CAMBRIDGE
UNIVERSITY PRESS

In vitro effects of the combination of serotonin, selenium, zinc, and vitamins D and E supplementation on human sperm motility and reactive oxygen species production

Yasemin Yilmazer[1] [iD], Elnaz Moshfeghi[2], Fadime Cetin[3] and Necati Findikli[4]

[1]Department of Molecular Biology and Genetics, Istanbul Sabahattin Zaim University, Istanbul, Turkey; [2]Department of Molecular Biology and Genetics, Yildiz Technical University, Istanbul, Turkey; [3]Department of Bioengineering, Yildiz Technical University, Istanbul, Turkey and [4]IVF Laboratory, Erasme Hospital, Brussels, Belgium

Summary

Infertility affects 15% of all couples worldwide and 50% of cases of infertility are solely due to male factors. A decrease in motility in the semen is considered one of the main factors that is directly related to infertility. The use of supplementation to improve the overall sperm quality has become increasingly popular worldwide. The purpose of this study was to evaluate whether sperm motility was affected by the combination of serotonin (5-HT), selenium (Se), zinc (Zn), and vitamins D, and E supplementation. Semen samples were incubated for 75 min at 37°C in medium containing varying concentrations of 5-HT, Se, Zn, vitamin D, and E. 5-HT (200 µM), Se (2 µg/ml), Zn (10 µg/ml), vitamin D (100 nM), and vitamin E (2 mmol) have also been shown to increase progressive sperm motility. Three different mixtures of supplements were also tested for their combined effects on sperm motility and reactive oxygen species (ROS) production. While the total motility in the control group was 71.96%, this was found to increase to 82.85% in the first mixture. In contrast the average ROS level was 8.97% in the control group and decreased to 4.23% in the first mixture. Inclusion of a supplement cocktail (5-HT, Se, Zn, vitamins D and E) in sperm processing and culture medium could create an overall improvement in sperm motility while decreasing ROS levels during the incubation period. These molecules may enhance the success of assisted reproduction techniques when present in sperm preparation medium.

Introduction

Sperm motility is a critical factor in the male reproductive system. Low sperm motility and viability, a measurement of sperm's capacity for fertilization, are indicative of poor semen quality, which is recognized as a major contributing factor to male infertility (Brugh and Lipshultz, 2004). Male factor infertility can be treated with assisted reproductive technologies (ARTs), such as intrauterine insemination (IUI) and *in vitro* fertilization (IVF) with or without intracytoplasmic sperm injection. Gradient separation (Percoll) and swim-up techniques are commonly used to process sperm specimens in IUI and *in vitro* fertilization (IVF; Akerlöf *et al.*, 1987). The use of a washing medium and centrifugation steps in these processes can result in low motile sperm recovery. In addition to the percentage of total motility, the type of sperm movement is also documented to influence fertilizing capacity (Auger *et al.*, 1994).

Physiologically, reactive oxygen species (ROS) are produced in human semen and sperm during spermatogenesis, mitochondrial activity, and capacitation (Agarwal *et al.*, 2014). However, excessive ROS production can disrupt the balance between oxidants and antioxidants in semen, leading to oxidative damage in various macromolecules within spermatozoa such as polyunsaturated acids in the plasma membrane, nucleic acids in DNA structure, and mitochondrial function (Aitken, 2017). This damage contributes to sperm dysfunction related to membrane integrity, resulting in impaired motility, viability, acrosome reaction, sperm–oocyte fusion, and DNA integrity, leading to difficulties in fertilization, implantation, and the maintenance of pregnancy (Alahmar, 2019).

It is known that neurotransmitters, which are responsible for carrying electrical signals between nerve cells, significantly affect the male reproductive systems. According to studies, varying degrees of secretion of neurotransmitters, especially serotonin, result in significant changes in the mating rate of males. Serotonin (5-HT or 5-hydroxytryptamine hydrochloride) is a neurotransmitter that is produced by the hydroxylation and decarboxylation of tryptophan in the body and is essential for almost all living organisms (Hull *et al.*, 2004; Hull and Dominguez, 2007). Meizel and Turner observed that serotonin induced an acrosomal reaction (AR) in capacitated sperm, implying the presence of a 5-HT-mediated receptor effect in the hamster

sperm (Meizel and Turner, 1983). When mammalian spermatozoa exhibit AR in their heads and hyperactivity in their flagella, they are ready for fertilization (Austin, 1977). For the sperm and egg plasma membranes to fuse, the acrosome must undergo an exocytotic event known as the AR to reach the zona pellucida (ZP) of the egg (Yudin *et al.*, 1988; Fujinoki, 2009). The force for spermatozoa to penetrate the ZP is created by the sperm flagellum's hyper-activation movement (Fujinoki *et al.*, 2001; Suarez and Ho, 2003). Overall, numerous studies so far have shown that serotonin can trigger the acrosome reaction (Austin, 1977; Meizel and Turner, 1982, 1983; Yudin *et al.*, 1988; Fujinoki *et al.*, 2001; Suarez and Ho, 2003; Fujinoki, 2009).

Serotonin regulates libido and sexual behaviour at the reproductive level in mammals through a hormonal mechanism; it is therefore crucial for reproductive organs. Serotonin plays reproductive roles by contracting the vas deferens, controlling cAMP, blood flow in the testicles, and producing testosterone (Parisi *et al.*, 1984). Serotonin promotes motility and raises the rate of spermatozoa fertilization in invertebrates. This enhanced motility caused by serotonin could be due to flagella activation via cAMP-dependent dynein phosphorylation (Bandivdekar *et al.*, 1992; Stephens and Prior, 1992). One study showed that serotonergic neurons control copulation behaviour in *Drosophila melanogaster* (Yilmazer *et al.*, 2016).

According to previous studies, dietary selenium can increase sperm quality in infertile individuals (Keskes-Ammar *et al.*, 2003; Moslemi and Tavanbakhsh, 2011). Also *in vitro* selenium supplementation is shown to improve sperm motility and viability (Ghafarizadeh *et al.*, 2018). Reactive oxygen species are reduced by zinc antioxidant effects (Bray and Bettger, 1990; Narasimhaiah *et al.*, 2018). High ROS concentrations can impair sperm function (Sikka, 2001). Sperm quality, including motility, morphology, concentration, and acrosome response in the serum and seminal plasma, may be influenced by vitamin D (Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2011; Ramlau-Hansen *et al.*, 2011; Jueraitetibaike *et al.*, 2019). Vitamin E may lessen the effects of oxidative stress, enhancing the viability of sperm for *in vitro* fertilization (Suleiman *et al.*, 1996; Greco *et al.*, 2005).

Some studies have shown the individually significant effect of serotonin, selenium, zinc, and vitamins D and E supplementation on sperm motility. Therefore, we decided to evaluate the combination of these supplementation effects on human sperm motility and ROS levels *in vitro* conditions. The aim of this study was to evidence the synergistic incidence of antioxidants and serotonin on the motility and ROS production of spermatozoa. The combined effect of antioxidants and serotonin is stronger than the effect alone.

Materials and methods

Sample collection

In this study, 150 normozoospermic men were included and their semen samples were analyzed according to the World Health Organization (2010) guidelines (World Health Organization, 2010) for the effectiveness of serotonin, selenium, zinc, and vitamins D and E on sperm motility. After 3–5 days of sexual abstinence, samples were collected via masturbation. Ejaculates were incubated for 30 min at 37°C for liquefaction. After that, a Makler counting chamber and 10 μl aliquot of semen were used to assess basal sperm characteristics including concentration and motility. All volunteers read and signed an informed consent form

before agreeing to take part in this study, which was approved by the Bezmialem Vakif University's Ethical Committee (Ethical Approval Number 15/166).

Sperm preparation

The three-layer density gradient technique was used for sperm preparation (Allamaneni *et al.*, 2005). The first step was to place 1 ml of 90% gradient medium at the bottom of a centrifuge tube, followed by 1 ml of 70% gradient medium and, finally, 1 ml of 50% gradient medium, to construct a gradient column. Then, 2 ml semen was added on top of the 50% layer, and the mixture was centrifuged at 300 rpm for 20 min. After centrifugation, the pellet was resuspended and washed with 3 ml of sperm-wash medium and then centrifuged for 10 min at 500 rpm. The supernatant was once more discarded, and the pellet was resuspended to the final volume in 0.55 ml of sperm-wash medium. Prepared sperm were then added and incubated in equal concentrations in different test groups as well as in the control group. For the control group, semen was incubated with HEPES-containing Modified Human Tubal Fluid (HTF) medium.

Sperm incubation with enriched medium

To evaluate the effectiveness of the supplements, serotonin, selenium, zinc, and vitamins D and E were added to HEPES-containing Modified HTF medium. The different doses of each supplements used are as follows: 25, 50, 100, 200, and 300 μM serotonin (5-HT, Santa Cruz-298707), 1, 2, and 4 μg/ml selenium (sodium selenite, Santa Cruz-253595A), 1, 10, and 20 μg/ml zinc (MERCK-108883), 50, 100, and 150 nM vitamin D 1α,25-dihydroxyvitamin D_3 (1,25D, Calbiochem-Milliopore-5.097210001), and 1, 2 and 3 mmol vitamin E (α-tocopherol, Sigma-Aldrich-258024). For each supplement group, 30 samples obtained from normozoospermic men were used. Three distinct combinations were formulated using an HTF-based sperm-washing medium enriched with HEPES buffer, guided by initial findings and varying concentrations of serotonin, vitamins, and minerals. These combinations are referred to as Mix 1, Mix 2, and Mix 3. Mix 1 consists of 100 μM serotonin, 2 μg/ml selenium, 10 μg/ml zinc, 100 nM vitamin D, and 2 mmol vitamin E. Mix 2 consists of 50 μM serotonin, 1 μg/ml selenium, 5 μg/ml zinc, 50 nM vitamin D, and 1 mmol vitamin E. Mix 3 consists of 25 μM serotonin, 1 μg/ml selenium, 1 μg/ml zinc, 50 nM vitamin D, and 1 mmol vitamin E. To determine the best combination, 30 normozoospermic men's samples were used in sperm motility and ROS experiments. The sperm motility of the prepared spermatozoa was then assessed after 75 min of incubation in each of these media at 37°C with 5% CO_2.

Assessment of sperm motility

After the incubation period, 10 μl of sperm suspension from each experimental group was applied to a Makler counting chamber (Sefi Medical Industries, Haifa, Israel), and motility parameters were assessed. A minimum of five microscopic fields was examined to determine the percentage of sperm motility, involving at least 200 spermatozoa for each sample. Following the criteria outlined by WHO in 2010, sperm motility was categorized into three groups: progressive motile (Group A, representing sperm that swim primarily in a straight line or large circles), non-progressive motile (Group B, encompassing all other patterns of active tail movements without progression, including swimming in small
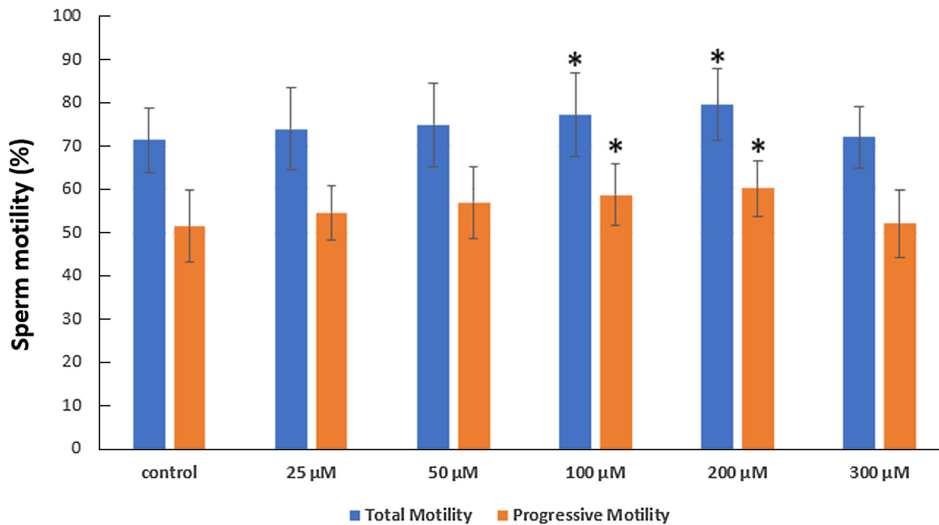
Figure 1. The impact of serotonin addition on sperm motility was investigated after 75 min of incubation. Mean standard deviation (SD). *$P < 0.05$ indicates a significant deviation from the control group.

circles), and immotile (Group C, indicating sperm cells with no observable movement). Total motility denotes the overall number of sperm cells in motion.

Determination of ROS levels in human spermatozoa

Within the scope of the ROS experiment, 2′,7′-dichlorofluorescin diacetate H2DCF-DA (H2-DCF, DCF; cat. no. D399, Invitrogen) fluorescent dye was used to detect intracellular H_2O_2 level in the spermatozoa by flow cytometry (CytoFLEX, Beckman Coulter Life Sciences, CA, USA).

Briefly, sperm samples were aliquoted into negative control and test groups and analyzed by direct flow cytometry without adding any dye to the control group (25×10^5 ml/spermatozoa). In each group, DCFDA (final concentration of 10 µM) was added to the sperm suspension (25×10^5 /ml spermatozoa). The mixture was incubated at 25°C for 30 min in the dark, and the labelled spermatozoa were analyzed by flow cytometry. In comparison with the untreated group, the ROS levels in sperm cells were measured as a percentage of fluorescence intensity (Guthrie amd Welch, 2006).

Statistical analysis

Three repeated-measures analysis of variance (ANOVA) was used to assess the effect of medium on sperm motility using the IBM SPSS Statistics 25 software package. Statistical significance was determined using one-way ANOVA (Tukey's post hoc) and paired *t*-test. A $P < 0.05$ value was considered statistically significant. The data were presented as mean ± standard deviation.

Results

Effect of 5-HT supplementation on sperm motility

After a 75-min incubation period, sperm motility was significantly increased in solution with 100 and 200 µM serotonin. The average total motility was found to increase from 71.36% (in the control group), to 73.90% in 25 µM, 74.89% in 50 µM, 77.16% in 100 µM, 79.44% in 200 µM and 71.95% in 300 µM serotonin-containing solution. Similarly, the average progressive motility increased from 51.37%, to 54.58% in 25 µM, 56.90% in 50 µM, 58.68% in 100 µM, 60.10% in 200 µM and 52.05% in 300 µM serotonin-containing solution (Figure 1).
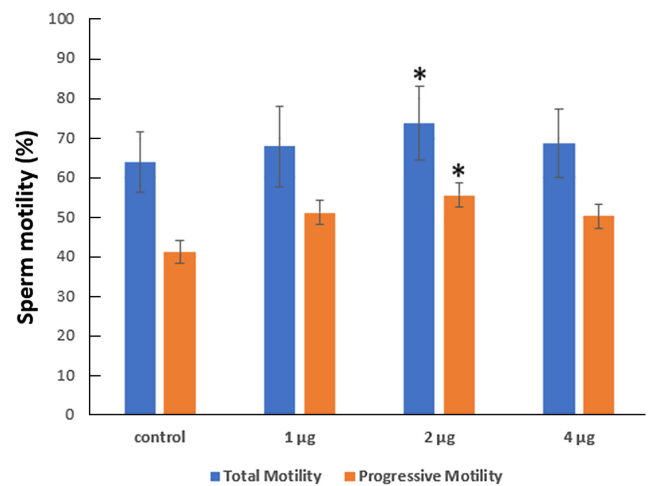

Figure 2. Following the 75 min of incubation, the effect of selenium addition on sperm motility was investigated. Mean ± standard deviation (SD). *$P < 0.05$ significant difference compared with the control group.

Effect of selenium supplementation on sperm motility

After 75 min of incubation, sperm motility was significantly increased in solution with 2 µg/ml selenium. The average of total motility was found to increase from 63.95% (in the control group) to 67.88% in 1 µg/ml, 73.61% in 2 µg/ml, and 68.60% in 4 µg/ml of selenium-supplemented solution. The average progressive motility was found to increase from 41.27% to 51.18% in 1 µg/ml, 55.58% in 2 µg/ml, and 50.36% in 4 µg/ml of selenium-supplemented solution (Figure 2).

Effect of zinc supplementation on sperm motility

The incubation of spermatozoa for 75 min in zinc-supplemented medium created an increase in the total motility from 64.30% (in the control group) to 67.55% in 1 µg/ml, 72.71% in 10 µg/ml and 68.87% in 20 µg/ml zinc-supplemented medium. The average progressive motility was found to increase from 37.99 to 44.14% in 1 µg/ml, 50.64% in 10 µg/ml and 40.65% in 20 µg/ml zinc-supplemented medium (Figure 3). Sperm motility was significantly increased in solution with 10 µg/ml zinc.
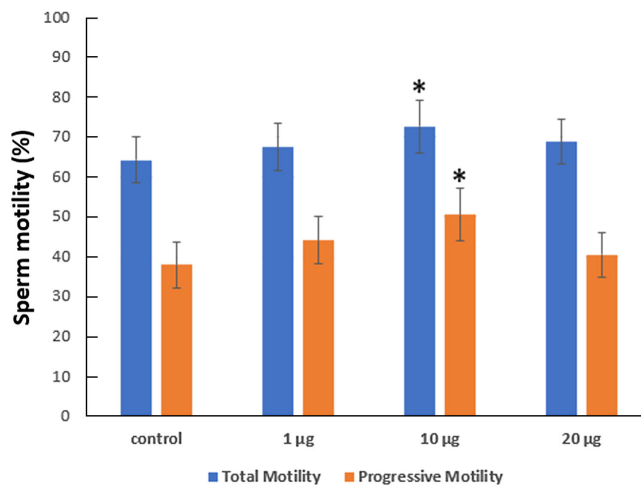
Figure 3. The function of zinc supplementation on sperm motility was investigated after 75 min of incubation. Mean standard deviation (SD). *$P < 0.05$ indicates a significant deviation from the control group.
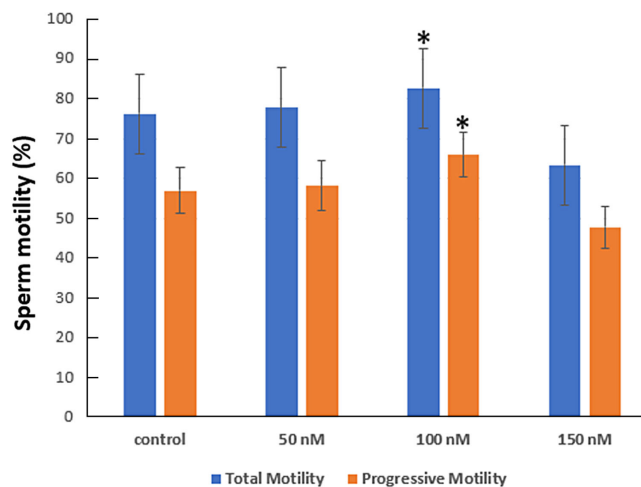

Figure 5. Vitamin E affecting supplementation on sperm motility was studied. Mean ± standard deviation (SD). *$P < 0.05$ significant difference compared with the control group.

Figure 4. After 75 min of incubation, the effect of Vitamin D supplementation on sperm motility was studied. Mean ± standard deviation (SD). *$P < 0.05$ significant difference compared with the control group.

Figure 6. After 75 min of incubation, the effect of solution that mixtures in different ratios on sperm motility was studied. Mean ± standard deviation (SD). *$P < 0.05$ significant difference compared with the control group.

Effect of vitamin D supplementation on sperm motility

At the end of the 75-min incubation period the average total motility decreased from 76.28% to 63.40% in 150 nM vitamin D solution, while it increased to 77.82% in 50 nM and 82.58% in 100 nM vitamin D-containing solution. The average progressive motility decreased from 56.93% to 47.76% in 150 nM vitamin D solution, but increased to 58.14% in 50 nM and 65.89% in 100 nM vitamin D-containing solution (Figure 4). Sperm motility was significantly increased in solution with 100 nM vitamin D.

Sperm motility of vitamin E

According to the control group, the average total motility decreased from 74.77% to 55.84% in 3 mmol vitamin E solution, whereas it increased to 75.15% in 1 mmol and 77.78% in 2 mmol vitamin E-containing solution. The average progressive motility was found to decrease from 55.21% to 46.04% in 3 mmol vitamin E solution, but increased to 55.95% in 1 mmol and 64.07% in 2 mmol vitamin E-containing solution (Figure 5). Sperm motility was significantly increased in solution with 2 mmol vitamin E.
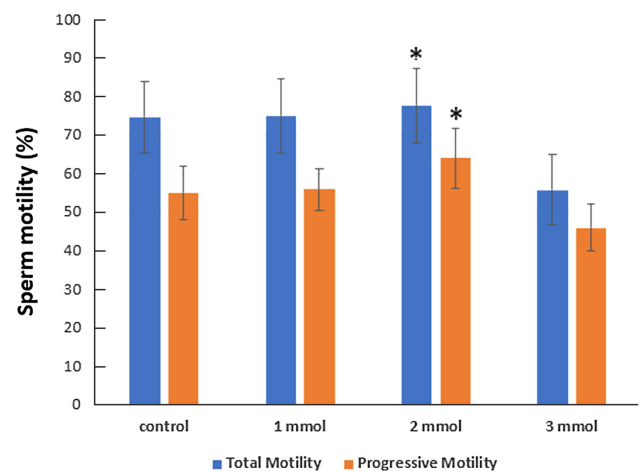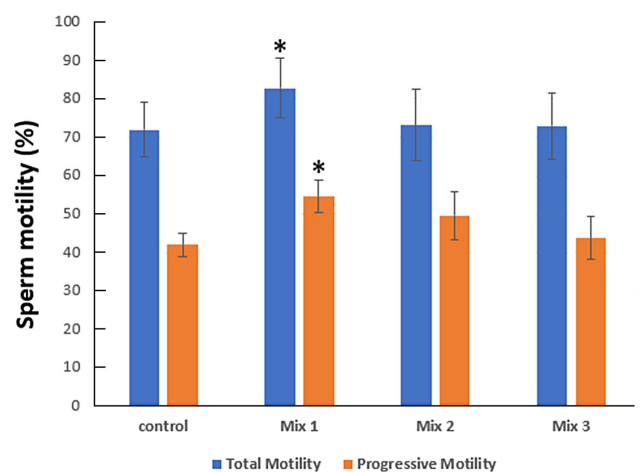
Effect of different mixtures of supplementations on sperm motility

Three different solutions were prepared with an HTF-based sperm-washing medium containing 10 ml of HEPES buffer and different concentrations of each of serotonin, vitamins, and minerals in each. After 75 min of incubation, while the average total motility was 71.96% in the control group (without supplement), it was found to increase to 82.85% in Mix 1, 73.20% in Mix 2 and 72.89% in Mix 3. The average progressive motility was found to increase from 41.94% to 54.52% in Mix 1, 49.60% in Mix 2 and 43.72% in Mix 3 (Figure 6). Finally, it was found that the mixture with the highest serotonin concentration (Mix 1) was superior to the others in terms of enhancing sperm motility.

Effect of different mixtures of supplementations on ROS production

A ROS experiment was performed using flow cytometry after 75 min of incubation. Sperm motility was highest in the Mix 1 group, and ROS production was also found to be lowest in Mix 1. After 75
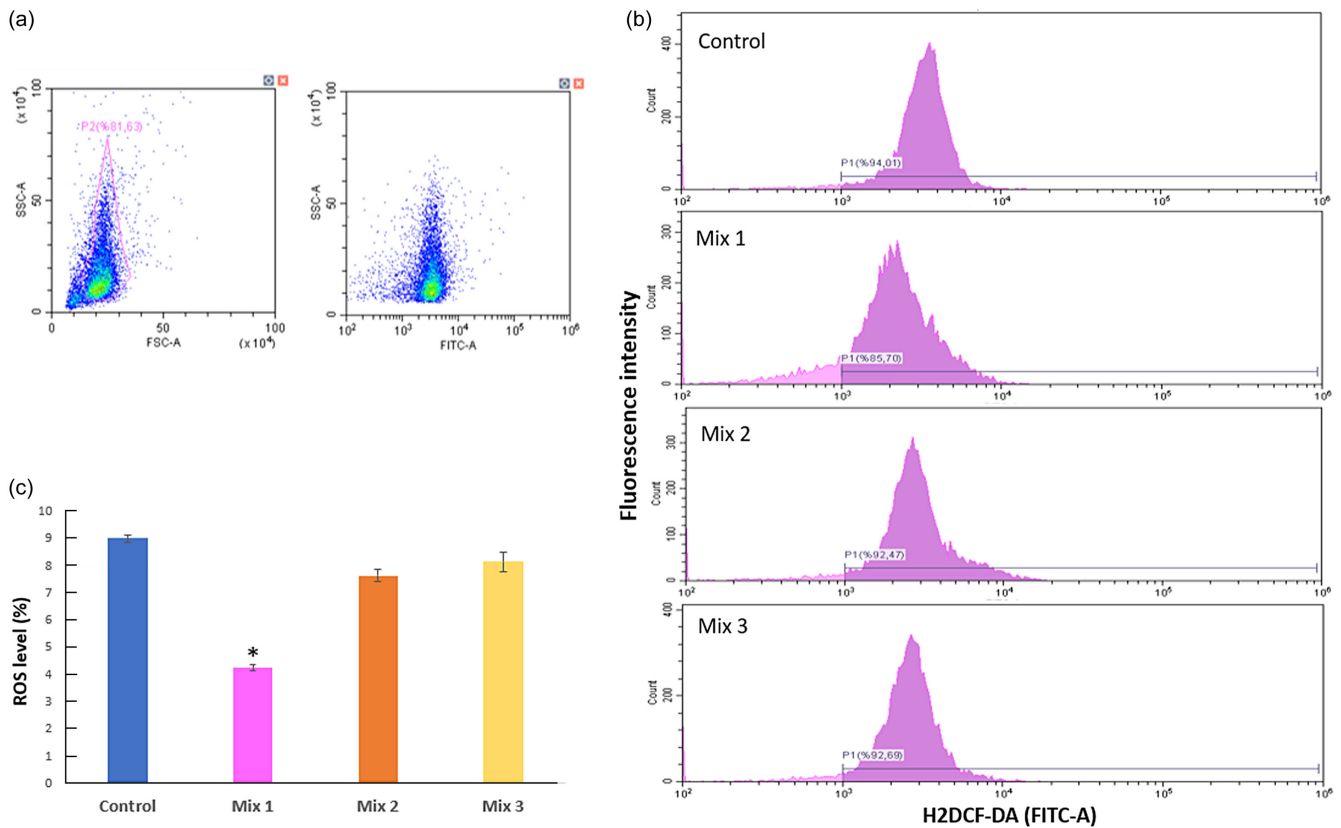
Figure 7. After 75 min of incubation, the effect of supplement mixtures in different ratios on ROS levels was studied. (a) Forward scatter dot plot used to select the respective population. (b) Histograms displaying fluorescence of fluorescein isothiocyanate (FITC) channel, used to measure fluorescence intensity of $2',7'$-dichlorofluorescin diacetate H2DCF-DA that is proportional to ROS production. Representative example of one sample ROS experiment. (c) Comparison of the average of ROS production after 75 min incubation of three mixtures. Mean ± standard deviation (SD). *$P < 0.05$ significant difference compared with the control group.

min of the incubation, while the average of ROS level was 8.97% in the control group (without supplement), it was decreased to 4.23% in the first, 7.62% in the second and 8.12% in the third mixture (Figure 7).

Discussion

Male fertility problems are becoming more prevalent around the world, as evidenced by a decrease in sperm concentration and spermatozoa motility (Levine *et al.*, 2017). Recent studies have shown that nutrients, vitamins, and minerals have essential roles in sperm motility and quality (Keskes-Ammar *et al.*, 2003; Aquila *et al.*, 2008, 2009; Ghafarizadeh *et al.*, 2018). The main ingredient of our mixture is serotonin, which is crucial for the growth and metabolism of spermatozoa while promoting sperm motility (Jimeńez-Trejo *et al.*, 2012). Furthermore, the serotonergic system plays multiple roles in the testes during spermatogenesis, insemination, the mating phase and the sperm preparation processes that occur in the caput epididymis (Jiménez-Trejo *et al.*, 2018). The 5-HT present in seminal fluid or uterine interacts with 5-HT receptors and 5-HT transporters on the surface of sperm to affect sperm physiology (Mann *et al.*, 1961).

In this study, it was found that after 75 min of incubation, samples treated with 200 µM serotonin, 2 µg/ml selenium, 10 µg/ml zinc, 100 nM vitamin D, and 2 mmol vitamin E solution had significantly increased progressive motility compared with the control group ($P < 0.05$). These results indicated that *in vitro* serotonin and antioxidant supplementation in the sperm-washing/

incubation medium could provide and create protective and beneficial effects on sperm motility according to our findings. The findings of this study also support previous research findings that antioxidant supplementation improves sperm motility in *in vitro* medium (Amenta *et al.*, 1992; Banihani *et al.*, 2012; Yun *et al.*, 2013). The absence of double or triple combinations of these ingredients in literature encouraged us to develop combinations with different dosages. According to our studies, sperm motility was highest in the Mix 1 group due to a positive correlation between sperm motility and serotonin content in Mix 1.

The overproduction of ROS can result in oxidative stress, leading to damage to proteins, DNA, and the plasma membrane of human spermatozoa (Bui *et al.*, 2018). In our experiments, we observed a reduction in ROS production after 75 min incubation with serotonin and antioxidant supplementation. With an increase in the serotonin content in Mix 1, there was a corresponding decrease in ROS production. While ROS production plays a crucial role in sperm capacitation (O'Flaherty and Matsushita-Fournier, 2017), excessive ROS levels can adversely affect sperm functions (Sikka, 2001). Studies have also shown previously that *in vitro* selenium supplementation protects sperm plasma membrane from oxidant damage (Ghafarizadeh *et al.*, 2018), and zinc exhibits antioxidant properties and has the potential to reduce ROS levels (Bray and Bettger, 1990). Vitamin E also serves as a protective agent against ROS-induced damage to spermatozoa (Verma and Kanwar, 1999).

In this study, the supplements used in the evaluated mixture solutions are selenium, zinc, vitamin D, and vitamin E. Another

crucial element is selenium (Se), which is required for male fertility maintenance. Selenium deficiency in the diet impairs spermatogenesis and increases oxidative stress. Normal spermatogenesis requires selenium. Here, low selenium levels in the seminal are associated with barren sperm quality and a higher risk of male infertility. Sperm quantity, motility, regular morphology, and vitality are all markedly correlated with seminal plasma selenium levels (Morbat *et al.*, 2018). However, Zn deficiency appears to have a greater influence on sperm movement. Some researchers have found a positive correlation between seminal fluid Zn and active sperm motility and an inverse correlation between immotile sperm motility nevertheless, sperm motility appears to be more affected by Zn deficiency. According to some studies, semen Zn has a positive correlation with active sperm motility and an inverse correlation with immotile sperm motility (Yamaguchi *et al.*, 2009). All these findings indicate that seminal plasma Zn levels are crucial for spermatogenesis, sperm maturation, sperm parameter quality, and spermatozoa's capacity to fertilize. Sperm motility is regulated by vitamin D in seminal plasma. When vitamin D is in its active form, it might improve sperm motility by activating the sperm mitochondrial respiratory chain and increasing intracellular calcium ion concentration via the cAMP/PKA pathway (Jueraitetibaike *et al.*, 2019). Vitamin E can help spermatozoa survive and make better sperm viability and motility. Vitamin E supplementation at sperm diluent may improve sperm quality by lowering lipid peroxidation of the sperm plasma membrane.

To sum up, our results show that serotonin, selenium, zinc, vitamin D, and vitamin E can be added to the commercial medium to improve sperm preparation for ARTs. These nutrients may also help to protect sperm quality during preparation and sampling. Further investigation is required to determine the effect of serotonin and antioxidant supplementation on fertility and embryo development in these patients during IVF or intra-cytoplasmic sperm injection (ICSI) procedures. Our findings will be applied to the development of a new, more effective sperm-wash medium containing serotonin and antioxidants.

References

Agarwal, A., Virk, G., Ong, C. and du Plessis, S. S. (2014). Effect of oxidative stress on male reproduction. *The World Journal of Men's Health*, **32**(1), 1–17. doi: 10.5534/wjmh.2014.32.1.1

Aitken, R. J. (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Molecular Reproduction and Development*, **84**(10), 1039–1052. doi: 10.1002/mrd.22871

Akerlöf, E., Fredricson, B., Gustafsson, O., Lundin, A., Lunell, N. O., Nylund, L., Rosenborg, L. and Pousette, A. (1987). Comparison between a swim-up and a Percoll gradient technique for the separation of human spermatozoa. *International Journal of Andrology*, **10**(5), 663–669. doi: 10.1111/j.1365-2605.1987.tb00367.x

Alahmar, A. T. (2019). Role of oxidative stress in male infertility: An updated review. *Journal of Human Reproductive Sciences*, **12**(1), 4–18. doi: 10.4103/jhrs.JHRS_150_18

Allamaneni, S. S., Agarwal, A., Rama, S., Ranganathan, P. and Sharma, R. K. (2005). Comparative study on density gradients and swim-up preparation techniques utilizing neat and cryopreserved spermatozoa. *Asian Journal of Andrology*, **7**(1), 86–92. doi: 10.1111/j.1745-7262.2005.00008.x

Amenta, F., Vega, J. A., Ricci, A. and Collier, W. L. (1992). Localization of 5-hydroxytryptamine-like immunoreactive cells and nerve fibers in the rat female reproductive system. *The Anatomical Record*, **233**(3), 478–484. doi: 10.1002/ar.1092330315

Aquila, S., Guido, C., Perrotta, I., Tripepi, S., Nastro, A. and Andò, S. (2008). Human sperm anatomy: Ultrastructural localization of 1α,25-dihydroxyvitamin D3 receptor and its possible role in the human male gamete. *Journal of Anatomy*, **213**(5), 555–564. doi: 10.1111/j.1469-7580.2008.00975.x

Aquila, S., Guido, C., Middea, E., Perrotta, I., Bruno, R., Pellegrino, M. and Andò, S. (2009). Human male gamete endocrinology: 1alpha,25-dihydroxyvitamin D_3 (1,25(OH)2D$_3$) regulates different aspects of human sperm biology and metabolism. *Reproductive Biology and Endocrinology: RB&E*, **7**, 140. doi: 10.1186/1477-7827-7-140

Auger, J., Serres, C., Wolf, J. P. and Jouannet, P. (1994). Mouvement des spermatozoïdes et fécondation [Sperm motility and fertilization]. *Contraception, Fertilite, Sexualite*, **22**(5), 314–318.

Austin, C. R. (1977). Mammalian fertilization mechanisms. *Nature*, **270**(5632), 84–84. doi: 10.1038/270084a0

Bandivdekar, A. H., Segal, S. J. and Koide, S. S. (1992). Binding of 5-hydroxytryptamine analogs by isolated *Spisula* sperm membrane. *Invertebrate Reproduction and Development*, **21**(1), 43–46. doi: 10.1080/07924259.1992.9672218

Banihani, S., Sharma, R., Bayachou, M., Sabanegh, E. and Agarwal, A. (2012). Human sperm DNA oxidation, motility and viability in the presence of L-carnitine during *in vitro* incubation and centrifugation. *Andrologia*, **44** (Suppl. 1), 505–512. doi: 10.1111/j.1439-0272.2011.01216.x

Blomberg Jensen, M., Bjerrum, P. J., Jessen, T. E., Nielsen, J. E., Joensen, U. N., Olesen, I. A., Petersen, J. H., Juul, A., Dissing, S. and Jørgensen, N. (2011). Vitamin D is positively associated with sperm motility and increases intracellular calcium in human spermatozoa. *Human Reproduction*, **26**(6), 1307–1317. doi: 10.1093/humrep/der059

Bray, T. M. and Bettger, W. J. (1990). The physiological role of zinc as an antioxidant. *Free Radical Biology and Medicine*, **8**(3), 281–291. doi: 10.1016/0891-5849(90)90076-U

Brugh, V. M. and Lipshultz, L. I. (2004). Male factor infertility: Evaluation and management. *The Medical Clinics of North America*, **88**(2), 367–385. doi: 10.1016/S0025-7125(03)00150-0

Bui, A. D., Sharma, R., Henkel, R. and Agarwal, A. (2018). Reactive oxygen species impact on sperm DNA and its role in male infertility. *Andrologia*, **50**(8), e13012. doi: 10.1111/and.13012

Fujinoki, M. (2009). Non-genomic regulation of mammalian sperm hyperactivation. *Reproductive Medicine and Biology*, **8**(2), 47–52. doi: 10.1007/s12522-009-0012-2

Fujinoki, M., Ohtake, H. and Okuno, M. (2001). Serine phosphorylation of flagellar proteins associated with the motility activation of hamster spermatozoa. *Biomedical Research*, **22**(1), 45–58. doi: 10.2220/biomedres.22.45

Ghafarizadeh, A. A., Vaezi, G., Shariatzadeh, M. A. and Malekirad, A. A. (2018). Effect of *in vitro* selenium supplementation on sperm quality in asthenoteratozoospermic men. *Andrologia*, **50**(2). doi: 10.1111/and.12869

Greco, E., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S. and Tesarik, J. (2005). Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *Journal of Andrology*, **26**(3), 349–353. doi: 10.2164/jandrol.04146

Guthrie, H. D. and Welch, G. R. (2006). Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *Journal of Animal Science*, **84**(8), 2089–2100. doi: 10.2527/jas.2005-766

Hull, E. M. and Dominguez, J. M. (2007). Sexual behavior in male rodents. *Hormones and Behavior*, **52**(1), 45–55. doi: 10.1016/j.yhbeh.2007.03.030

Hull, E. M., Muschamp, J. W. and Sato, S. (2004). Dopamine and serotonin: Influences on male sexual behavior. *Physiology and Behavior*, **83**(2), 291–307. doi: 10.1016/j.physbeh.2004.08.018

Jimeńez-Trejo, F., Tapia-Rodríguez, M., Cerbón, M., Kuhn, D. M., Manjarrez-Gutiérrez, G., Mendoza-Rodríguez, C. A. and Picazo, O.

(2012). Evidence of 5-HT components in human sperm: Implications for protein tyrosine phosphorylation and the physiology of motility. *Reproduction*, **144**(6), 677–685. doi: 10.1530/REP-12-0145

Jiménez-Trejo, F., Coronado-Mares, I., Boeta, M., González-Santoyo, I., Vigueras-Villaseñor, R., Arriaga-Canon, C., Herrera, L. A. and Tapia-Rodríguez, M. (2018). Identification of serotoninergic system components in stallion sperm. *Histology and Histopathology*, **33**(9), 951–958. doi: 10.14670/HH-11-989

Jueraitetibaike, K., Ding, Z., Wang, D. D., Peng, L. P., Jing, J., Chen, L., Ge, X., Qiu, X. H. and Yao, B. (2019). The effect of vitamin D on sperm motility and the underlying mechanism. *Asian Journal of Andrology*, **21**(4), 400–407. doi: 10.4103/aja.aja_105_18

Keskes-Ammar, L., Feki-Chakroun, N., Rebai, T., Sahnoun, Z., Ghozzi, H., Hammami, S., Zghal, K., Fki, H., Damak, J. and Bahloul, A. (2003). Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Archives of Andrology*, **49**(2), 83–94. doi: 10.1080/01485010390129269

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R. and Swan, S. H. (2017). Temporal trends in sperm count: A systematic review and meta-regression analysis. *Human Reproduction Update*, **23**(6), 646–659. doi: 10.1093/humupd/dmx022

Mann, T., Seamark, R. F. and Sharman, D. F. (1961). On the question of the occurrence and metabolism of 5-hydroxytryptamine and related indole compounds in mammalian semen. *British Journal of Pharmacology and Chemotherapy*, **17**(2), 208–217. doi: 10.1111/j.1476-5381.1961.tb01280.x

Meizel, S. and Turner, K. O. (1982). Stimulation of the hamster sperm acrosome reaction by biogenic amines. *Biology of Reproduction*, **26** (Suppl. 1), 83A.

Meizel, S. and Turner, K. O. (1983). Serotonin or its agonist 5-methoxytryptamine can stimulate hamster sperm acrosome reactions in a more direct manner than catecholamines. *The Journal of Experimental Zoology*, **226**(1), 171–174. doi: 10.1002/jez.1402260120

Morbat, M. M., Hadi, A. M. and Hadri, D. H. (2018). Effect of selenium in treatment of male infertility. *Experimental Techniques in Urology and Nephrology*, **1**(5). doi: 10.31031/ETUN.2018.01.000521

Moslemi, M. K. and Tavanbakhsh, S. (2011). Selenium-vitamin E supplementation in infertile men: Effects on semen parameters and pregnancy rate. *International Journal of General Medicine*, **4**, 99–104. doi: 10.2147/IJGM.S16275

Narasimhaiah, M., Arunachalam, A., Sellappan, S., Mayasula, V. K., Guvvala, P. R., Ghosh, S. K., Chandra, V., Ghosh, J. and Kumar, H. (2018). Organic zinc and copper supplementation on antioxidant protective mechanism and their correlation with sperm functional characteristics in goats. *Reproduction in Domestic Animals*, **53**(3), 644–654. doi: 10.1111/rda.13154

O'Flaherty, C. and Matsushita-Fournier, D. (2017). Reactive oxygen species and protein modifications in spermatozoa. *Biology of Reproduction*, **97**(4), 577–585. doi: 10.1093/biolre/iox104

Parisi, E., de Prisco, P., Capasso, A. and del Prete, M. (1984). Serotonin and sperm motility. *Cell Biology International Reports*, **8**(2), 95. doi: 10.1016/0309-1651(84)90075-4

Ramlau-Hansen, C. H., Moeller, U. K., Bonde, J. P., Olsen, J. and Thulstrup, A. M. (2011) Are serum levels of vitamin D associated with semen quality? Results from a cross-sectional study in young healthy men. *Fertility and Sterility*, **95**(3), 1000–1004. doi: 10.1016/j.fertnstert.2010.11.002

Sikka, S. C. (2001). Relative impact of oxidative stress on male reproductive function. *Current Medicinal Chemistry*, **8**(7), 851–862. doi: 10.2174/0929867013373039

Stephens, R. E. and Prior, G. (1992). Dynein from serotonin-activated cilia and flagella: Extraction characteristics and distinct sites for cAMP-dependent protein phosphorylation. *Journal of Cell Science*, **103**(4), 999–1012. doi: 10.1242/jcs.103.4.999

Suarez, S. S. and Ho, H. C. (2003). Hyperactivated motility in sperm. *Reproduction in Domestic Animals*, **38**(2), 119–124. doi: 10.1046/j.1439-0531.2003.00397.x

Suleiman, S. A., Ali, M. E., Zaki, Z. M. S., El-Malik, E. M. A. and Nasr, M. A. (1996). Lipid peroxidation and human sperm motility: Protective role of vitamin E. *Journal of Andrology*, **17**(5), 530–537. doi: 10.1002/j.1939-4640.1996.tb01830.x

Verma, A. and Kanwar, K. C. (1999). Effect of vitamin E on human sperm motility and lipid peroxidation *in vitro*. *Asian Journal of Andrology*, **1**(3), 151–154.

World Health Organization. (2010). *World Health Organization laboratory manual for the examination and processing of human semen*. World Health Organization, Swtizerland.

Yamaguchi, S., Miura, C., Kikuchi, K., Celino, F. T., Agusa, T., Tanabe, S. and Miura, T. (2009). Zinc is an essential trace element for spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, **106**(26), 10859–10864. doi: 10.1073/pnas.0900602106

Yilmazer, Y. B., Koganezawa, M., Sato, K., Xu, J. and Yamamoto, D. (2016). Serotonergic neuronal death and concomitant serotonin deficiency curb copulation ability of *Drosophila* platonic mutants. *Nature Communications*, **7**, 13792. doi: 10.1038/ncomms13792

Yudin, A. I., Gottlieb, W. and Meizel, S. (1988). Ultrastructural studies of the early events of the human sperm acrosome reaction as initiated by human follicular fluid. *Gamete Research*, **20**(1), 11–24. doi: 10.1002/mrd.1120200103

Yun, J. I., Gong, S. P., Song, Y. H. and Lee, S. T. (2013). Effects of combined antioxidant supplementation on human sperm motility and morphology during sperm manipulation *in vitro*. *Fertility and Sterility*, **100**(2), 373–378. doi: 10.1016/j.fertnstert.2013.04.015