected, this does not preclude the possibility of cellular changes at a functional or molecular level in response to certain stress signals. Furthermore, Nazanin et al. (2024) identified increased expression of proinflammatory markers—such as inducible nitric oxide synthase, cyclooxygenase-2, and toll-like receptor-4—in spermatogonia following moderate and high-intensity exercise, but not after low-intensity activity. Celino et al. (2011) described that spermatogonia may possess regulatory features and protective mechanisms that contribute to the stability in their numbers observed across different experimental conditions. These findings suggest that spermatogonia may be less responsive in quantity to physiological stressors such as exercise, possibly due to their role as stem cells and their inherent capacity to maintain homeostasis. The unchanged spermatogonia count observed in this study may reflect the activation of such characteristics, or it may indicate that the level or duration of the exercise conditions applied was not sufficient to cause a measurable impact on this specific cell population. However, these possibilities remain to be explored by further studies to gain a better understanding of the resilience of spermatogonia.

Bcl-2 is expressed in late-stage spermatocytes and spermatids, but not in spermatogonia and early spermatocytes (Beumer et al., 2000; Oldereid et al., 2001). This enlightens the findings in this study that the changes in Bcl-2 affect the other germ cells but not the spermatogonia. On the other hand, despite its absence in spermatogonia, Bcl-2 regulates the apoptosis of spermatogonia for the sake of the subsequent normal spermatogenesis (Furuchi et al., 1996). This indicates that the level of Bcl-2 produced by the exercise intensities in this study did not disrupt the spermatogonia apoptotic balance to reduce its count.

Aniline blue staining is a simple, easy, and inexpensive method to assess sperm chromatin quality (Dutta et al., 2021). During sperm maturation, histones in chromatin are replaced by protamine to increase chromatin condensation and protect sperm DNA from damage (Dutta et al., 2021). Failure of this process is associated with male infertility (Agarwal et al., 2014). Sperm with immature chromatin contains many histones with lysine residues and therefore reacts positively to aniline blue staining, marked by a blue-stained sperm head. In contrast, mature sperm chromatin contains many protamines with cysteine residues that react negatively and are unstained (Pourmasumi et al., 2019). Moreover, sperm of infertile men have unstable nuclei containing aberrant DNA with many histones that are also stained with aniline blue (Foresta et al., 1992).

The absence of notable changes in the chromatin quality across all groups of this study suggests that the exercise protocol employed did not impair sperm DNA maturation. Moreover, Maleki et al. (2017) found that moderate and high-intensity continuous training as well as high-intensity interval training improve sperm DNA integrity in healthy male subjects. In obese groups, exercise intervention significantly improved sperm protamine deficiency (Nematollahi et al., 2019). The prevention and alleviation of oxidative stress with the correct dose of exercise intensity may also preserve and enhance the integrity of sperm DNA chromatin.

The lack of significant change in chromatin quality among groups in this study may be due to the intervention period of 28 days, which is shorter than a full spermatogenesis cycle of 35.5 days, consisting of eight days for spermatogonia A to become primary spermatocytes; 12.5 days for meiosis of primary and secondary spermatocytes; nine and a half days for the spermatid phase, and five and a half days for the maturation phase (Dillasamola, 2021). Therefore, sperm sampled post-intervention may not reflect the

treatment's full effect on maturation, as the sperm that was influenced by the treatment from the beginning of spermatogenesis had not reached the maturation phase. Another study that was conducted for ten weeks found abnormalities in sperm maturation and increased apoptosis in the high-intensity exercise group (Shokri et al., 2014). Meanwhile, Samadian et al. (2019a, 2019b) found that moderate-intensity exercise for six weeks improved the sperm chromatin quality as well as upregulating testicular SOD, Bcl-2, and inhibited apoptosis.

This study demonstrated that low-intensity exercise in increasing SOD and Bcl-2 activity, which improved sperm quality, as indicated in increasing spermatogenic cell count compared to other intensities and control. Moderate-intensity exercise had higher SOD and Bcl-2 activity, and higher spermatogenic cells than the control, although the difference was not significant. Despite the increasing SOD and Bcl-2 expression, high-intensity exercise had lower spermatogenic cell count than the control group although the difference was not significant. These findings were considerable as the effect sizes of this study varied from moderate to large. Other previous similar research did not present the effect sizes of the results. Therefore, this study recommends low-intensity exercise as the optimal exercise intensity for improving sperm quality and advises avoiding high-intensity exercise that may reduce sperm quality.

While the findings provide valuable insight into the role of exercise intensity, this study has several limitations to be acknowledged. The duration of intervention was shorter than a complete spermatogenesis cycle, so the mature sperm assessed may not fully reflect the impact of the exercise treatments, particularly regarding chromatin integrity and DNA maturation. Besides, this study used only one exercise modality of swimming with load, and the effects of other exercise modalities (e.g., swimming without load, wheel running, treadmill running) were not assessed, limiting the generalizability of the findings across various physical activity types. Additionally, the design of fixed exercise interval, frequency, and duration in this study does not assess the effects of other interval, frequency, or duration combinations, which may also influence oxidative stress and reproductive function. Moreover, the evaluation of immediate post-intervention outcomes provides no information on whether the observed effects on SOD, Bcl-2, or spermatogenic cell counts persist or reverse over time. Also, other biomarkers of antioxidants, apoptosis, inflammation, and hormones involved in oxidative stress and spermatogenesis regulation were not measured, limiting mechanistic insights. A single duration of four weeks was used to compare the effects of various exercise intensities. Finally, the use of animal models in this fundamental research limits direct extrapolation to humans. Therefore, the findings of this study should be interpreted with caution and warrant further studies in humans to validate the results.

Future research with longer intervention duration and outcomes evaluation is needed to capture full spermatogenic cycles and determine long-term impacts of the exercise on fertility. Moreover, further studies on other exercise protocols (type, interval, duration, frequency) are necessary to reveal the full impacts of exercise on fertility to determine lifestyle recommendations for improving and preserving fertility. Furthermore, exploration on a broader panel of oxidative stress and apoptosis markers, hormonal assessments (testosterone, LH, FSH), as well as molecular pathways such as genomic and epigenetic changes in sperm, is needed to understand the underlying mechanisms more comprehensively and explore long-term impacts on offspring health and transgenerational effects. The findings of this study have potential implications for the development of prevention and treatment strategies for male infertility, particularly through lifestyle modifications such as regular low-intensity exercise, while caution is needed with high-intensity exercise. Nevertheless, the findings of this study still need to be validated before they can be applied in clinical practice to other species, including humans, by adjusting the dosage of exercise intensity and considering the differences in reproductive physiology between species.

Conclusions

This study demonstrated that low-intensity exercise intensities effectively enhanced testicular SOD and Bcl-2 expression, and increased spermatogenic cell count, suggesting its potential in improving sperm quality in animal models, but these findings need further validation in humans to confirm the clinical relevance. Moderate-intensity exercise showed similar but less pronounced benefits to low-intensity exercise, while high-intensity exercise appeared detrimental despite elevated antioxidant and anti-apoptotic markers. Notably, no chromatin quality impairment was detected, underscoring the potential

safety of short-term exercise interventions. These findings provide preliminary evidence on the potential benefit of low-intensity exercise for male fertility. However, given the limitations of this study, including the short intervention duration, use of a single exercise modality, and reliance on an animal model, further research is needed to validate these results and determine the clinical relevance.

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