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Effect of resveratrol on sperm motility in subjects affected by idiopathic asthenozoospermia: An *in vitro* study

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ABSTRACT

Asthenozoospermia (AZS) is responsible for about 80 % of male infertility cases. Oxidative stress (OS) seems to be involved in cases of AZS otherwise termed "idiopathic," so antioxidant molecules have gained increasing interest in the treatment of infertility. In the present study, the *in vitro* effects of two different concentrations (12 and 30 μM) of resveratrol (RSV), a potent natural antioxidant, on sperm motility and OS counteragent of 154 subjects with AZS were evaluated. After 1 hour at 37 °C, the control group and the group treated with 12 μM of RSV showed a slight increase in progressive motility (PM) and a simultaneous decrease in non-progressive motility (NP). Conversely, the group treated with 30 μM of RSV showed a significant decrease in all motility parameters. Moreover, a significant decrease in Dichlorofluorescein (DCF) fluorescence intensity from controls (248.14 ± 111.16 a.u.) was observed both in group treated with 12 μM (152.47 ± 110.59 a.u., $p < 0.0001$) and 30 μM of RSV (128.06 ± 94.21 a.u., $p < 0.0001$). These findings support the hypothesis that excessive ROS reduction may lead to redox unbalance that could paradoxically worsen the seminal parameters of subjects with AZS when treated with excessive doses of antioxidants.

1. Introduction

Infertility is a condition of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse (Vander Borght and Wyns, 2018). From the male perspective, infertility is most commonly caused by abnormal semen analysis (including total absence of spermatozoa or low sperm count and anomalies in morphology and/or motility of the sperms) or ejaculatory dysfunction (WHO, 2019/2021).

A reduction in progressive motility (PR) of sperms below 32 % and/or a reduction in total motility (TM = PR + non-progressive motility - NP) below 40 % is defined as asthenozoospermia (AZS) (World Health Organization, 2010). AZS, with varying degrees of severity, is responsible for up to 80 % of infertility cases in men (Tu et al., 2020). Furthermore, AZS may be related to genitourinary tract infections (Delli Muti et al., 2022) anti-spermatozoa antibodies (ASA), varicocele or other anatomical changes that may involve the genitourinary tract, endocrinological and/or systemic diseases, and environmental factors (Balercia et al., 2002; Delli Muti et al., 2023). When the cause of impaired sperm motility cannot be clearly identified, the definition “idiopathic AZS” is frequently used, and oxidative stress (OS) could be involved in such cases (Tirabassi et al., 2017). Indeed, spermatozoa require oxygen to survive and perform their functions, and physiological levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are required to maintain normal cellular functions (Tirabassi et al., 2017; Park and Pang, 2021). In this purpose, studies have shown that low levels of ROS are important for the capacitation and hyperactivation of the acrosomal reaction, and ultimately the ability of the spermatozoon to fertilize the egg cell (Alahmar, 2018; Dutta et al., 2019). Definitely, spermatogenesis is a process closely dependent on energy metabolism, since any alteration in the regulation of energy production in these cells impairs the normal development of spermatogenesis and, consequently, male fertility (Han et al., 2017).

On the other hand, levels of ROS and RNS exceeding the antioxidant systems can lead to OS, witch in turn is linked to cellular damage and the onset of several diseases, including cancer (Di Meo et al., 2016; Tossetta

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and Marzioni, 2023). Indeed, spermatozoa are particularly susceptible to OS due to their high membrane content of polyunsaturated fatty acids and intracellular deficiency of antioxidant enzymes (Sanocka and Kurpisz, 2004; Hamilton et al., 2022). Thus, since ROS at the sperm level have both physiological and pathological roles, an antioxidant system is needed to maintain constant ROS levels in seminal plasma. In fact, antioxidants act as free radical scavengers in order to protect sperm against ROS and several enzymatic (superoxide dismutase – SOD -, catalase – CAT -, and glutathione peroxidase – GPX) and non-enzymatic molecules (vitamin C, vitamin E, taurine, carnitine, lycopene…) are found in semen (Zini et al., 2009). Natural compounds are biological substances that can be found in plants, bacteria, fungi, and marine organisms, and are widely used as supplements to counteract inflammation, OS and cancer (Alba et al., 2023; Perugini et al., 2019; Drasar and Moravcova, 2004; Karre et al., 2013). Among these, resveratrol (RSV, trans 3,5,4′ trihydroxystilbene) is a naturally occurring non-flavonoid polyphenol that belongs to the stilbene family. It has been shown to exert innumerable biological activities, both *in vitro* and *in vivo*, so this molecule is receiving considerable attention from the scientific community (Tossetta et al., 2022). RSV acts on ROS either by chelating the metals, which catalyze the reaction of its formation, or by transforming the radical into a stable species via a termination reaction (Kovacic and Somanathan, 2010). Several studies confirmed that RSV may counteract excessive ROS production, mitochondrial redox state imbalance, and lipid peroxidation (Liu et al., 2013; Liguori et al., 2018). Studies conducted in animal models showed that RSV is capable of leading to the reduction of ROS levels, resulting in the increase of antioxidant capacity, the increase of membrane potential and the inhibition of cytochrome C released from the inner mitochondrial membrane (Moridi et al., 2015; Zhang et al., 2019). In addition, due to its synergistic activity exerted at both the cellular and tissue levels, RSV can be considered an endothelium-protective factor, counteracting the main pathophysiological processes leading to vascular damage and related degenerative diseases (Wallerath et al., 2002). At the level of the male reproductive system, RSV appears to have phytoestrogenic activity on the androgen receptor (AR) through inhibition of its dimerization (Streicher et al., 2014) and IL-6-induced transcriptional activity in prostate cancer cells (Lee et al., 2014). At the testicular level, RSV activity appears to involve some transcription factors that influence AR function (Smith and Walker, 2014). In addition, several studies have shown that RSV could increase sperm quality in humans due to its ability to cross the blood-testicular barrier (Garcez et al., 2010; Collodel et al., 2011). Therefore, RSV could be an important compound to reduce the levels of ROS that negatively influence sperm function. Thus, the present study aims to evaluate *in vitro* the impact of RSV, at different concentration, on sperm motility in subjects with idiopathic infertility, an area where research remains limited. While the antioxidant properties of RSV are well documented, its potential to influence sperm motility through mechanisms involving oxidative stress modulation has not been fully explored. Furthermore, this study addresses an important clinical question by examining whether excessive antioxidant supplementation could lead to reductive stress, a condition characterized by an imbalance in redox homeostasis that might impair cellular function. By highlighting both the benefits and potential risks of RSV, this study could provide new insights into its application in reproductive medicine and contribute to the understanding of oxidative and reductive stress in male infertility.

2. Materials and methods

2.1. Patients

In the present study, 154 asthenozoospermic patients (aged 18–60 years, mean age 34 years) referring to the Andrology Unit of the Clinic of Endocrinology - Azienda Ospedaliero-Universitaria delle Marche in the period from January 2021 to January 2022 for infertility problems lasting 18 months at least were enrolled. All study participants had normal sperm morphology (> 4 %) and no evidence of leukocytospermia (white cells count $<1 \times 10^6$/mL in the ejaculate), sperm concentration $> 15 \times 10^6$/mL, progressive motility < 35 % and sperm viability > 58 %, according to criteria established in the WHO guidelines (World Health Organization, 2010). At the time of enrollment, semen cultures were negative for microbial infections, including Chlamydia and Mycoplasma urealyticum infections, as was the search for anti-spermatozoa adherent antibodies (MAR-test). The hormone profile (gonadotropins, testosterone, estradiol and prolactin) was also in the normal range. Patients had no evidence of anatomic abnormalities of the genital tract, including varicocele (after scrotal echodoppler), or clinical history of cryptorchidism, testicular torsion, or genital tract infections. Absence of systemic diseases (hypertension, diabetes mellitus, hypercholesterolemia) or drug treatment in the 3 months prior to enrollment in the present study, absence of smoking habits, alcohol intake, and lack of occasional exposure to chemical agents were additional elements for inclusion in the present study. In addition, participants were not taking any supplement containing antioxidants, and were following a standard Mediterranean Diet. Since no specific causes for reduced sperm motility were found, patients were classified as having idiopathic AZS.

This study was exempted from ethical review and approval as written informed consent was obtained from all participants, with documentation in line with the general authorization from the Italian Data Protection Authority for processing personal data for scientific research purposes (http://www.garanteprivacy.it (accessed on 11th January 2021)). Additionally, all information related to human materials was managed using anonymous numerical codes, and all samples were processed in accordance with the Declaration of Helsinki as revised in 2013 (http://www.wma.net (accessed on 11th January 2021)).

2.2. Experimental design and semen analysis

Semen analysis was performed according to the fifth edition of the WHO manual (World Health Organization, 2010): semen samples were obtained by masturbation following 2–7 days of abstinence from sexual intercourse. Samples were collected in sterile containers and allowed to liquefy at 37 °C for 30 minutes. Ejaculated volumes were determined by specimen weight, and a semen density of 1.0 g/mL was assumed, as recommended by the WHO 2021 manual for semen analysis. Each semen sample was divided into three aliquots: one remained untreated, while the other two were incubated with RSV (Sigma, St. Louis, MO, USA) dissolved in DMSO, to a final concentration of either 12 μM and 30 μM, respectively, for 1 hour at 37 °C, to ensure physiological conditions. Sperm concentration, motility (PR, NP, and TM) and viability were assessed using a Makler counting chamber, at baseline and following incubation with RSV. Sperm concentration was determined by counting sperm cells in a defined area of the chamber and calculating the total concentration per milliliter. Motility was evaluated by observing sperm movement under a microscope and classifying sperm as progressive (PR), non-progressive (NP), or total motility (TM). Viability was assessed using the eosin-nigrosin staining method, where live sperm cells remain unstained, while dead cells appear red, with a minimum of 200 sperm cells analyzed. The procedures followed the guidelines outlined in the WHO 2021 manual for semen analysis. These procedures were applied consistently to all three aliquots by two operators in order to reduce analysis time.

The concentration of RSV and incubation time were chosen according to literature (Collodel et al., 2011; Cui et al., 2016). At the end of incubation, spermatozoa were evaluated for total and progressive motility (according to WHO 2010) and Viability Assessment, TBARS levels, ROS production and the total antioxidant capacity by means of ORAC assay.

2.3. Viability assessment

After treatment, sperm viability was assessed using the eosin-nigrosin staining method, following WHO guidelines, to ensure that any changes in motility or ROS levels were not affected by a loss of cell viability. In brief, 20 μL of the sperm sample was combined with 20 μL of the staining solution, and the mixture was placed on a glass slide. The sample was then examined under a light microscope at 40 × magnification. Viable sperm cells remained unstained, while dead sperm cells appeared red. A minimum of 200 sperm cells were analyzed, and the percentage of stained sperm cells was calculated.

2.4. Measurement of lipoperoxides (thiobarbituric acid reactive substances, TBARS)

The thiobarbituric acid reactive substances (TBARS) determination kit is provided by Cayman (Cayman Chemical Company, Ann Arbor, MI) and allows determination of lipid peroxidation in the sample. MDA-TBA adducts, formed by the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA) at high temperatures (100 °C) and under acidic conditions, are measured on 96-well microplates using a microplate reader (Synergy HT, Bio-tek, Winooski, VT, USA) at a wavelength of 530 nm. TBARS levels are expressed in mM using MDA as the standard.

2.5. Dichlorodihydrofluorescein diacetate assay

ROS production was evaluated using the fluorescent probe 2,7-dichlorodihydrofluorescin diacetate (H_2DCFDA) as previously described (Tannous et al., 1999). H_2DCFDA is a nonfluorescent compound that diffuses rapidly across the plasma membrane; within cells it is hydrolyzed to 2′-7′-dichlorofluorescein (DCF) through cytosolic esterases and is oxidized to intracellular fluorescent DCF if ROS are present (Kooy et al., 1997). The free base of H_2DCFDA was prepared daily, as reported previously (Tannous et al., 1999), at a stock concentration of 10 mM. The reagent, once prepared, is kept in the dark until used. Samples were incubated for 45 min at RT with 50 μl of 25 μM H_2DCFDA. Fluorescence was measured in a microplate reader (Synergy HT, Bio-tek, Winooski, VT, USA) at an excitation wavelength of 475 nm and an emission wavelength of 520 nm. The detected fluorescence intensity can be related to the levels of ROS found in the sample. ROS levels are expressed as arbitrary units (a.u.).

2.6. Oxygen radical absorbance capacity (ORAC)

The ORAC assay is a fluorescence method that measures the ability of an antioxidant molecule to prevent the loss of the fluorescence signal of the fluorescein probe by scavenging peroxyl radicals generated by the thermal decomposition of 2,2′-azobis (2-methylpropylamide) dihydrochloride (AAPH). The reaction was carried out in a sodium phosphate buffet at pH7.4. The calibration curve was prepared by measuring standard solutions of different Trolox concentrations, from 25 to 500 μM, and a PBS (phosphate-buffered saline) solution was used as a blank. The ORAC assay was performed on standard solutions and samples, all analyzed in replicates. Fluorescence emission was recorded every 5 minutes for 3 h at ex485/em530 nm. The final ORAC values were calculated using the net area under the decay curves (AUC) and the ORAC capacity of the samples were expressed as μM Trolox equivalents.

2.7. Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 26.0 (SPSS Inc., Chicago, IL) and Graphpad Prism version 8.4.3 (GraphPad Software, Inc., San Diego, CA) for Microsoft Windows. Normal distribution for continuous variables was assessed using the Kolmogorov-Smirnov test for normality. All the included variables were non-normally distributed. Data are shown as mean ± standard deviation (SD) and/or median and interquartile range (IQR). Changes from baseline were assessed by Wilcoxon's test, whereas comparison between groups was performed by Kruskall-Wallis test with post hoc Mann-Whitney U tests. Categorical variables were compared using Chi-square test. Bivariate correlations were investigated by Spearman's test. In this purpose, 0–0.19 is regarded as very weak, 0.2–0.39 as weak, 0.40–0.59 as moderate, 0.6–0.79 as strong and 0.8–1 as very strong correlation. Values of $p < 0.05$ were considered statistically significant.

3. Results

A total of 154 samples were included in the present study. Baseline characteristics of semen samples, according with the 5th edition of the WHO manual are reported in Table 1.

3.1. Comparison of motility parameter before and after treatment with resveratrol

After 1 hour at 37 °C and administration of RSV at the concentration of 12 and 30 μM, significant changes from baseline in both PM and NP were observed in all the groups of samples. Notably, the control group and the group treated with 12 μM of RSV showed a slight increase in PM and a simultaneous decrease in NP, whereas TM was unchanged. Conversely, the group treated with 30 μM of RSV showed a significant decrease in all motility parameters. Results of pre-post comparison are shown in Table 2 and Fig. 1.

In addition, of the 154 samples included in the study, 48 (31.2 %) fell under the diagnosis of AZS at baseline. After 1 hour, the percentage was not different for the control group and the group treated with 12 μM of RSV (respectively 29.2 % −45-, and 30.5 % - 47). The group treated with higher doses of RSV showed a slight increase in samples with AZS (61, 39.6 %), but difference was not statistically significant ($p = 0.196$) (Fig. 2).

3.2. Comparison of oxidative stress markers after treatment with resveratrol

Differences in DCF fluorescence intensity and TBARS measure were evaluated between the 3 groups after 1 hour incubation at 37 °C. Interestingly, a significant decrease in DCF fluorescence intensity from control group (248.14 ± 111.16 a.u.) was observed both in group treated with 12 (152.47 ± 110.59 a.u., $p < 0.0001$) and 30 μM of RSV (128.06 ± 94.21 a.u., $p < 0.0001$) (Fig. 3), but difference between the two different concentrations of RSV was not statistically significant. Conversely, no significant changes in TBARS levels were observed (Table 3).

Table 1
Basal semen parameters.

	Mean ± SD	Median (IQR)	Normal values
pH	7.8 ± 0.2	-	≥ 7.2
Sperm concentration (x 10^6/mmc)	69.0 ± 52.2	62.50 (28.0–90.3)	> 15
PR (%)	36.9 ± 13.0	40.0 (30.0–45.0)	> 32
NP (%)	7.0 ± 2.9	5.0 (5.0–10.0)	-
TM (%)	43.9 ± 12.5	45.0 (35.0–51.3)	> 40
Vitality (%)	65.2 ± 18.7	71.0 (48.3–80.8)	> 58

NP= non-progressive motility; PR= progressive motility; TM= total motility.

Table 2
Changes from baseline (motility parameters).

	Control group	p-value	RSV 12 μM	p-value	RSV 30 μM	p-value
PR (%)	Δ = +1.10 ± 6.88	p = 0.005	Δ = +1.20 ± 7.24	p = 0.013	Δ = −1.99 ± 8.89	p = 0.019
NP (%)	Δ = −1.01 ± 3.16	p < 0.0001	Δ = −1.40 ± 2.94	p < 0.0001	Δ = −1.44 ± 2.93	p < 0.0001
TM (%)	Δ = +0.09 ± 6.50	p = 0.292	Δ = −0.23 ± 7.24	P = 0.775	Δ = −3.45 ± 8.78	p < 0.0001

NP= non-progressive motility; PR= progressive motility; TM= total motility; Δ = mean change from baseline

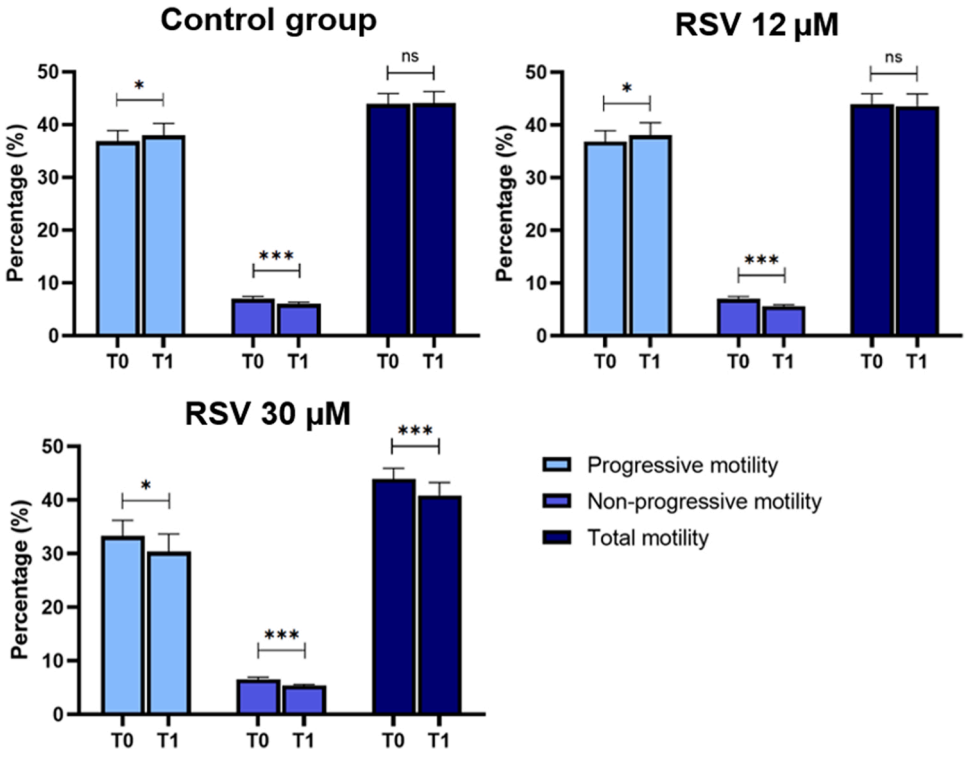

Fig. 1. Motility parameters before and after resveratrol treatment. RSV=resveratrol; ns=not significant. *p < 0.05; ***p < 0.0001.

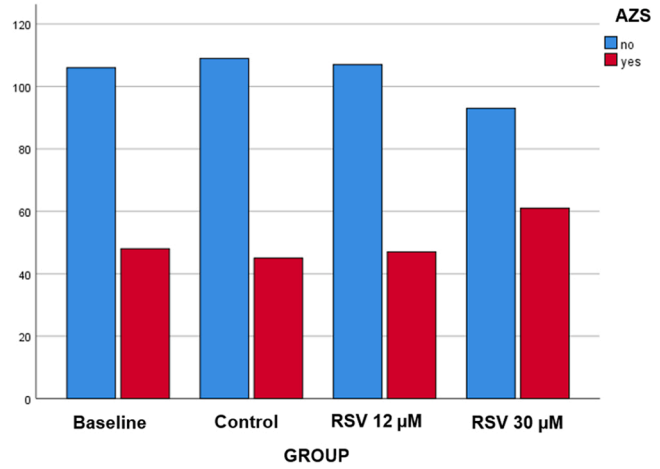

Fig. 2. Bar graph of asthenozoospermia (AZS).

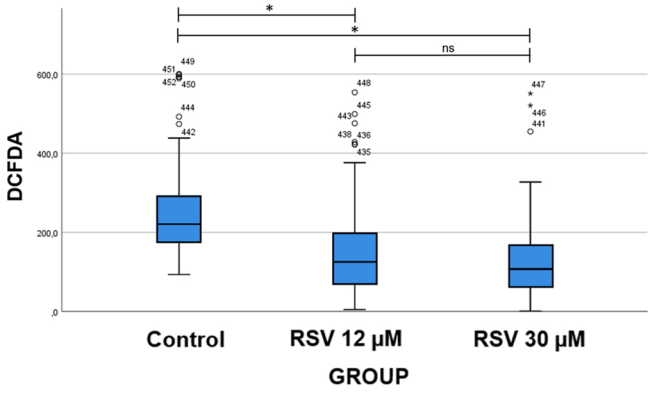

Fig. 3. DCF fluorescence intensity between groups. * p < 0.0001.

3.3. Bivariate analysis between oxidative stress markers and motility parameters

Results of bivariate analysis are shown in Tables 4 and 5. Interestingly, a significant positive correlation between TBARS and both PR and TM emerged. In addition, the correlation was even stronger, but it was confirmed only in the control group, when the 3 groups were analyzed separately.

According with these findings, samples with AZS compared with samples without AZS showed significantly lower values of TBARS (18.45 ± 9.81 mM vs. 20.29 ± 9.00 mM, p = 0.01), whereas DCF fluorescence intensity (179.13 ± 130.03 a.u. vs. 171.30 ± 110.87 a.u., p = 0.935) and ORAC levels (28790.78 ± 12214.33 μM Trolox equivalent vs. 30754.32 ± 12206.67 μM Trolox equivalent, p = 0.164) were substantially similar.

An additional analysis revealed no significant correlation between

Table 3
Differences in oxidative stress markers between groups.

	Control group	RSV 12 μM	RSV 30 μM	p-value
DCF fluorescence intensity (a. u.)	248.14 ± 111.16*,°	152.47 ± 110.59*	128.06 ± 94.21°	*,°p < 0.0001
TBARS (mM)	20.55 ± 9.46	19.35 ± 9.17	18.84 ± 9.03	p = 0.158
ORAC μM Trolox equivalent	29531.42 ± 12170.09	30480.02 ± 11555.84	30287.96 ± 12652.00	p = 0.698

DFCDA= Dichlorofluorescein; ORAC= Oxygen radical absorbance capacity; TBARS= Thiobarbituric acid reactive substances.

Table 4
Group comparison after 1 hour.

	DCF fluorescence intensity (a.u.)	TBARS (mM)	ORAC (μM Trolox equivalent)
PR (%)	r = −0.002	r = 0.200*	r = 0.074
NP (%)	r = −0.001	r = −0.017	r = −0.020
TM (%)	r = −0.000	r = 0.203*	r = 0.084

DCF= Dichlorofluorescein; NP= non-progressive motility; ORAC = Oxygen radical absorbance capacity; PR= progressive motility; TM= total motility; TBARS= Thiobarbituric acid reactive substances. *p < 0.0001.

participant age and oxidative stress markers at any time point and for any concentration of RSV (p > 0.05; data not shown).

4. Discussion

RSV is a polyphenol that derives from food sources such as wine and berries with several biological properties, including anti-inflammatory, antiglycation and, above all, antioxidant activity (Galiniak et al., 2019). Interestingly, anti-cancer effects have also been reported *in vitro* for a wide range of RSV concentrations (1–500 μM) (Yousef et al., 2017). However, all that glitters is not gold, and *in vitro* citotoxicity for RSV concentrations exceeding 100 μM has also been reported in human-derived renal epithelial cells (Kristl et al., 2009).

As stated before, the balance between pro-oxidants and antioxidants (or "cellular redox") in semen is crucial for sperm cells, which are particularly susceptible to OS. Cellular pro-oxidants include ROS, mainly represented by superoxide anione (O_2^-), hydrogen peroxide (H_2O_2) and are neutralized by enzymatic (SOD, and CAT) and non-enzymatic antioxidants (e.g., reduced glutathione – GSH, acorbic acid, and tocopherol). In particular, GPX neutralizes H_2O_2 by riducing it in H_2O through the conversion of GSSG to GSH by using NADPH as a cofactor (Sadeghi et al., 2023). Due to its potent antioxidant action, its potential positive effect on spermatogenesis has been a matter of great interest. In animal models, the administration of RSV demonstrated protective effects on sperms against gonadotoxic agents, including vancomycin (Alshehri, 2023), bisphenol A (Bordbar et al., 2023), busulfan (Hafezi et al., 2022), nicotine (Francisco et al., 2022), and metals (Mitra et al., 2022). In human samples, RSV showed optimal protective effect against lipid peroxidation (LPO) at the concentrations of 15 (Collodel et al., 2011) and 25 μM (Shabani Nashtaei et al., 2017).

In the present study, we investigated whether the administration of two different concentrations of RSV could have an ameliorative effect on sperm motility for patients suffering from male infertility. One hundred and fifty-four semen samples were evaluated, 48 of which (31.2 %) fell within the diagnosis of AZS according with the 2010 WHO manual (PR < 32 % and/or TM < 40 %). Previous *in vivo* evidence showed that the administration of oral supplements containing 150 mg of RSV improved sperm motility (48.3 % ± 13.8 vs. 59.0 % ± 12.8, p = 0.0001) in men affected by idiopathic male infertility (Illiano et al., 2020). Similarly, RSV was able, *in vitro*, to counteract the detrimental effects of benzo-α-pyrene on sperm motility both at the concentration of 15 and 45 μM in a sample of 30 normozoospermic, healthy, non-smoker men (Alamo et al., 2019).

In our study, surprisingly, the addition of RSV had no positive effects and indeed, at the highest concentrations it was even detrimental to motility in our semen samples. Actually, we found that the administration of 12 μM of RSV has not protective effects on sperm motility, leading to changes in PR and NP similar to those observed in the control sample. Conversely, the administration of 30 μM of RSV resulted in significantly lower TM compared with the control sample. In addition, PM values were significantly lower after incubation with RSV 30 μM for one hour. In accordance with this, the percentage of samples with AZS was completely overlapping between those at baseline (48, 31.2 %) and after 1 hour in the control group (45, 29.2 %) an in the group treated with 12 μM of RSV (47, 30.5 %), whereas the number of samples with AZS was higher (61, 39.6 %), albeit difference was not statistically significant (p = 0.196). In line with some of our findings, Garcez et al. investigated the effects of high concentrations of RSV (0.1, 1 and 10 mM) in cryopreservation medium on semen samples from fertile and infertile men. Interestingly, they also reported that the addition of RSV was not able to prevent the post-thawing reduction in sperm motility, despite the addition of RSV successfully prevented lipid damage induced by cryopreservation assessed by TBARS measure (Garcez et al., 2010). Similarly, the intraperitoneal administration of RSV led to a dose-dependent decrease in sperm motility in mice, but this time with a concurrent rise in levels of GSSG and a decrease in CAT and SOD, suggesting a shift in the redox status and consequent OS (Ranawat et al., 2014). Paradoxically, redox unbalance can arise as a result of both increased GSH and GSSG, that cause reductive or oxidative stress, respectively, which are both associated with mitochondrial dysfunction and subsequent ROS leakage (Sadeghi et al., 2023).

In our study, ROS levels significantly decreased in samples treated with RSV, as demonstrated by a lower DCF fluorescence intensity after one hour with 12 μM (152.47 ± 110.59 a.u.) and 30 μM RSV (128.06

Table 5
Bivariate correlations between oxidative stress markers and motility parameters divided by groups of treatment.

	Control group			RSV 12 μM			RSV 30 μM		
	DCF fluorescence intensity	TBARS	ORAC	DCF fluorescence intensity	TBARS	ORAC	DCF fluorescence intensity	TBARS	ORAC
PR (%)	r = −0.092	r = 0.282*	r = 0.087	r = 0.280*	r = 0.149	r = 0.098	r = 0.037	r = 0.125	r = 0.026
NP (%)	r = −0.007	r = −0.032	r = 0.075	r = −0.008	r = −0.009	r = 0.029	r = −0.065	r = −0.045	r = −0.050
TM (%)	r = −0.107	r = 0.291**	r = 0.116	r = 0.291**	r = 0.153	r = 0.103	r = 0.031	r = 0.123	r = 0.027

DCF= Dichlorofluorescein; NP= non-progressive motility; ORAC = Oxygen radical absorbance capacity; PR= progressive motility; TM= total motility; TBARS= -Thiobarbituric acid reactive substances.
* p < 0.05;
** p < 0.0001.

± 94.21 a.u.), compared with control samples (248.14 ± 111.16 a.u.). Conversely, we did not observe any significant difference in LPO indexes comparing controls and samples treated with RSV at the concentrations of 12 and 30 μM. As stated before, low levels of ROS are required in semen to ensure normal sperm cell functions such as sperm capacitation, sperm hyperactivation, and acrosome reaction (Salvio et al., 2021). For this reason, excessive ROS reduction, which can occur nowadays because of easy access to commercial antioxidant products, which are widely available without the need for a prescription, could lead to unfavorable consequences. This was hypothesized more than twenty years ago by Halliwell, who first proposed the "antioxidant paradox" theory, according to which suppression of localized oxidation could disrupt vital processes for the cell and promote, under specific conditions, apoptosis (Halliwell, 2000). This theory has been further explored in recent years, demonstrating that decreasing physiological ROS levels drive spermatozoa towards a condition named "reductive stress", which is surprisingly as harmful as OS because impairs the functioning of DNA repair systems that are involved in correcting the physiological DNA double strand breaks that develop during the physiological chromatin rearrangement that occurs during spermiogenesis, leading to DNA damage in mature spermatozoa (Sadeghi et al., 2023).

A weak positive correlation between PR, TM, and TBARS levels emerged. This could be due to increase OS due to hyperactivation and capacitation of motile spermatozoa that may increase ROS production (Kim and Parthasarathy, 1998). Indeed, a recent study by Castellini et al. reported that the administration of n-3-enriched diets to rabbits was able to significantly improve motility rate and track speed of spermatozoa, leading to a concurrent increase in TBARS levels (Castellini et al., 2019). This could be indirectly confirmed by the observation that TBARS levels decrease, albeit not significantly, in samples treated with increasing RSV concentrations. In addition, the correlation between PR, RM and TBARS levels was confirmed only in the control group, but not in samples treated with RSV, further suggesting that RSV may buffer the effect of ROS by neutralizing OS.

The results of the present study should be considered in light of several limitations, since the effects of RSV may extend beyond direct antioxidant activity. In fact, recent studies suggest that its impact may influence mitochondrial function, gene expression, and epigenetic regulation. These effects could also explain the observed improvements in sperm motility. For instance, RSV has been reported to modulate mitochondrial biogenesis and enhance mitochondrial function, which are crucial for cellular energy production, including in sperm cells (Zhang et al., 2024; Gherardi et al., 2022). Moreover, RSV has been found to interact with various signaling pathways such as SIRT1, which plays a key role in mitochondrial regulation and the cellular response to stress (Dutta et al., 2024). RSV may exert epigenetic effects by modifying histone acetylation patterns and DNA methylation, further influencing gene expression in a manner that could optimize sperm motility (Tian et al., 2023; Erdoğan et al., 2023).

In conclusion, in the present study we observed that RSV at the highest concentrations does not appear to preserve sperm motility. In addition, RSV doses of 30 μM may be accompanied by a reduction in motility by altering the redox balance of spermatozoa. Our results, therefore, support the existence of a redox stress that could paradoxically worsen the seminal picture of subjects with AZS when treated with excessive doses of antioxidants.

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CRediT authorship contribution statement

Nicola Delli Muti: Investigation, Formal analysis. **vignini Arianna:** Writing – review & editing, Conceptualization. **Alia Sonila:** Methodology, Formal analysis. **Ciarloni Alessandro:** Methodology, Data curation. **Balercia Giancarlo:** Writing – review & editing, Supervision. **Salvolini Eleonora:** Supervision. **Di Paolo Alice:** Investigation, Formal analysis. **Membrino Valentina:** Investigation, Formal analysis. **Salvio Gianmaria:** Writing – original draft, Supervision, Methodology.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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