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Investigation of the effect of serotonin-activated semen washing medium on sperm motility at the molecular level: a pilot study

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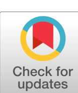
Summary

In Assisted Reproductive Technologies (ART), efficient sperm preparation is vital for successful fertilization, with washing media enhancing the process. This pilot study examines the molecular-level impact of a new serotonin-containing sperm-washing medium (Prototype) on sperm motility and ROS metabolism, comparing it with commercially available media (Origio and Irvine). Semen samples from thirty-one individuals underwent preparation using the swim-up method post-semen analysis. Each sample was separately washed with the Prototype, Origio and Irvine mediums. ROS formation was determined through flow cytometric, and AT2R and PRDX2 protein levels, associated with sperm motility, were assessed via Western blot. Statistical evaluation compared the findings among the three outlined media. Significant differences were found among three washing media in terms of total and progressive motility. The Prototype medium showed the highest increase in both total (66%) and progressive motility (59%), while the control group exhibited the lowest increases (41% and 27.7%, respectively). Regarding ROS levels, the prototype (11.5%) and Origio (10.7%) groups demonstrated a notable decrease, contrasting with Irvine (25.8%). Molecular assessment revealed a significant elevation in AT2R protein levels in the prototype medium (59%), compared to other media. Additionally, an increase in PRDX2 protein levels was observed in the prototype medium, although this didn't reach statistical significance. Serotonin-activated washing media for sperm preparation can be a suitable choice for selecting high-quality sperm in ART. A broader molecular analysis with a larger sample size is required to explore the mechanisms and effectiveness of using a serotonin-containing sperm-washing medium in routine ART.

Introduction

Male infertility is a multifactorial disorder characterized by the failure of pregnancy to occur in a clinically fertile woman and usually affects 30–50% of infertile couples (Aziz et al., 2010) Since the effective motility of sperm has a significant impact on its ability to reach and fertilize an ovum, low sperm motility is an important determinant of male infertility (Vaughan et al., 2019). Possible variables that can negatively affect sperm motility include hormonal imbalances, genetic factors, infections, lifestyle choices such as excessive smoking and alcohol use, exposure to increased heat, and underlying medical conditions including varicoceles and certain drugs. The first step in analyzing sperm quality is a basic semen analysis, which involves examining important sperm measures such as concentration, motility, morphology and vitality (Aziz et al., 2010; De Los Santos et al., 2016).

ART, which involves meticulous sperm selection, enhances the likelihood of a successful reproductive outcome (Kim et al., 2015). Spermatozoa preparation processes include basic washing techniques that have evolved into separation methods based on a variety of concepts, such as migration, filtering and density gradient centrifugation. The standard swim-up approach, known for its simplicity and cost-effectiveness, is one of the first techniques used (Abraham et al., 2016). An additional or complementary method for preparing sperm for in vitro ART, beyond the aforementioned sperm separation procedures, can involve in vitro treatment of spermatozoa to enhance their functionality (Raad et al., 2019). An ejaculate, in addition to live spermatozoa, typically contains seminal liquid, epithelial cells, immature and necrotic sperm cells and blood cells (and in some, also bacteria). These component products of toxic or bioactive substances such as decapacitation factors and free oxygen radicals may impair the fertilization rate of the egg. In sperm preparation, washing media is used to remove sperm from this seminal fluid, enhancing sperm activity and bolstering the likelihood of successful pregnancy. During the capacitation step, a pivotal phase in the fertilization process, elevated



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levels of intracellular calcium, bicarbonate and hydrogen peroxide activate adenylate cyclase. This activation leads to the production of cyclic adenosine monophosphate (cAMP) and the phosphorylation of specific proteins. The research suggests that the supplementation of the environment with elements like magnesium, HEPES, calcium, ascorbic acid and bicarbonate can substantially enhance the outcomes of fertilization processes (Al-Dujaily et al., 2017; Aziz et al., 2010; De Los Santos et al., 2016; Fanaei et al., 2014; Kim et al., 2015). Additionally, reactive oxygen species (ROS) are produced by both spermatozoa and leukocytes in ejaculate and are natural by-products of various biological activities (Agarwal et al., 2014). ROS can modulate flagellar movement by altering the pattern of flagellar movement, the primary mechanism responsible for sperm propulsion, or by regulating the activation of many signalling pathways and ion channels involved in the regulation of sperm motility (Vigolo et al., 2022). However, excessive ROS is dangerous for sperm functions (Aprioku, 2013). Oxidative stress refers to a condition in which the body's capacity to counteract and eliminate the harmful effects of ROS is impaired. To protect male germ cells from the effects of ROS and boost their motility for enhanced yield, several chemicals can be put into the washing medium (Ménézo et al., 2014; Morielli & O'Flaherty, 2015). To address this objective, we have formulated an innovative washing medium that incorporates serotonin as a potent ingredient to enhance sperm function.

Serotonin (5-hydroxytryptamine; C10H12N2O or 5-HT), belonging to the indoleamine family, plays an active role in numerous physiological processes (Berger et al., 2009). While serotonin is well-recognized as a crucial neurotransmitter in the central nervous system (CNS), its influence extends beyond the CNS, impacting various mammalian systems including the gastrointestinal, cardiovascular and pulmonary systems (De Ponti, 2004; MacLean et al., 2000; Neumann et al., 2023; Pourhamzeh et al., 2022). In mammals, serotonin is present in both male and female reproductive organs, distributed throughout the testis alongside its receptors and enzymes involved in biosynthesis (Fujinoki, 2011; Jimeńez-Trejo et al., 2012; Jiménez-Trejo et al., 2013). This presence is essential for testosterone synthesis and proper sperm development. Blocking serotonin's effects has been reported to disrupt hormonal communication between the testicles and the pituitary (Díaz-Ramos et al., 2018; Xu et al., 2022). Moreover, serotonin is implicated in supporting sperm flagellar movement through the phosphorylation of cAMP (Frungieri et al., 2002).

In this study, we focused on investigating the effect of the prototype washing media used before ART process on both sperm motility and ROS levels in sperm cells, in comparison to two commercially available wash media (Origio, Irvine). We also investigated the expression levels of two sperm proteins (angiotensin II type 2 receptor (AT2R) and Peroxiredoxin 2 (PRDX2)) that are previously documented to be associated with sperm motility, to uncover the molecular mechanisms underlying these reactions (O'Flaherty, 2014a).

Materials and methods

Patient collections and semen analysis

The semen samples were obtained from 31 male participants who were partners of couples undergoing intracytoplasmic sperm injection (ICSI) or in vitro fertilization (IVF) at the Atakent Acıbadem In Vitro Fertilization Center in Istanbul, Turkey. The selection of a small sample size consisting of 31 cases aimed to explore initial trends and collect preliminary data. The specimens were collected into a sterile container by masturbation after a sexual abstinence period of 2 to 5 days, and evaluated according to the guideline set (5th edition) by the World Health Organization (Gottardo & Kliesch, 2011). The fresh semen samples were liquefied for 10 min at 37°C with 5% CO_2 or 30 min at room temperature, and then processed. Subsequently, it was then subjected to routine andrological analyses to determine the concentration, pH, volume, viscosity, mobility and morphology. Makler Chamber was used to determine concentration and mobility for all samples undergoing pre- and post-evaluation steps. Furthermore, sperm morphological assessments of all samples were performed as Kruger's Strict Criteria and by the same operator. Before obtaining their signed consent, the subjects were informed in detail regarding the purpose of the research.

Spermatozoa preparation

Sperm washing refers to the procedure of isolating the viable and motile sperm from the remaining components of the semen sample. Besides containing live sperm, the ejaculate also contains enzymes, proteins and fluids that are unsuitable for intrauterine insemination (IUI) or directly proceeding with IVF (Fácio et al., 2016; Kim et al., 2015). As mentioned previously, our washing medium and two commercial sperm-washing media, Origio (ORIGIO ® Sperm Wash), and Irvine (Irvine Scientific, USA), were used to obtain a meaningful comparison for the sperm-washing process. During this phase, the semen specimen was split into four equal parts, and each part was processed with a dedicated washing medium. The control group (pre-wash) comprises semen specimens that have undergone no processing procedures. During swim-up, the tubes were first centrifuged at 1500x g for 10 min. Then, after the seminal fluid was removed, the selected medium was slowly added on top of the pellet and the tube was incubated for 20 min. At this stage, most motile sperm will swim into the upper layer in the added medium. Finally, a Makler counting chamber and a 10-µl aliquot of each semen sample collected from the upper layer were used to evaluate sperm characteristics, including concentration and motility for each aliquoted semen.

Sperm ROS detection

The intracellular ROS were identified using cell-permeable probe H2DCFDA ((H2-DCF, DCF) Cat No: D399), a fluorescein derivative. It is a chemically reduced version of fluorescein that is employed in cells as an indication of ROS (Aziz et al., 2010). The oxidation of this probe is detected with a flow cytometer to monitor the increase in fluorescence by employing fluorescein (green fluorescence) excitation sources and filters. The nonfluorescent H2DCFDA is transformed to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) when acetate groups are cleaved by intracellular esterases (An esterase is a hydrolase enzyme that, by a process known as hydrolysis, breaks down esters into an acid and an alcohol) and oxidation (a process that occurs when atoms or groups of atoms lose electrons). Although the oxidation of H2DCFDA is not directly susceptible to singlet oxygen, it can indirectly contribute to the production of DCF via reactions with cellular substrates that give peroxyl products and peroxyl radicals. Each semen sample was divided into four equal portions and individually suspended and incubated with available culture medium. The control group (4th group) was prepared using Phosphate-buffered saline (PBS) as a medium. Subsequently, each sperm suspension was divided into test and negative control

groups (25×10^5/ml spermatozoa). The test groups received the addition of H2DCF-DA (10 μM) and were incubated at 25°C in the dark for 30 min. Following incubation, the intensity of DCF fluorescence in cells was measured using a flow cytometer (Vaughan et al., 2019) (CytoFLEX, Beckman Coulter Life Sciences, CA, USA). Following that, we assessed the ROS value by enumerating positively and negatively stained spermatozoa, presenting the findings as the percentage of sperm cells manifesting ROS activity.

Western blotting assay

The semen was collected and lysed in radio-immuno precipitation assay buffer (Thermo Fisher Scientific, Waltham, USA) containing 1 mM phenyl methyl sulfonyl fluoride, and 1 mM sodium orthovanadate as a protease inhibitor, and the proteins (50 μg) in the lysate was separated by 12% SDS-PAGE. Following that, the isolated proteins were semi-dry transferred to PVDF membranes (Thermo Fisher Scientific, Waltham, USA) and the membranes were blocked by incubation with a 5% skim milk solution for 90 minutes. Then, the membranes were incubated with the different primary antibodies and concentrations (AT2R; Recombinant Anti-Angiotensin II Type 2 Receptor antibody (1:500, ab92445), PRDX2; PRX II Antibody (1:200, sc-515428) and β-actin (1:300, Abcam, Cambridge, MA) for each receptor at +4°C overnight. Subsequently, the membrane was washed and incubated with a horseradish peroxidase-conjugated (HRP) secondary antibody (1:3000, Abcam, Cambridge, MA) at room temperature for 2 hours. Then, the membrane was incubated with the chemiluminescent substrate (Thermo Fisher Scientific, Super Signal West Pico PLUS Chemiluminescent Substrate) for 5 min, and then viewed a cooled CCD camera.

A Western blot stripping protocol was performed for repeated use of the PVDF membranes to which semen proteins were transferred for each antibody. The blot was incubated in a stripping buffer at +50°C for 30 min. After removing the stain from the stripping buffer, it was washed for 5 min using washing buffer (TBS buffer containing 0.05% Tween 20 detergent). After this, the membrane was stripped, and the second probing experiment was started. Western blot image quantification was performed by Image J software.

Results

Semen analysis

Table 1 presents the initial semen volume, sperm concentration and motility results of the samples included in the analysis (Before the washing step with different media).

Effect of different washing media on sperm motility

The total motility obtained from different groups is summarized in Fig. 1A. Our pre-wash group had the lowest motility (41%), which was statistically significant among the four groups. While the newly-developed prototype medium exhibited the most significant increase in total motility (66%), the level of difference observed with respect to the results obtained from other commercial media was not statistically significant. The biodistribution chart shows that the deviant values range from 30 to 82, while the deviant values of the medium-treated groups are similar. As seen in Fig. 1B, the pre-washing specimen, similar to total motility, shows a lower value of progressive motility (28%) compared to semen samples washed with different media used in the study. This reduction is statistically significant. The prototype environment, with bias values in biodiversity distribution ranging from 20 to 75, demonstrates a notable rise in progressive motility (59%) as compared to Origio (56%) and Irvine (48%).

The effect of various washing media on ROS production

ROS levels were assessed using flow cytometry (Escada-Rebelo et al., 2020). The DCFH-DA probe was added to the semen mixture, following a 30-min incubation at 37°C with three distinct media, separately (Fig. 2A). Based on the distribution of biodiversity, the Prototype and Origio cohorts displayed comparatively minimal deviation, while the Irvine and pre-wash groups exhibited higher deviation values. According to the data shown in Fig. 2B-C, there is a notable discrepancy in ROS values between the Prototype (11.5%) and Origio (10.7%) groups versus the Irvine (25.8%) and Pre-wash (29.5%) groups, signifying statistical significance. Nevertheless, there is no discernible statistical disparity between these two groups. Notably, both the Prototype and Origio media groups demonstrated the highest sperm motility alongside the lowest ROS production levels, marking a significant association between levels of reactive oxygen species and sperm motility, as similar to the literature.

The effect of various washing media on sperm AT2R and PRDX proteins

AT2R protein expression was found to be elevated in spermatozoa exposed to a prototype medium, amounting to 34.5%, a statistically significant p-value of less than 0.05 as compared to commercial items (Fig. 3A). Sperm cells treated with both the control group (18.6%) and other mediums (Origio and Irvine) showed lower levels of AT2R protein than the prototype medium. Also, Fig. 3A shows that the significance became even more noticeable with a p-value of less than 0.001 as compared to the pre-wash control group. Upon a closer inspection of the distribution chart, a clear differentiation is seen between the values of 10 and 43. However, the prototype group has been shown to exhibit a distribution that spans from 22 to 43.

Figure 3B illustrates that the PRDX2 protein exhibits elevated levels within the prototype group compared to other groups, albeit without statistical significance. The pre-wash group demonstrates values closely resembling those of the other groups. The biodiversity distribution graph highlights significant outlier values, particularly evident within three distinct groupings.

Discussion

Male fertility hinges greatly on sperm quality and motility, crucial factors for successful fertilization in ART (Levine et al., 2017). Therefore, assessment of low viability and motility of sperm when performing ART such as IUI and ICSI, IVF is the first step in addressing male factor infertility, as these factors are vital components of the male reproductive system ((Baldini et al., 2021; Oseguera-López et al., 2019). In these techniques, the process of sperm washing stands as a vital necessity for the success of fertilisation (Henkel & Schill, 2003; Zollner et al., 2001). Seminal plasma containing sperm can produce harmful substances that diminish sperm viability such as seminal fluids, cells and bacteria. Sperm cell preparation prior to ART allows motile and morphologically normal spermatozoa to be effectively isolated from other cell types such as leukocytes and bacteria, as well as potentially hazardous chemicals (Baldini et al., 2021; Escada-Rebelo et al., 2020; Levine et al., 2017). Effective

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Table 1. Initial characteristics of the semen samples included in the study

Patient NO	Volume (ml)	Concentration (xMillion/ml)	Total M.	(+4)	(+3)	(+2)	(+1)
1	2.0	50	50%	0%	30%	20%	50%
2	2.5	80	43%	0%	31%	12%	25%
3	3.0	100	40%	5%	15%	20%	60%
4	3.0	110	30%	0%	22%	8%	70%
5	2.0	70	55%	1%	31%	23%	45%
6	2.5	80	30%	0%	20%	10%	70%
7	2.0	45	25%	0%	5%	20%	75%
8	2.0	40	25%	0%	10%	15%	75%
9	2.0	62	50%	10%	23%	17%	50%
10	2.5	70	60%	0%	40%	20%	40%
11	3.0	100	35%	2%	23%	10%	65%
12	2.5	50	40%	0%	25%	15%	60%
13	1.5	10	30%	5%	15%	10%	70%
14	2.0	70	40%	0%	25%	15%	60%
15	2.7	100	30%	0%	18%	12%	70%
16	2.5	80	55%	0%	30%	25%	45%
17	2.0	45	45%	0%	30%	15%	55%
18	1.8	16	25%	0%	15%	10%	75%
19	2.0	30	40%	10%	35%	10%	60%
20	1.8	12	40%	0%	31%	9%	60%
21	2.0	50	40%	0%	25%	15%	60%
22	3.2	100	40%	0%	30%	10%	60%
23	3.0	90	60%	10%	35%	15%	40%
24	2.5	86	55%	8%	23%	24%	45%
25	1.5	10	30%	0%	20%	10%	70%
26	2.5	90	60%	10%	40%	10%	40%
27	2.0	40	50%	0%	35%	15%	50%
28	2.2	50	50%	5%	25%	20%	50%
29	3.5	130	40%	10%	20%	10%	60%
30	2.0	40	25%	2%	18%	5%	75%
31	3.0	100	45%	10%	25%	10%	58%
Mean	**2.3**	**64.7**	**41.4%**	**2.8%**	**24.8%**	**14.2%**	**58%**
±SD	*0.5*	*31.6*	*10.9%*	*4.1%*	*8.3%*	*5.2%*	*12.6%*

procedures for eliminating seminal plasma are required to prepare and select normal and healthy sperm for ART therapy. For this purpose, centres performing ART employ two procedures: swim-up and density gradient centrifugation. These techniques rely on sperm movement and density, respectively. These processes involve the use of washing medium and centrifugation stages. Furthermore, the utilization of various media for sperm preparation is known to be beneficial for ART programmes (De Los Santos et al., 2016). Here, our study focuses on evaluating the impact of our novel sperm-washing medium in comparison to two commercially available media. A pivotal characteristic of our novel medium lies in its inclusion of serotonin, essential for spermatozoa development and metabolism, thereby potentially augmenting sperm motility (Jimeńez-Trejo et al., 2012; Dogan et al., 2024).

The findings of the study show that both total and progressive motility of sperm after sperm washing with three different media were significantly increased ($p < .05$) compared to the control group (washed with PBS only), revealing the importance of the selection of the media in sperm washing before ART. While a statistically significant high increase was observed between our prototype medium and the control group, relative increase is still observed when compared to commercial media. As it is generally known, the higher the motility is, the higher the Adenosine Triphosphate (ATP) requirement would be. Serotonin in our

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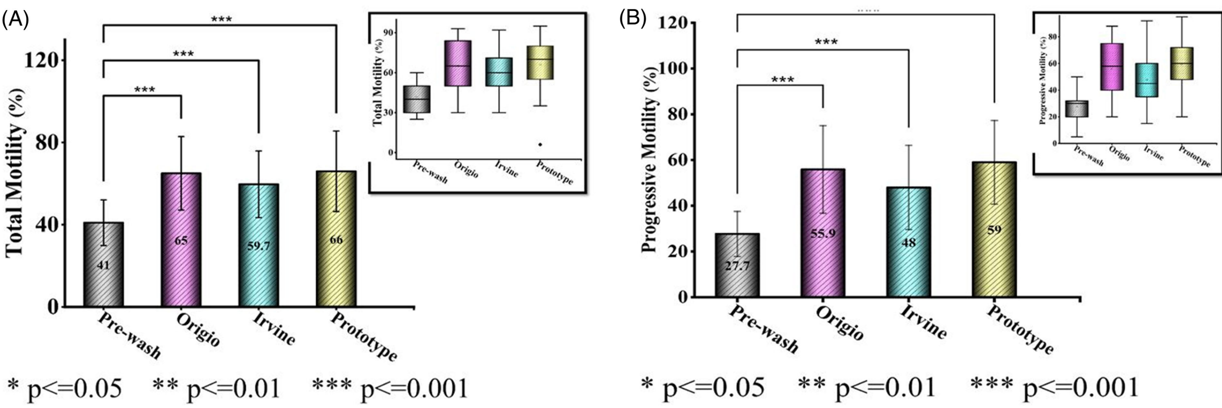

Figure 1. The effects of different washing mediums on sperm motility. (A) Total motility. (B) Progressive motility.

Figure 2. Following a 30-minute incubation at 37°C with DCFH-DA probe, the effects of various mediums on ROS levels were assessed using flow cytometry. (A) A forward and scatter dot plot was employed to identify the relevant population. (B) Histograms were utilized to display the fluorescence of the FITC channel, which measured the intensity of 2′,7′-dichlorofluorescin diacetate H2DCF-DA, a marker proportional to ROS production. An illustrative instance of a ROS experiment conducted on a single sample; (C) A comparison of the mean ROS generation during a 30-minute incubation period in three distinct media. Mean ± standard deviation. *p < 0.05 denotes a statistically significant difference when compared to the control group.

medium is known to affect blood flow in the testes as well as testicular development, cAMP formation and testosterone synthesis, indicating that any abnormalities in its metabolism can negatively affect reproductive health (Frungieri et al., 2002). Serotonin is thought to enhance sperm flagellar motility, which may be explained by its interaction with cAMP to phosphorylate (dynein phosphorylation mechanism) it (Stephens & Prior, 1992). In this mechanism, serotonin stimulates adenylate cyclase, an enzyme that synthesizes cAMP, triggering a signalling cascade that leads to an increase in intracellular cAMP levels. This increase in

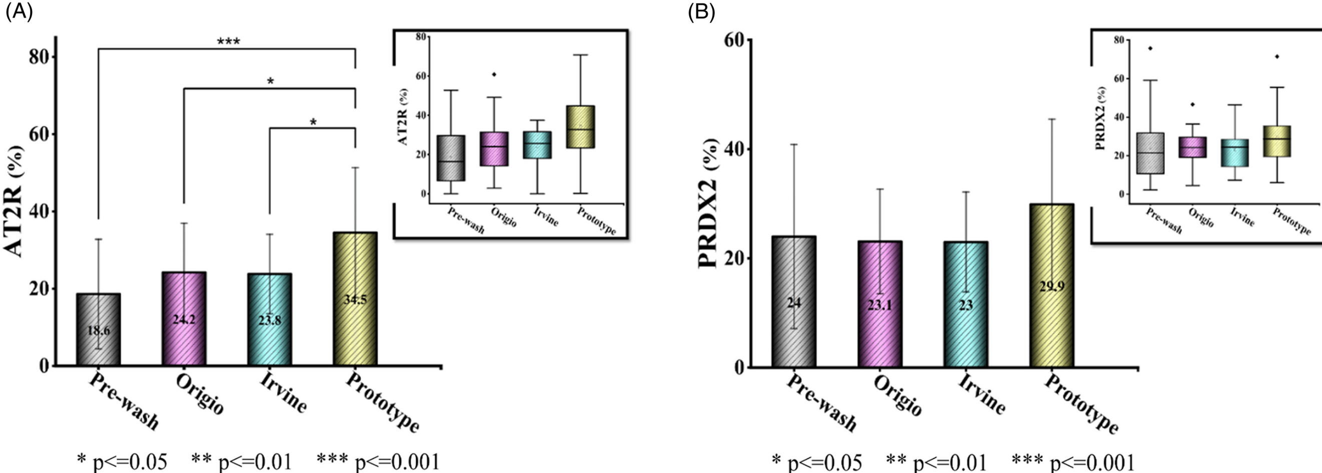

Figure 3. The effects of different washing media on protein expression. (A) Angiotensin II type 2 receptor (AT2R). (B) Peroxiredoxin 2 (PRDX2).

cAMP can have a wide range of physiological effects, including activation of protein kinases and other signalling pathways that affect cell motility (Chakraborty & Saha, 2022; Yilmazer et al., 2016).

Also, recent data have shown that the ATP required for sperm motility is generated by glycolysis, a process that occurs throughout the central portion of the sperm and that the frequency of flagella beating is closely related to the rate at which dynein hydrolyses ATP (Miki, 2007; Mukai & Okuno, 2004). Serotonin increases glycolytic flux by activating 6-phosphofructo-1-kinase (PFK), modulating the binding of the enzyme to the membrane cytoskeleton (Assouline-Cohen et al., 1998). Furthermore, the effect of 5-HT on sperm motility may be attributed to various molecular levels, including promoting intracellular calcium concentration (Torres-Flores et al., 2008) or an acrosomal reaction (Fujinoki, 2011).

Optimal levels of reactive oxygen species are essential for the proper functioning of sperm, whereas high levels of ROS can have detrimental effects on sperm cells, such as reduced sperm motility. High amounts of ROS can trigger oxidative stress, potentially damaging the sperm membrane, DNA, mitochondria and some cellular components (Bui et al., 2018; Jixiang et al., 2022). Consequently, this can lead to impaired sperm motility and reduced fertility. The antioxidant defense systems of sperm help to maintain the balance of reactive oxygen species, which requires precise regulation (Shahar et al., 2011). Because ROS production in small amounts is important for sperm function such as sperm capacitation. According to our findings, after 30 min incubation with various washing media, we observed a significant decrease in ROS production in our serotonin-containing medium (11.5%) and Origio (10.7%), which is in line with the literature that ROS levels decrease as serotonin content increases (Yilmazer et al., 2024). However, the reason for this decrease needs to be elucidated by molecular mechanisms. Recent studies have shown that oxidative stress negatively affects the ability of spermatozoa to acquire fertilizing ability (Fang & Zhong, 2020; Nowicka-bauer & Nixon, 2020), thus explaining why men with high levels of ROS in semen are infertile.

Angiotensin II is part of the renin-angiotensin system (RAS), a complex enzyme-protein chain that regulates kidney function, blood pressure, vascular tone and fluid balance, and it increases the percentage of motile sperm. To initiate this system, Angiotensinogen (AGT) is cleaved by renin to create Angiotensin I (Ang I). The angiotensin-converting enzyme (ACE) hydrolyses inactive Ang I to produce Ang II, a highly active peptide form (FORESTA et al., 1991). Ultimately, Ang II interacts with Ang II type 1 and 2 receptors (AT1R and AT2R) to carry out its functions (Pascolo et al., 2020). Male fertility is determined by a series of complicated and highly structured procedures that rely on the complex coordination of communication systems, particularly the RAS. According to literature, the male reproductive system has its own local RAS, with Renin believed to be produced by Leydig cells in the testes. Among other components, Ang I and Ang II, along with two types of ACE, somatic (sACE) and testis (tACE), are involved (FORESTA et al., 1991). The presence of Angiotensin II type 1 receptor (AT1R) has been noted in developing spermatids within the sperm tail, mature spermatozoa and sperm motility. Additionally, the angiotensin II type 2 receptor (AT2R) has been found in sperm and is associated with sperm concentration and motility (Gianzo et al., 2016; Gianzo & Subirán, 2020; Vinson et al., 1995; Wennemuth et al., 1999). Also, it was demonstrated that asthenozoospermic samples exhibit lower levels of AT2R expression in comparison to normozoospermic samples (Gianzo et al., 2016; Pascolo et al., 2020). In our study, sperm samples washed with a medium containing serotonin showed significantly higher levels of ATR2 compared to the other three groups, providing compelling evidence that it could contribute to sperm motility. In addition, reaching a much higher AT2R level with our developing medium compared to the commercially available Origio (24.2%) and Irvine (23.8%) environments is promising in terms of commercialization potential. Additionally, considering the effect of the serotonin-supplemented medium on sperm motility and ROS production, the increase in AT2R is consistent.

PRDXs, with six members (PRDX1 to 6), are a unique antioxidative protein family that includes specific cysteine residues that aid in the break of hydroperoxides (Hampton & O'Connor, 2016). One or two Cys residues at the active site help their activity (O'Flaherty, 2014a) and are defined by their ability to catalyze peroxide reduction using thioredoxin as an electron donor. PRDXs are original SH-dependent, selenium- and heme-free peroxidases that are found in almost all living organisms. These proteins, in contrast to traditional antioxidant enzymes, carry out a variety of biological functions, such as scavenging reactive oxygen species to protect cells from OS and controlling ROS-dependent pathways (Wood et al., 2003). PRDXs are abundant and differently

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distributed throughout the human spermatozoon and are thought to be the first line of defense against oxidative stress damage in human spermatozoa. Excessive levels of ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2\bullet^-$), the hydroxyl radical (HO•), nitric oxide (NO•) and peroxynitrite ($ONOO^-$) in spermatozoa, which are usually formed in the sperm mitochondria (Koppers et al., 2008) or by a mixture of them (NO• and O• produce ONOO−) and become harmful by-products of cellular metabolism (Ferramosca et al., 2013), are linked to male infertility (Agarwal et al., 2006; Aitken & Baker, 2006; Liu et al., 2016). Also, PRDX2 is the fastest-regenerating redox protein among the six members of the PRDX family and has a higher H_2O_2 neutralization efficiency (Liu et al., 2016). PRDX2, also referred to as peroxiredoxin-2, serves as a pivotal constituent of the antioxidant enzyme pathway in sperm cells, playing a pivotal role in ROS scavenging generated during critical physiological processes such as sperm maturation, capacitation and the acrosome reaction. The sperm's flagellum, plasma membrane, and head (acrosome, nucleus and equatorial region) all contain PRDX2 (O'Flaherty, 2014b). PRDX2's enzymatic activity is dependent on the thioredoxin system. PRDX2 aids in the reduction of peroxides, highly reactive chemicals that can harm biological components such as proteins, lipids and DNA, by converting them to water (H_2O) and other less hazardous molecules (O'Flaherty, 2014a). Functioning as a thiol-specific antioxidant enzyme, PRDX2 exploits its active cysteine residue to efficiently counteract peroxides and detoxify ROS, significantly contributing to maintaining redox equilibrium in sperm cells. Through ROS neutralization, PRDX2 aids in preserving sperm functionality and integrity, highlighting its indispensable role in shielding sperm cells from oxidative stress-induced impairment within the intricate landscape of male reproductive physiology (O'Flaherty, 2014a, 2014b; Wood et al., 2003). According to the findings, our developing medium, from the washing mediums applied to the sperms washed with the swim-up technique, was found to significantly reduce the ROS level and relatively increase the amount of PRDX2 protein in semen cells, not statistically significant. However, after treatment of semen samples with Origio and Irvine media, there was no increase in PRDX2 protein, even the results were relatively less than the control group. The observed rise in PRDX2 levels following exposure to the prototype medium can be attributed that the prototype medium promotes the interaction between PRDX2's active cysteine residue and oxidized forms of oxygen molecules, resulting in a decrease in ROS levels. Conversely, exposure to Origio and Irvine media does not induce a comparable increase in PRDX2 levels. This discrepancy indicates that these media may impact ROS levels through pathways different from those affected by the prototype medium. The results emphasize the possibility of improving sperm-washing techniques, particularly by using enhanced media, to positively impact important factors related to sperm function as well as specific difficulties experienced in the ART field.

Serotonin-activated washing media for sperm preparation could potentially offer a novel approach to selecting high-quality sperm for ART programmes. By conducting more comprehensive molecular analyses with a larger sample size in future studies, one can delve deeper into understanding the mechanisms behind this process and further validate its efficacy. It's crucial to continue research in this area to improve the success rates of ART procedures and ultimately help individuals and couples achieve their reproductive goals.

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Approval for this work was obtained from the Ethics Committee of Acibadem Mehmet Ali Aydinlar University (ATADEK-2021-01/26), and informed consent has been obtained from all participants.

Competing interests. The authors declare that there are no conflicts of interest.

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