
The oxidation-reduction potential as a
method for the determination of oxidative
stress in semen samples and the examination
of the lifestyle factors inducing it

Doctoral (PhD) thesis

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LIST OF ABBREVIATIONS

AMP	adenosine monophosphate
BMI	body mass index
CAT	catalase
DFI	DNA Fragmentation Index
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
ETC	electron transport chain
FSH	follicle-stimulating hormone
G6PD	glucose-6-phosphate dehydrogenase
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-S-transferase
H ₂ O ₂	hydrogen peroxide
KSH	Hungarian Central Statistical Office
LH	luteinizing hormone
LPO	lipid peroxidation
MDA	malondialdehyde
NADPH	nicotinamide adenine dinucleotide phosphate
NAG	neutral alpha-glucosidase
NBT	nitro blue tetrazolium
O ₂ ^{•-}	superoxide anion radical
•OH	hydroxyl radical
ORP	oxidation-reduction potential
OS	oxidative stress
PBS	phosphate-buffered saline
PMN	polymorphonuclear leukocyte

PSA	prostate-specific antigen
RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
TAC	total antioxidant capacity
TBA	thiobarbituric acid
t-BuOOH	tert-butyl hydroperoxide
WHO	World Health Organization

1. INTRODUCTION

1.1. Epidemiology and etiology of infertility

Infertility is defined as the failure to achieve pregnancy after one year of regular, unprotected sexual intercourse. Male infertility is diagnosed when the reproductive dysfunction of the male partner is identified as the underlying cause of the infertile relationship. Infertility affects approximately 15% of couples of reproductive age worldwide, with male factors contributing to more than half of all cases (Cox et al., 2022). According to the World Health Organization (WHO), male fertility has been in continuous decline since the mid-20th century and currently affects more than 30 million men globally (Siddiq et al., 2002). Idiopathic infertility accounts for 25-30% of all cases, in which classical sperm parameters - concentration, motility, and morphology - remain below the WHO reference values (Hamada et al., 2012).

Lifestyle and environmental factors associated with modern civilization, such as sedentary behaviour, obesity, smoking, alcohol consumption, psychological stress, and exposure to environmental toxins, further deteriorate male fertility (Skakkebaek et al., 2022). Oxidative stress (OS), which results from an imbalance between excessive reactive oxygen species (ROS) production and insufficient antioxidant defense, has been identified in 30-80% of male infertility cases and affects more than 90 million men worldwide (Agarwal et al., 2019). Both endogenous factors - including obesity, infections, and varicocele - and exogenous factors, such as smoking, radiation, air pollution, and endocrine disruptors, contribute to the development of OS (Pavuluri et al., 2024).

Endocrine disruptors - including pesticides, plastics, phytoestrogens, and mycotoxins - can exert oestrogen-like or antiandrogenic effects, leading to hormonal imbalance and oxidative damage, thereby adversely affecting fertility in both sexes (Czarnywojtek et al., 2021).

1.2. Oxidative stress

Free radicals are ions, atoms, or molecules containing an unpaired electron in their outer orbital, which makes them highly reactive. In healthy tissues, they are natural by-products of normal cellular metabolism and play essential roles in cellular signaling, spermatogenesis, oocyte maturation, and embryonic development; however, their excessive accumulation may lead to cellular damage (Rahman et al., 2007).

OS occurs when the level of ROS exceeds the antioxidant defense capacity of the cell. The major ROS include the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl

radical ($\bullet\text{OH}$), which are primarily generated during mitochondrial respiration (Gille et al., 1995). Among them, the $\bullet\text{OH}$ radical is the most reactive, capable of oxidizing nearly all cellular macromolecules - proteins, lipids, and nucleic acids - and no specific enzymatic defense exists against it (Wang et al., 1998).

Antioxidants constitute the primary intracellular defense mechanism against free radical-induced damage. The cellular antioxidant system can be classified into two main groups: non-enzymatic components (glutathione - GSH, vitamins C and E, coenzyme Q10, carotenoids, zinc, selenium) and enzymatic components (superoxide dismutase - SOD, catalase - CAT, glutathione peroxidase - GPx, glutathione reductase - GR). Together, these systems maintain redox homeostasis, neutralize ROS, and regenerate oxidized glutathione (Pompella et al., 2003).

Excessive ROS generation can result in extensive oxidative damage to proteins, lipids, and DNA. The most widely recognized marker of oxidative DNA damage is 8-hydroxy-2'-deoxyguanosine (8-OHdG), which can cause mutations and aberrant gene expression (Pisoschi et al., 2015). Lipid peroxidation (LPO) produces unstable radicals that trigger chain reactions within cellular membranes; its end products, such as malondialdehyde (MDA), can be detected clinically (Siddiqui et al., 2008). Protein oxidation, particularly affecting cysteine and methionine residues, results in structural and functional alterations, leading to impaired enzymatic activity and disrupted signaling pathways (Li et al., 2009).

OS plays a central role in the pathogenesis of numerous diseases, including neurodegenerative disorders (Alzheimer's and Parkinson's disease), cardiovascular diseases (hypertension, heart failure), metabolic disorders (diabetes, obesity), malignancies, and ocular conditions (cataract). Through similar mechanisms, OS is also a key contributing factor in the development of male infertility (Kowalczyk et al., 2022).

1.3. The role of ROS in the regulation of spermatogenesis

ROS are not solely detrimental; they play a fundamental role in the regulation of spermatogenesis and sperm function. At low concentrations, ROS promote sperm maturation, motility, capacitation, hyperactivation, the acrosome reaction, and recognition of the zona pellucida (O'Flaherty et al., 2006).

During capacitation, cyclic AMP-dependent signaling pathways are activated, accompanied by a decrease in membrane cholesterol and phospholipid content, an increase in membrane fluidity, and altered tyrosine phosphorylation of proteins (Du Plessis et al., 2015). Low levels of H_2O_2

stimulate this process, whereas excessive antioxidant activity (e.g., catalase treatment) inhibits it, underscoring the physiological importance of ROS.

Hyperactivation is mediated by the $O_2^{\bullet-}$, while elevated SOD activity suppresses it. Similarly, low concentrations of $O_2^{\bullet-}$ and H_2O_2 are required for the acrosome reaction. Membrane fluidity - determined by the content of polyunsaturated fatty acids and the activity of phospholipase A_2 - is also regulated by ROS and is essential for sperm-oocyte fusion (Ávila et al., 2022).

1.4. Methods for measuring OS in andrology

Several laboratory techniques are available for the assessment of OS, targeting either the direct measurement of ROS or the indirect evaluation of oxidative damage and antioxidant capacity. The most common direct methods include chemiluminescence, the NBT test, cytochrome-c reduction, flow cytometry, and electron spin resonance (Ávila et al., 2022). The chemiluminescent method uses luminol or lucigenin assays to sensitively measure the light emission induced by ROS, but it is an expensive and technically sensitive procedure (Agarwal et al., 2015). The NBT test is a simple spectrophotometric method based on the formation of diformazan proportional to ROS levels, providing information on the oxidative activity of sperm cells (Agarwal et al., 2008). Flow cytometry quantifies intracellular ROS using fluorescent markers, but due to its high equipment requirements, it is not suitable for routine clinical application (Guthrie et al., 2010).

Among indirect methods, the most widely used are LPO markers such as malondialdehyde (MDA) and isoprostanes. MDA is determined using the thiobarbituric acid (TBA) test, showing a strong correlation with sperm motility, DNA integrity, and fertilization potential (Tavilan et al., 2005). Newer biomarkers, including isoprostan ($8\text{-iso-PGF}_2\alpha$) and C11-BODIPY, are promising but not yet fully validated for clinical use (Aitken et al., 2007).

The measurement of oxidation-reduction potential (ORP) using the MiOXSYS system provides an integrated assessment of redox balance. The static ORP (sORP) reflects the ratio between oxidants and antioxidants, while normalization to sperm concentration enables direct comparison between samples (Garcia-Segura et al., 2020).

Although several indirect indicators such as leukocytospermia, hyperviscosity, and reduced sperm viability are associated with OS (Kopa et al., 2005), the assessment of OS has not yet become part of routine andrological diagnostics due to the limited standardization and clinical validation of existing methods.

1.5. Protection of ejaculate against OS

The antioxidant defense of spermatozoa relies primarily on the seminal plasma, which contains high concentrations of non-enzymatic antioxidants (vitamins C and E, L-carnitine, zinc, selenium, coenzyme Q10) and enzymatic components such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPx) (O'Flaherty et al., 2022). Reduced glutathione (GSH) plays a central role in maintaining redox homeostasis and neutralizing ROS.

In contrast, the intrinsic antioxidant capacity of spermatozoa is limited: they exhibit low GSH activity and lack several key antioxidant enzymes (CAT, SOD), as well as thioredoxin, peroxiredoxin, and mitochondrial GPx4. This incomplete defense system leads to enhanced LPO and membrane damage, particularly in sperm membranes rich in polyunsaturated fatty acids (Jones et al., 1979).

An additional vulnerability arises from the absence of active DNA repair mechanisms in spermatozoa. After fertilization, the restoration of DNA integrity in the zygote depends entirely on the oocyte's repair machinery, which declines with maternal age, thereby increasing the risk of genetic errors and impaired fertility (Smith et al., 2013).

1.6. Lifestyle factors as sources of OS

Research over recent decades have clearly demonstrated that environmental and lifestyle factors significantly contribute to the decline in both male and female fertility, partly through the enhancement of OS (Smith et al., 2020).

Smoking is one of the strongest inducers of OS. Cigarette smoke contains thousands of compounds - including nicotine and carbon monoxide - that promote the formation of free radicals, leading to DNA fragmentation, sperm damage, and reduced fertilization potential. Similarly, oxidative injury to oocytes accelerates their aging process (Davis et al., 2018).

Alcohol consumption increases ROS generation via ethanol and acetaldehyde metabolism, enhancing LPO and impairing sperm motility and genetic integrity. Chronic excessive alcohol intake can induce chromosomal abnormalities and DNA strand breaks, thereby reducing fertility in both sexes (Guthauser et al., 2014).

Obesity is associated with chronic low-grade inflammation, which elevates ROS production and disrupts hormonal balance. In men, this results in impaired spermatogenesis and increased sperm DNA fragmentation, while in women it contributes to ovulatory dysfunction and menstrual irregularities (Garcia et al., 2021).

Pesticides and industrial chemicals exert oxidative and endocrine-disrupting effects, directly damaging reproductive tissues and gamete DNA. Prolonged exposure has been linked to embryonic developmental abnormalities and decreased fertility (Parker et al., 2020).

Phytoestrogens, especially soy-derived isoflavonoids, may influence oxidative balance through hormonal modulation. Excessive intake may exacerbate oxidative damage, although their overall impact on fertility remains unclear (Zhang et al., 2020).

The use of mobile phones and other electromagnetic devices may also enhance ROS production. Prolonged exposure to electromagnetic fields can cause DNA fragmentation, reduced sperm motility, and impaired fertilization outcomes (Muzzongiro et al., 2021).

Psychological stress - including anxiety, depression, and chronic tension - stimulates free radical formation via cortisol-mediated oxidative pathways. These processes can lead to oxidative damage in both oocytes and spermatozoa, thereby compromising reproductive outcomes (Karunyam et al., 2023).

2. OBJECTIVES

Several factors contributing to infertility are associated with increased production of ROS and the development of OS. Numerous clinical studies have confirmed the relationship between impaired sperm parameters and OS, in which environmental and lifestyle factors also play a role. Currently, there is no standardized, validated protocol for the diagnosis and treatment of OS in routine andrological practice.

Measurement of the ORP provides a new, objective method for assessing the oxidant-antioxidant balance; it is a rapid, reproducible, and user-friendly approach, although its routine application has not yet become widespread.

Main research questions:

1. Are classical sperm parameters (concentration, motility, morphology) significantly poorer in cases with elevated ORP values compared to those with normal ORP values? Are there observable correlations between these parameters?
2. How does correction of measured ORP values for sperm concentration affect the assessment of the OS status of the sample?
3. Does artificially induced intracellular ROS generation manifest in classical sperm parameters (concentration, motility, morphology) and in ORP values?
4. How does the ORP value correlate with other well-established fluorescent ROS indicators?
5. After removal of the seminal plasma - thus eliminating its protective effect - are spermatozoa more sensitive to artificially induced OS, and to what extent is this reflected in the deterioration of classical sperm parameters and in ORP values?
6. To what extent are lifestyle factors known from the literature to induce OS present among patients undergoing infertility treatment? How does the prevalence of these factors compare with national data reported by the Hungarian Central Statistical Office (KSH)?

3. MATERIALS AND METHODS

3.1. Sample collection and basic analyses

Participants provided semen samples by masturbation after 3 days of sexual abstinence. Samples were processed within 1 hour after liquefaction. Semen analysis - including sperm concentration, total and progressive motility, vitality, and DNA fragmentation - was performed using the SCA SCOPE system (Microptic SL, Barcelona, Spain), while MiOXSYS (Caerus Biotech, Vilnius, Lithuania) was used for ORP measurement. Inclusion criteria were as follows: sperm concentration ≥ 30.0 M/mL, vitality $\geq 70\%$, ORP value ≤ 1.36 mV/ 10^6 /mL, and semen volume ≥ 3.0 mL. Samples were divided into two groups: native semen and centrifuged, washed spermatozoa ($400\times g$ for 10 min, PBS wash). Experiments were conducted using samples from 10-10 volunteers, and data from 183 patients were analysed retrospectively to assess the relationship between sperm count and ORP values. Sample collection was voluntary and based on written informed consent, carried out at the Dunamenti Reproductive Center, Tapolca site.

3.2. Artificial ROS induction

To validate ORP measurement, three OS inducers were applied in vitro: menadione ($O_2^{\bullet-}$ generation), H_2O_2 as a direct oxidant, and tert-butyl hydroperoxide (t-BuOOH) to induce LPO. The samples were treated at 300 μ l per tube in a concentration range of 1-5 mM at 37 °C for 2 hours. Dimethyl sulfoxide (DMSO) was used for the dilutions and the control, with a final concentration consistently maintained at 5%. The oxidative state was assessed using the MiOXSYS system, while intracellular ROS production was measured with the CellROX Green fluorescent probe (excitation 485 nm, emission 520 nm). The measured ORP value was not normalized to the cell concentration, as the cell concentration used during the parallel treatments was constant (20.0 M/mL). Semen parameters were re-evaluated before and after each exposure.

All experiments were conducted with approval from the Regional Research Ethics Committee of the University of Pécs Clinical Center (approval no. 8754-PTE/2021).

3.3. Questionnaire survey

Between 2020 and 2021, a total of 200 couples undergoing infertility treatment at the Dunamenti Reproductive Center Tapolca site completed a questionnaire focusing on lifestyle factors. The survey evaluated nutritional habits, harmful addictions, body mass index (BMI), known diseases, and the level of psychological stress. The collected data were compared with national data from KSH.

The survey was approved by the Chief Medical Officer of Hungary, based on the expert opinion of the Human Reproduction Committee (permit no. 23584-8/2020/EÜIG).

3.4. Chemicals

All chemicals used in the experiments were obtained from Sigma-Aldrich (Merck), except for the CellROX Green reagent, which was purchased from Thermo Fisher Scientific.

3.5. Statistical analysis

Data are presented as mean $\pm$ standard deviation. The Shapiro-Wilk test was used to assess normality, and Mann-Whitney tests were applied for non-parametric comparisons (using GraphPad InStat 7.0 software) The questionnaire study was descriptive in nature; therefore, no statistical tests were performed.

4. RESULTS

4.1. Effect of sperm count on classical semen parameters and ORP

During the analyzed period, semen samples were divided into two groups based on sperm concentration: oligozoospermic (<16.0 M/mL) and normozoospermic (>16.0 M/mL). In the oligozoospermic group, both total and progressive motility, as well as vitality, were significantly lower compared to the normozoospermic group (total motility: $25.8 \pm 14.1\%$ vs. $37.4 \pm 17.8\%$; progressive motility: $19.7 \pm 13.1\%$ vs. $28.8 \pm 15.0\%$; vitality: $60.5 \pm 8.7\%$ vs. $67.4 \pm 11.6\%$). The normalized ORP values were markedly higher in the oligozoospermic group (8.5 ± 13.9 mV/10⁶/mL vs. 1.0 ± 0.9 mV/10⁶/mL), indicating an increased level of OS. Similarly, sperm DNA fragmentation was also elevated in this group ($20.5 \pm 10.6\%$ vs. $16.8 \pm 10.0\%$). However, there was no significant difference in the non-normalized native ORP values between the two groups (38.5 ± 16.6 mV vs. 40.6 ± 25.5 mV) (Table 1).

		Concentration	Motility	Progressive motility	Vitality	ORP	ORP	DFI	
		n	M/mL	%	%	%	mV/10 ⁶ /mL	mV	%
<16 M/mL	69	8.3±4.1	25.8±14.1	19.7±13.1	60.5±8.7	8.5±13.9	38.5±16.6	20.5±10.6	
>16 M/mL	90	61.6±58.8***	37.4±17.8***	28.8±15.8***	67.4±11.6***	1.0±0.9**	40.6±25.5	16.8±10.0	

Table 1. Distribution of semen parameters according to sperm concentration.

** $p < 1\%$, *** $p < 0.1\%$. p -values were calculated using the Mann-Whitney test.

4.2. Effect of artificial ROS induction on classical sperm parameters in native samples

In native semen samples, treatment with H₂O₂ at concentrations of 1 mM, 2 mM, and 5 mM for 2 hours resulted in a 12.1%, 25.3%, and 64.1% decrease in sperm viability, respectively. Total motility decreased by 45.9%, 65.7%, and 84.1%, while progressive motility declined by 52.0%, 82.2%, and 100.0%, respectively, with increasing concentrations of the oxidizing agent. Subsequent treatment with menadione at 1 mM, 2 mM, and 5 mM caused decreases in viability of 17.0%, 40.0%, and 51.9%, and reductions in total motility of 85.1%, 95.4%, and 100.0%, while progressive motility declined by 16.8%, 100.0%, and 100.0%, respectively.

Finally, exposure to t-BuOOH at 1 mM, 2 mM, and 5 mM concentrations led to decreases in sperm viability of 14.5%, 26.4%, and 41.2%, total motility by 72.7%, 81.6%, and 86.7%, and progressive motility by 75.2%, 88.8%, and 94.1%, respectively (Figure 1).

Treatment with high-concentration DMSO appeared to cause no measurable toxic effects, although a potential additive effect cannot be excluded.

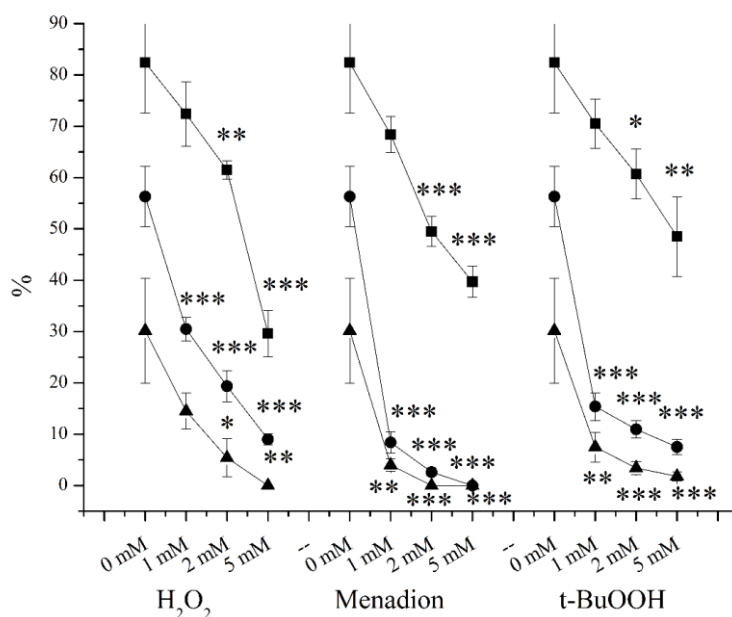


Figure 1. Exposure of native semen samples for 2 hours at 37 °C to H_2O_2 , menadione, and t-BuOOH. Sperm viability (■), total (●), and progressive motility (▲). * $p < 5\%$; ** $p < 1\%$; *** $p < 0.1\%$. p -values were calculated using the Mann-Whitney test.

4.3. Effect of artificial ROS induction on classical sperm parameters in seminal plasma-free samples

After centrifugation and washing, spermatozoa separated from their seminal plasma and resuspended in PBS showed a pattern similar to that observed in native samples.

Exposure to H_2O_2 at 1 mM, 2 mM, and 5 mM for 2 hours led to decreases in sperm viability of 53.7%, 83.0%, and 88.4%, respectively. Regarding total and progressive motility, even the lowest concentration caused a complete (100.0%) loss, except for 1 mM, where total motility decreased by 94.1%.

Treatment with menadione at 1 mM, 2 mM, and 5 mM resulted in reductions in viability of 53.6%, 76.8%, and 93.9%, respectively, and in 100% loss of both total and progressive motility at all concentrations.

Finally, t-BuOOH treatments at 1 mM, 2 mM, and 5 mM led to decreases in sperm vitality of 54.3%, 74.3%, and 83%, respectively, along with 100.0% reductions in both total and progressive motility (Figure 2).

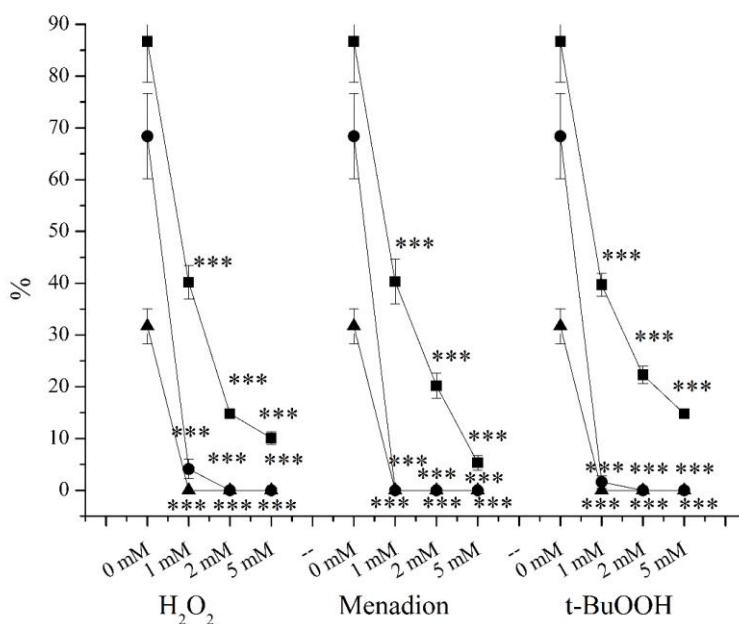


Figure 2. Exposure of plasma-free semen samples for 2 hours at 37 °C to H_2O_2 , menadione, and t-BuOOH. Sperm viability (■), total (●), and progressive motility (▲). *** $p < 0.1\%$. p -values were calculated using the Mann-Whitney test.

4.4. Effect of artificial ROS induction on the ORP in native semen samples

2 hours after treatment of semen samples with different concentrations (1 mM, 2 mM, 5 mM), the following increases in ORP were observed after normalization to sperm concentration 10^6 : 0.98-fold, 1.13-fold, and 1.42-fold increases after H_2O_2 exposure; 3.73-fold, 4.41-fold, and 5.02-fold increases after menadione treatment; and 1.06-fold, 1.25-fold, and 1.57-fold increases following t-BuOOH treatment (Figure 3).

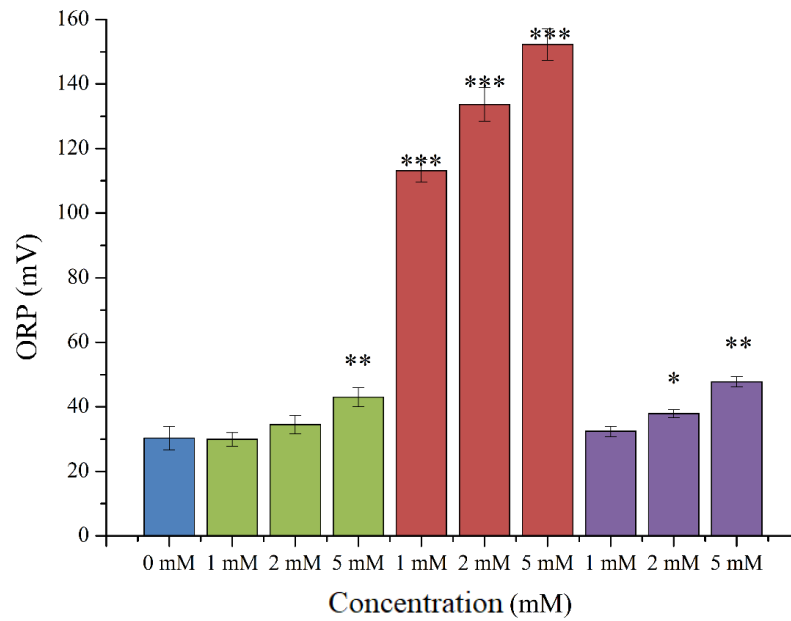


Figure 3. Exposure of native semen samples for 2 hours at 37 °C to H₂O₂ (green bars), menadione (red bars), and t-BuOOH (purple bars). ORP were determined using the MiOXSYS Analyzer, and the results are expressed in mV. * $p < 5\%$; ** $p < 1\%$; *** $p < 0.1\%$. p -values were calculated using the Mann-Whitney test.

4.5. Effect of artificial ROS induction on the ORP in seminal plasma-free semen samples

2 hours after treatment of seminal plasma-free sperm samples with H₂O₂ at concentrations of 1 mM, 2 mM, and 5 mM, the ORP increased 1.00-fold, 1.09-fold, and 1.12-fold, respectively. Following menadione incubation at the same concentrations, 1.23-fold, 1.27-fold, and 1.27-fold increases were observed, while t-BuOOH treatment caused 1.20-fold, 1.40-fold, and 1.48-fold rises in ORP values (Figure 4).

All results were expressed in millivolts (mV) and normalized to sperm concentration $\times 10^6$. The removal of seminal plasma and replacement with PBS led to a substantial increase in the baseline ORP value: while the native sample showed an average ORP of 30.3 mV, the plasma-free samples reached 201.9 mV.

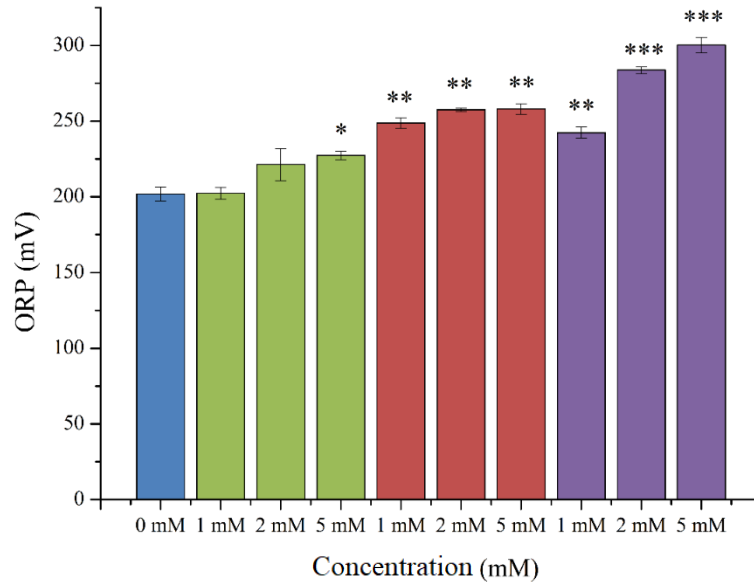


Figure 4. Exposure of plasma-free semen samples for 2 hours at 37 °C to H₂O₂ (green bars), menadione (red bars), and t-BuOOH (purple bars). ORP were determined using the MiOXSYS Analyzer, and the results are expressed in mV. * $p < 5\%$; ** $p < 1\%$; *** $p < 0.1\%$. p -values were calculated using the Mann-Whitney test.

4.6. Correlation of ORP values with other well-known fluorescent ROS indicators

The non-quantitative determination of OS showed results consistent with the MiOXSYS measurements. In comparison with the control, fluorescence intensity increased under all three treatment conditions (Figure 5). However, the degree of fluorescence increase did not correlate with the magnitude of mV changes measured by the MiOXSYS method.

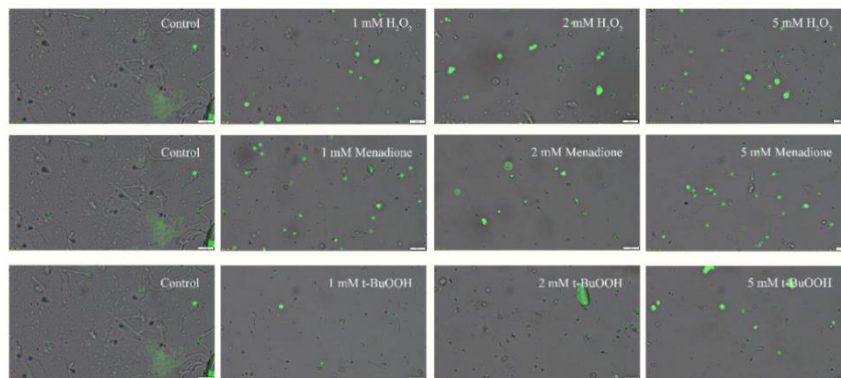


Figure 5. Schematic representation of OS induction in native sperm samples after 2-hour exposure to 1 mM, 2 mM, and 5 mM H₂O₂; 1 mM, 2 mM, and 5 mM menadione; and 1 mM, 2 mM, and 5 mM t-BuOOH.

Similar fluorescent changes were observed in the plasma-free samples (Figure 6), comparable to those detected in the native semen samples.

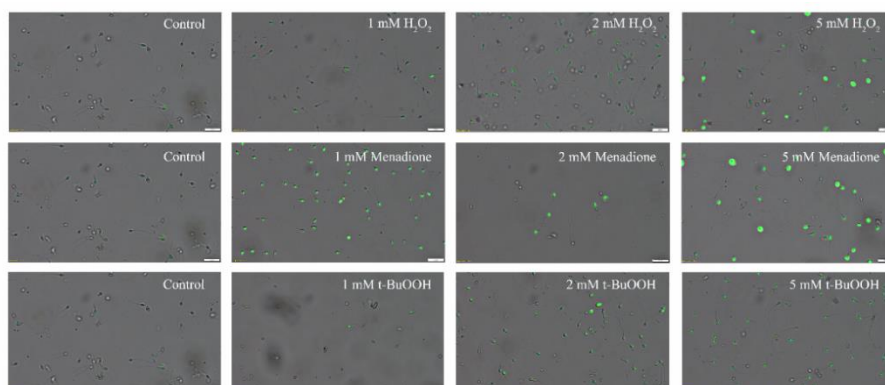


Figure 6. Schematic representation of OS induction in plasma-free sperm samples after 2-hour exposure to 1 mM, 2 mM, and 5 mM H₂O₂; 1 mM, 2 mM, and 5 mM menadione; and 1 mM, 2 mM, and 5 mM t-BuOOH.

The aim of our non-quantitative microscopic analysis was merely to confirm the successful induction of OS using a well-established detection method.

4.7. Examination of lifestyle factors causing OS

4.7.1. Evaluation of Body Mass Index

The Body Mass Index (BMI) of the participants was calculated based on their body weight and height according to the following formula:

$$\text{BMI} = \frac{\text{body weight (kg)}}{\text{height}^2 (\text{m}^2)}$$

Among the male participants, the average BMI was 28.2, with a median value of 27.7 (Figure 7). A total of 79.2% of the men had a BMI value above 25.0. These results indicate that overweight is a common condition within the examined population.

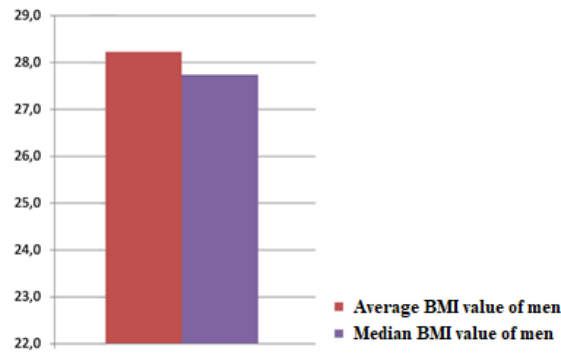


Figure 7. BMI values of men participating in the study

4.7.2. Evaluation of smoking habits among men

Among the respondents, 26.0% of men were regular smokers. Of these, 12.0% smoked fewer than 10 cigarettes per day, 10.0% smoked between 11 and 20, while 4.0% consumed more than one pack daily (Figure 8). The average number of cigarettes smoked per day among smokers was 13.4.

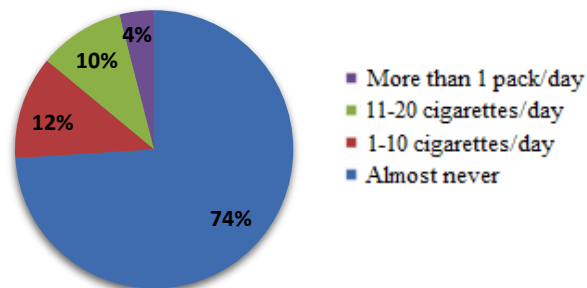


Figure 8. Smoking habits of men participating in the study

4.7.3. Evaluation of alcohol consumption habits among men

Among the male participants, 46.0% identified themselves as complete abstainers, while 4.0% reported drinking alcohol almost daily. Weekend drinkers accounted for 36.0% of the respondents, and 14.0% consumed alcohol 3-4 times per week (Figure 9).

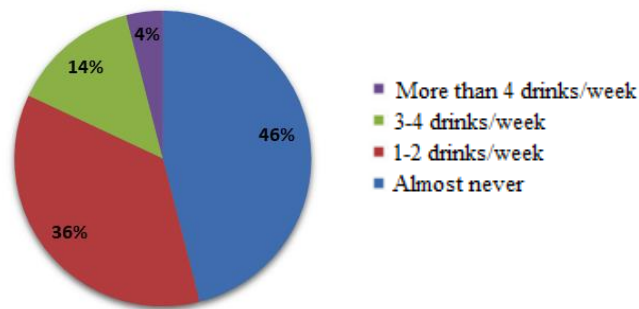


Figure 9. Alcohol consumption habits of men participating in the study

4.7.4. Evaluation of dietary habits among men

Only 2.0% of the male participants reported never consuming vegetables or fruits, whereas 29.0% consumed them daily. In contrast, 31.0% included vegetables or fruits in their diet 1-2 per week, and 38.0% consumed them 3-4 times per week (Figure 10).

Regarding supplementation, 59.0% of men used vitamin supplements daily, while 20.0% almost never used any dietary supplements. Additionally, 14.0% took vitamins 1-2 per week, and 7.0% used them 3-4 times per week (Figure 11).

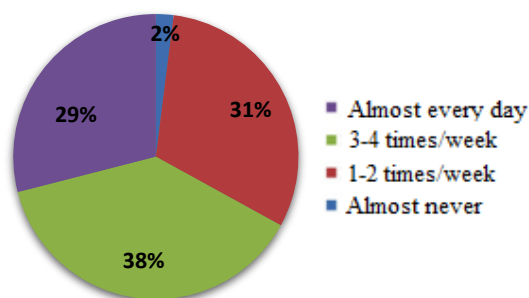


Figure 10. Vegetable and fruit consumption habits of men participating in the study

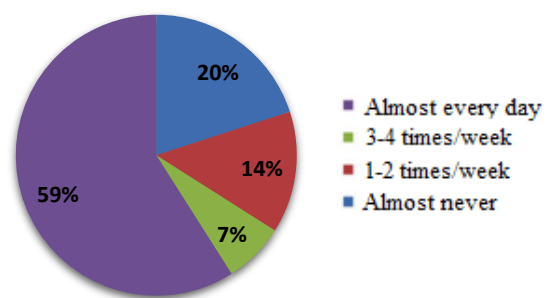


Figure 11. Vitamin consumption habits of men participating in the study

4.7.5. Evaluation of physical activity habits among men

Regarding exercise habits, 8.0% of the surveyed men reported engaging in daily physical activity, while 44.0% stated that they did not participate in any sports activities. Additionally,

33.0% exercised 1-2 per week, and 15.0% engaged in physical training 3-4 times per week (Figure 12).

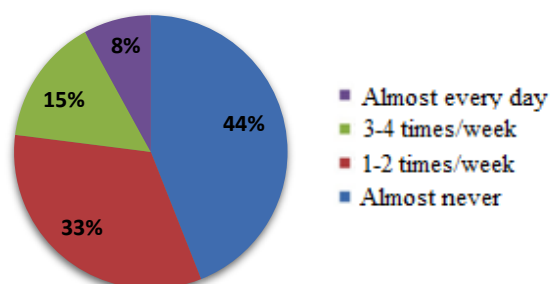


Figure 12. Physical activity habits of men participating in the study

4.7.6. Evaluation of psychological health among men

23.0% of the men reported a persistent sense of tension, most caused by work-related demands (24.0%) and stress related to childbearing (15.0%). Additionally, 55.0% experienced periodic stress due to infertility difficulties, while the workplace was a source of stress for 64.0%, and general lifestyle for 69.0%. In contrast, 30.0% almost never felt stressed about conceiving a child, 11.0% were free from work-related stress, and 7.0% considered their life completely stress-free (Figure 13). Furthermore 12.5% of respondents reported depressed mood, and 5.7% received psychological support.

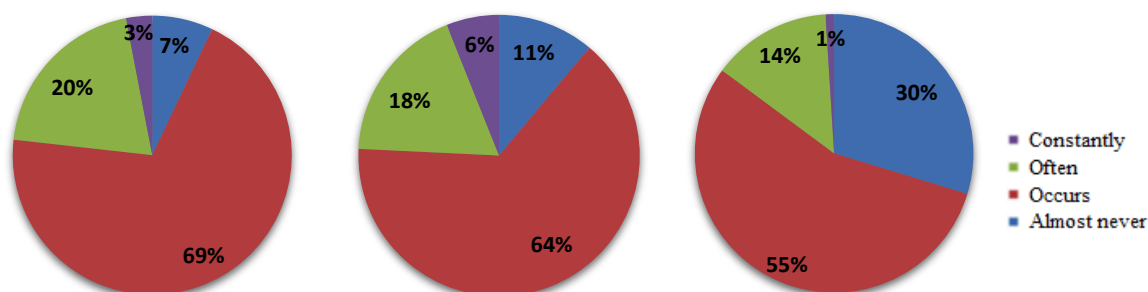


Figure 13 a, b, c. General, work-, and childbearing-related stress levels among men participating in the study

5. DISCUSSION AND CONCLUSIONS

5.1. Relationship between sperm count and ORP

Several studies have confirmed that elevated ORP levels negatively affect assisted reproduction outcomes - reducing blastocyst formation, implantation rate, and live birth rates. ORP correlates with male age, sperm DNA fragmentation, as well as sperm count, motility, and morphology (Henkel et al., 2022). Our retrospective data supported these findings: oligozoospermic samples showed lower motility, progressive motility, and vitality, together with higher DNA fragmentation and increased normalized ORP values. However, uncorrected ORP values did not differ significantly between groups, emphasizing the importance of sperm concentration normalization for proper ORP interpretation. Based on our results, sperm concentrations below $10 \times 10^6/\text{mL}$ may distort ORP reliability. Conversely, samples above $50 \times 10^6/\text{mL}$ typically exhibited low ORP values ($<1.36 \text{ mV}$), suggesting the absence of OS under normozoospermic conditions. These findings indicate that sperm concentration is a critical correction factor in the clinical interpretation of ORP, particularly in low-count samples, to ensure accurate assessment of OS.

5.2. Examination of the effect of sperm count on ORP

Using the MiOXSYS system, we measured the total range of intracellular ROS. It is assumed that a higher sperm count is associated with greater intracellular ROS production. To induce OS, three agents with different mechanisms were used: -to increase $\text{O}_2^{\bullet-}$ generation, H_2O_2 as a direct oxidative stressor, and t-BuOOH to induce LPO.

5.2.1. Menadione exposure

The main sources of ROS and $\text{O}_2^{\bullet-}$ in spermatozoa are the mitochondrial electron transport chain and membrane-bound NADPH oxidase (Ayad et al., 2022). Additional ROS may be generated via cytochrome P450, xanthine oxidase, or peroxisomal activity. Menadione, a synthetic water-soluble form of vitamin K, is a well-known OS inducer. Through one-electron reduction, it forms unstable semiquinone radicals, which generate ROS in the presence of oxygen. Inhibition of the NAD(P)H-quinone oxidoreductase 1 (NQO1) enzyme amplifies this process (de Souza et al., 2022). The resulting superoxide radicals can react with metal ions (e.g., Fe), damaging Fe-S protein complexes and producing further oxidative injury via the Fenton reaction. They can also inactivate antioxidant enzymes such as catalase. Experimental data

confirm that menadione dose-dependently increases ROS production and decreases sperm motility, viability, and mitochondrial activity. Depletion of GSH, loss of membrane potential, and reduced ATP generation are clear indicators of menadione-induced OS (Zhu et al., 2022).

5.2.2. H₂O₂ exposure

H₂O₂ forms in cells through disproportionation of O₂^{•-}, either spontaneously or via SOD. It is also produced in peroxisomes during amino acid and glucose oxidation. Because H₂O₂ is a neutral molecule, it freely crosses cell membranes, causing oxidative damage even far from its site of formation (Gille et al., 1995). It oxidizes cysteine and methionine residues in proteins, impairing their function, and contributes to the formation of the highly reactive [•]OH (Sraran et al., 1991). H₂O₂ induces OS, DNA and protein damage, and LPO in cells. In spermatozoa, H₂O₂ exposure significantly reduces viability and motility, impairs capacitation and oocyte-penetration ability, and enhances membrane LPO, confirming its key role in OS mediation (Pujinato et al., 2021).

5.2.3. LPO induced by t-BuOOH exposure

During LPO, the polyunsaturated fatty acids of the cell membrane undergo oxidative degradation initiated mainly by hydroxyl [•]OH. This process forms lipid and peroxyl radicals, generating a chain reaction that produces new lipid hydroperoxides and reduces membrane fluidity and permeability (Sidiqui et al., 2008). The t-BuOOH, a lipid hydroperoxide analog, is widely used experimentally to induce OS. Its toxicity arises from the butoxyl radicals formed via Fenton-type reactions (Girotti et al., 1998). Spermatozoa are particularly sensitive to LPO because their antioxidant defense (SOD, GPx, thioredoxins, peroxiredoxins) is limited and catalase is completely absent. Experimental data show that t-BuOOH treatment increases MDA levels (a LPO marker), decreases antioxidant activity (SOD, GSH), and reduces motility, concentration, and DNA integrity (Kumar et al., 2007).

In our study, all three agents - t-BuOOH, H₂O₂, and menadione - produced detectable ORP changes, with menadione exerting the strongest oxidative effect.

5.3. Influence of seminal plasma on ORP

Seminal plasma, which constitutes approximately 95% of the ejaculate, is a secretion with a complex composition that plays a fundamental role in nourishing spermatozoa, maintaining their motility, and protecting them against oxidative damage (Nieschlag et al., 2010). Its high

antioxidant content (vitamins C and E, coenzyme Q10, zinc, selenium) and enzymatic defense system (CAT, SOD, GPx, GR) maintain redox homeostasis by balancing ROS production (Gupta et al., 2021). In our experiments, removal of seminal plasma significantly increased the impact of OS: plasma-free samples showed 6-7-fold higher baseline ORP values and markedly reduced motility and viability. This indicates that in the absence of the antioxidant capacity of seminal plasma, sperm cells are more vulnerable to oxidative injury. Our findings confirm that seminal plasma is a critical protective factor against extracellular ROS and strongly influences the interpretation of ORP measurements.

5.4. Lifestyle factors causing OS in the studied male population

Participants were aged 23-66 years, consistent with the national average of men seeking infertility treatment. Andrological causes accounted for 46% of infertility cases. Based on BMI, 79.2% of men were overweight or obese, exceeding the national average (65.2%). Physical inactivity was common: only 20.0-23.0% exercised regularly. Daily fruit and vegetable consumption was below the national average (29.0% vs. 35.6-49.0%), though 59.0% reported daily vitamin supplementation. Alcohol consumption exceeded national averages, while smoking prevalence (26.0%) was slightly lower than the Hungarian mean (27.7%). In terms of mental health, 12.5% of men reported depressive mood, and 15.0% experienced high stress, both lower than population data. According to the literature, obesity, sedentary lifestyle, smoking, and excessive alcohol intake all promote OS and impair spermatogenesis. Obesity causes hormonal dysfunction (low testosterone, high estrogen), Sertoli- and Leydig cell impairment, and inflammation, leading to reduced sperm count and quality. Smoking induces oxidative DNA damage (Davis et al., 2018), while alcohol reduces gonadotropin and testosterone levels and impairs motility and morphology (Kerr et al., 1993). Chronic stress elevates cortisol, suppressing the hypothalamic-pituitary-gonadal axis, and reducing testosterone and spermatogenic efficiency (Nargund et al., 2015). Our findings confirm that these lifestyle factors - particularly obesity, physical inactivity, smoking, and stress - are closely associated with male infertility and increased OS.

5.5. Therapeutic possibilities for OS

Reducing elevated ORP levels can be achieved through antioxidant therapy, which, according to several studies, improves sperm quality and fertilization success. Common antioxidants include vitamins A, C, and E, L-carnitine, N-acetylcysteine, coenzyme Q10, omega-3 fatty

acids, lycopene, zinc, selenium, and folic acid. Systematic reviews show that combined antioxidant therapy has a favorable effect on spermatological parameters and assisted reproduction outcomes, although clinical evidence remains inconsistent (Li et al., 2021). Therapeutic efficacy depends on dose and duration: low-dose or short-term therapy may be ineffective, while excessive intake may cause “reductive stress”, disrupting redox balance (Henkel et al., 2019). Currently, OS diagnostics are not standardized; results from ROS, TAC, and ORP measurements are not directly comparable, and no uniform reference range exists (Agarwal et al., 2021). Consequently, routine clinical use of antioxidants is not yet recommended. Future research should focus on standardized, large-scale studies to establish the clinical usefulness of antioxidant therapy and ORP measurement in the diagnosis and treatment of male infertility.

6. THESES

1. The ORP shows a clear association with sperm concentration, sperm viability, and both total and progressive motility.
2. The relationship between ORP and sperm concentration can be misleading, as according to the manufacturer's recommendation, ORP values should be normalized to sperm count. Our findings indicate that this normalization may distort the measurements, particularly when sperm concentration is below 10 M/mL or above 50 M/mL. To better understand the correlation between sperm count and ORP, further standardized studies are needed. Accordingly, in our research we evaluated non-normalized native ORP values to more accurately assess the direct relationship between redox status and sperm concentration.
3. To induce intracellular OS, three compounds with different mechanisms were used: menadione (to promote $O_2^{\bullet-}$ formation), t-BuOOH (to trigger LPO), and H_2O_2 (as a direct oxidizing agent). All three agents increased OS in a concentration-dependent manner, resulting in decreased viability, reduced total motility, and lower progressive motility. The treatments also caused a significant rise in ORP values, indicating the presence of OS.
4. The in vitro-induced OS was confirmed not only by changes in ORP values and spermatological parameters, but also by a fluorescent ROS marker, consistent with data from the literature.
5. Since approximately 95% of semen consists of seminal plasma, which contains antioxidant components, its removal markedly worsened the effects of OS. The deterioration of sperm parameters was considerably more severe in plasma-free samples, confirming the protective role of seminal plasma.
6. The analysis of lifestyle factors associated with OS revealed that the overall health status of our patients was unfavorable, closely corresponding to national epidemiological data.

7. PUBLICATIONS

Publications forming the basis of the dissertation:

A Balló, P Czétány, KS Busznyákné, L Márk, N Mike, A Török, Á Szántó, G Máté Oxidoreduction Potential as a Method to Determine Oxidative Stress in Semen Samples
International Journal of Molecular Sciences. 2023 Jul 26; 24(15):11981. **Q1, IF: 4.9**

G Máté, **A Balló**, Á Szántó, Zs Kopa, A Török
Survey of Lifestyle Habits Among Men and Women Undergoing Infertility Treatment.
Orvosi Hetilap. 2024; 165(36):1423-1432. **Q3, IF: 0.8**

Total impact factor (for the dissertation): **5.7**

Cumulative impact factor: **37.8**

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Additional publications:

A Balló, KS Busznyákné, P Czétány, L Márk, A Török, Á Szántó, G Máté Estrogenic and Non-Estrogenic Disruptor Effect of Zearalenone on Male Reproduction: A Review.
International Journal of Molecular Sciences. 2023 Jan 13; 24(2):1578. **Q1, IF: 4.9**

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Magyar Sebészet. 2013; 66(5):289.

P Czétány, **A Balló**, L Márk, A Török, Á Szántó, G Máté An Alternative Application of Magnetic-Activated Cell Sorting: CD45 and CD235a Based Purification of Semen and Testicular Tissue Samples. International Journal of Molecular Sciences. 2024 Mar 24; 25(7):3627. **Q1, IF: 4.9**

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P Czétány, S Gitta, **A Balló**, A Sulc, G Máté, Á Szántó, L Márk Application of Mass Spectrometry Imaging in Uro-Oncology: Discovering Potential Biomarkers. *Life (Basel)*. 2022 Mar 3; 12(3):366. **Q2, IF: 3.2**

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P Czétány, **A Balló**, Á Szántó Introduction and Early Results of Microdissection Testicular Surgery for Testicular Tumors in Pécs. *Magyar Urológia*. 2024; 36(3):139-142.

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Zs Kopa, **A Balló** Male Infertility. In: Nyirády P. (ed.) *Urology for Medical Students, Residents, and Specialists*. 2019. ISBN: 978-963-331-490-6, pp. 319-327.

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