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In vitro supplementation of lycopene to bovine spermatozoa: effects on motility, viability and superoxide production*

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This study was designed to assess the effects of lycopene (LYC) on motility, viability and superoxide production of bovine spermatozoa during short-term (0h, 2h, 6h) and long-term (12h, 24h) *in vitro* culture periods. Semen samples were collected from 20 adult breeding bulls and diluted in saline supplemented with 2000, 1000, 500, 200, 100, 50, 10, 5 and 1 µM/L LYC. Spermatozoal motion parameters were examined using the SpermVision™ Computer Assisted Semen Analyzer (CASA) system. Cell viability was measured using the metabolic activity MTT assay, while the nitroblue-tetrazolium (NBT) test was used to assess intracellular superoxide formation. The CASA analysis revealed that 100 - 2000 µM/L LYC were able to prevent a rapid decline of spermatozoal motion (P<0.001 with respect to 200-2000 µM/L LYC; P<0.01 given 100 µM/L LYC). At the same time, supplementation of 1000 and 2000 µM/L LYC led to a significant preservation of cell viability (P<0.05 in the case of Time 0h and 2h; P<0.01 at Time 6h and 12h; P<0.001 with respect to Time 24h). All applied LYC concentrations significantly reduced the intracellular superoxide production, particularly at Times 12h (P<0.01 in the case of 1000 and 2000 µM/L LYC; P<0.05 with respect to 1-500 µM/L LYC) and 24h (P<0.001 in the case of 1000 and 2000 µM/L LYC; P<0.01 with respect to 500 µM/L LYC; P<0.05 given 1-200 µM/L LYC). The results indicate that lycopene may enhance spermatozoal activity and protect against cellular deterioration resulting from exposure to the *in vitro* environment.

KEY WORDS: lycopene / spermatozoa / bulls / motility / viability / oxidative stress

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Increasing evidence proves that oxidative damage plays a crucial role in the pathogenesis of male infertility. Overgeneration of reactive oxygen species (ROS) and a decreased antioxidant capacity of semen frequently observed in animals and humans are two major factors leading to oxidative stress (OS) [Aitken *et al.* 1991, Tremellen 2008, Bansal and Bilaspuri 2010]. At the same time, the characteristic cellular structure of spermatozoa leaves them to be particularly vulnerable to oxidative damage. OS may often result in alterations to the sperm structure or function, which are related to a decreased reproductive performance [Agarwal *et al.* 2014a,b].

The benefits of antioxidant supplementation on spermatozoal physiology and fertility have become the spotlight for diverse researchers [Donnelly *et al.* 1999, Bansal and Bilaspuri 2010, Agarwal and Sekhon 2012]. Substantial *in vitro* research has been conducted to show that biologically active substances including resveratrol, quercetin or tocopherols may reduce seminal OS and offer protection to male germ cells by neutralizing ROS [Donnelly *et al.* 1999, Taepongsorat *et al.* 2008, Tvrdá *et al.* 2015]. However, insufficient information is available on the effectiveness of carotenoids, particularly lycopene (LYC) – a potent singlet oxygen quencher [Rao and Rao 2007]. It is hypothesised that LYC could reduce the incidence of OS extinguishing ROS, and thus decrease the chances of oxidative insults to spermatozoa. Improvement of sperm activity and antioxidant characteristics reported in previous preliminary studies [Uysal and Bucak 2007, Rosato *et al.* 2012, Bucak *et al.* 2014] indicate a possible involvement of LYC in the reduction of seminal OS, increasing the chances of a normal sperm cell fertilizing the egg [Durairajanayagam *et al.* 2014].

In order to provide more insight into a possible role of LYC in male reproduction, this study was designed to assess the effects of different LYC concentrations on bovine spermatozoal motility, viability and superoxide production during a 24 hour *in vitro* culture.

Material and methods

Bovine semen samples were collected from 20 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). Each sample had to meet the basic criteria imposed on the corresponding breed. The samples were obtained on a regular collection schedule using an artificial vagina. Immediately after collection the samples were transferred to the laboratory and processed at room temperature (22-25°C).

Each individual sample was diluted in saline (PS; sodium chloride 0.9% w/v; Bieffe Medital, Italy) with different LYC concentrations (Sigma-Aldrich, St. Louis, USA; solubilised in butylated hydroxytoluene/BHT; Sigma-Aldrich, St. Louis, USA; A – 2000; B – 1000; C – 500; D – 200; E – 100; F – 50; G – 10; H – 5; I – 1 μM/L) using a dilution ratio of 1:40. The samples were cultured at room temperature (22-25°C). Specific analyses were carried out at cultivation Times 0, 2 and 6h (models suitable for a short-term *in vitro* culture) as well as 12 and 24h (models suitable for a long-term *in vitro* culture). The starting point for the assessment Times was the moment the sample was exposed to the medium.

Motility and progressive motility analyses were carried out using the CASA (Computer Assisted Semen Analyzer) system equipped with the SpermVision™ program (MiniTub, Tiefenbach, Germany) and the Olympus BX 51 microscope (Olympus, Japan). Each sample was placed into a Makler Counting Chamber (depth 10 μm, Sefi-Medical Instruments, Israel) and the percentage of motile (motility >5 μm/s; MOT) and progressively motile spermatozoa (motility >20 μm/s; PROG) was evaluated. A total of 1000-1500 cells were assessed in each analysis.

Viability of the cells exposed to LYC *in vitro* was evaluated by the metabolic activity (MTT) assay [Mosmann 1983]. This colorimetric assay measures the conversion of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. Formazan can then be measured spectrophotometrically at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data are expressed as percentages of the control (i.e. optical density of formazan from cells not exposed to LYC). Results from the analyses were collected during five repeated experiments at each concentration.

The nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of superoxide radical [Esfandiari *et al.* 2003]. This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of nitroblue tetrazolium chloride (2,2′-bis(4-Nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride; Sigma-Aldrich, St. Louis, USA) and the superoxide radical. Formazan can be measured spectrophotometrically at a measuring wavelength of 620 nm against 570 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed as percentages of the control (i.e. optical density of formazan from cells not exposed to LYC). Results from the analyses were collected during five repeated experiments at each concentration.

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated as first. One-way ANOVA with Dunnett's post test was used for statistical evaluations. The level of significance was set at $^{A}(P<0.001)$, $^{B}(P<0.01)$ and $^{C}(P<0.05)$, respectively.

Results and discussion

Mammalian spermatozoa have been characterised as cells exceptionally sensitive to ROS attacks, due to their specific structural and biochemical properties as well as a high risk of exposure to a variety of environments [Ball *et al.* 2001, Aitken and Sawyer 2003]. Therefore, antioxidants have been proposed as supplements to media used for sperm processing and *in vitro* incubation [Bansal and Bilaspuri 2010]. In order to shed more light on the mechanisms of action of LYC on the male gamete, this study was designed to investigate the effects of LYC supplementation on bovine spermatozoa exposed to an *in vitro* environment.

Table 1. Spermatozoal motility (%) in the absence (Ctrl) or presence (A-I) of lycopene during different time periods (mean±SEM; n=20)

Time (h)	Group									
	Ctrl	A	B	C	D	E	F	G	H	I
0	90.08±1.27	93.69±0.73	92.43±0.87	93.28±0.82	92.39±1.25	91.36±0.56	91.94±1.01	92.09±0.81	91.77±0.99	90.37±0.82
2	82.57±0.72	89.74±1.69^{B}	89.49±1.49^{B}	87.38±1.66	87.35±1.03	87.68±1.98	86.31±1.31	85.04±1.74	84.04±1.10	84.02±1.74
6	71.13±1.38	85.80±1.44^{A}	84.08±1.43^{A}	82.17±1.66^{A}	81.05±1.31^{A}	80.36±1.09^{B}	78.74±1.89^{C}	78.32±1.66^{C}	78.55±3.26	77.24±1.47
12	62.98±1.90	76.80±1.71^{A}	75.35±2.95^{A}	75.48±1.49^{A}	72.61±1.90^{A}	71.93±2.34^{B}	71.63±1.90^{B}	72.47±1.54^{B}	70.77±1.89^{C}	67.78±2.86
24	49.14±1.72	64.59±2.08^{A}	63.97±3.08^{A}	61.35±3.46^{A}	60.95±3.12^{A}	60.58±2.86^{B}	58.69±2.92	57.92±2.42	56.91±2.53	55.90±2.63

^{A}(P<0.001); ^{B}(P<0.01); ^{C}(P<0.05). Ctrl – 0; A – 2000; B – 1000; C – 500; D – 200; E – 100; F – 50; G – 10; H – 5; I - 1 μM/L LYC.

Table 2. Spermatozoal progressive motility (%) in the absence (Ctrl) or presence (A-I) of lycopene during different time periods (Mean±SEM; n=20)

Time (h)	Group									
	Ctrl	A	B	C	D	E	F	G	H	I
0	82.13±1.16	85.13±1.24	83.21±1.34	84.23±0.99	84.39±1.35	82.88±1.20	83.28±1.63	83.55±1.31	82.14±1.35	82.70±1.54
2	77.74±1.64	85.90±1.25^{B}	85.13±1.99^{B}	82.63±1.38	81.70±1.41	80.16±2.10	79.77±1.26	80.64±1.53	78.93±1.72	78.21±1.40
6	69.39±2.15	77.49±1.65^{B}	77.63±1.39^{B}	76.35±1.69^{B}	75.49±1.79^{C}	75.26±1.55^{C}	75.01±1.09	73.47±1.66	72.03±1.65	72.14±1.67
12	55.20±1.53	69.74±1.52^{A}	68.29±1.27^{A}	68.11±2.05^{A}	67.56±2.28^{A}	65.16±2.04^{B}	65.59±2.37^{B}	65.59±1.67^{B}	63.95±1.45^{C}	63.23±2.85
24	40.66±3.11	58.58±1.50^{A}	58.64±2.06^{A}	56.60±1.42^{A}	56.36±1.46^{A}	52.53±2.95^{A}	52.88±1.50^{A}	50.21±1.32^{B}	50.53±1.09^{B}	50.43±2.08^{B}

^{A} (P<0.001); ^{B}(P<0.01); ^{C}(P<0.05). Ctrl – 0; A – 2000; B – 1000; C – 500; D – 200; E – 100; F – 50; G – 10; H – 5; I - 1 μM/L LYC.

The CASA assessment showed a gradual decrease of spermatozoal motion parameters in all groups over the course of a 24h *in vitro* culture (Tab. 1, Tab. 2). No significant changes were recorded with respect to the initial MOT or PROG between the control and experimental groups. After 2h of culture a significant difference was found in experimental groups A and B, supplemented with the highest doses of the carotenoid while examining both MOT (Tab. 1) and PROG (Tab. 2). After 6h a significantly higher MOT was recorded in the groups administered with 10-2000 μM/L LYC (Tab. 1). At the same time, a significantly higher PROG was detected in experimental groups A-E (Tab. 2). Examination at 12h showed that both motion parameters were significantly increased in the groups supplemented with 5-2000 μM/L LYC (Tab. 1, Tab. 2). At the end of the experiments (24h), a significantly higher spermatozoal MOT was observed in groups A-E in comparison with the control (Tab. 1). Furthermore, LYC supplementation led to a significantly improved PROG in all the experimental groups when compared to the control (Tab. 2).

The MTT assay revealed that an immediate LYC administration (Time 0h) led to a significant improvement of cell viability in experimental groups A and B (Fig. 1). At 2h it was revealed that 200-2000 μM/L LYC had a significant vitality-promoting effect on bovine spermatozoa (Fig. 1) when compared to the control. The concentration array with significantly favourable effects on the mitochondrial activity further extended at 6h (Fig. 1). After 12h of the *in vitro* experiments cell viability remained significantly improved in groups A-G (Fig. 1) and this advantageous effect remained statistically significant towards the end of the *in vitro* culture (Time 24h; Fig. 1).

Interpretion of our results indicates that LYC supplemented to saline may serve as an effective motility promoting and mitochondrial protecting substance. The axonemal structures localised in the sperm midpiece are rich in mitochondria generating energy vital for spermatozoal motion [Garner and Hafez 1993]. *In vitro* cultures gradually decrease the sperm metabolism and induce irreversible changes leading to axonemal damage, subsequently followed by a decline of motility parameters and mitochondrial membrane potential related to ATP depletion [De Lamirande and Gagnon 1992, Cummins *et al.* 1994]. Motility is at the same time a vital prerequisite for the spermatozoa to penetrate through the cumular cells of the ovum in order to reach the *zona pellucida* and fuse with the female gamete [Garner and Hafez 1993]. Kasai *et al.* [2002] and Martinez-Pastor *et al.* [2004] already emphasised a strong relationship between the motion parameters and mitochondrial activity of the sperm cell.

Contrary to our study, Mangiagalli *et al.* [2012] observed that *in vivo* administration of 0.1 or 0.5 g/L LYC had no significant impact on the motility rate or forward progressive motility in fresh rabbit semen. Although LYC supplementation showed no association with an improvement of *in vivo* male reproductive performance, it was revealed that the carotenoid had a significant dose-dependent positive effect on sperm motility and viability in samples stored for 24 h at 5°C. Similar protective properties of LYC on spermatozoal survival were found in chilled fowl and bull cryopreserved semen [Mangiagalli *et al.* 2007, Bucak *et al.* 2014]. Correspondingly to our observations,

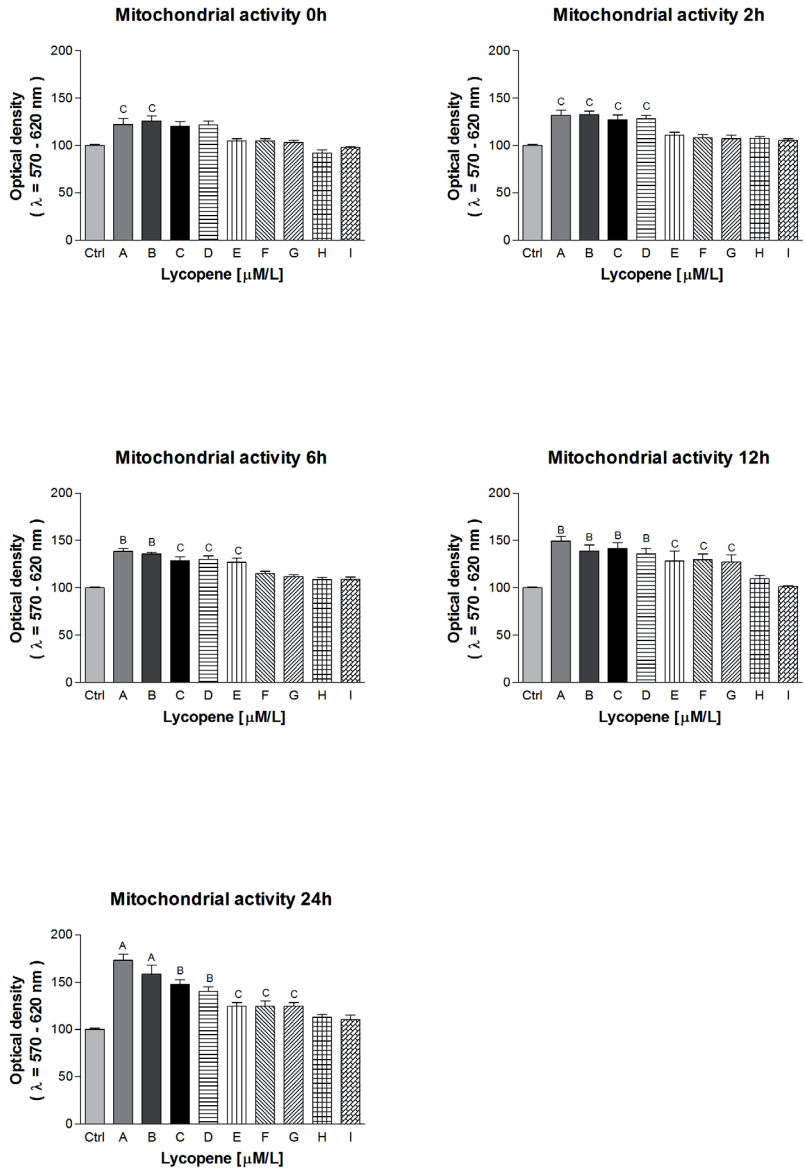

Fig. 1. The effect of various doses of lycopene on the viability of bovine spermatozoa (n=20) at 0h, 2h, 6h, 12h and 24h. Each bar represents mean (±SEM) optical density as the percentage of controls, which symbolise 100%. The data were obtained from five independent experiments. The level of significance was set at ^{A}(P<0.001); ^{B} (P<0.01); ^{C}(P<0.05). Ctrl – 0; A – 2000; B – 1000; C – 500; D – 200; E – 100; F – 50; G – 10; H – 5; I – 1 μM/L LYC.

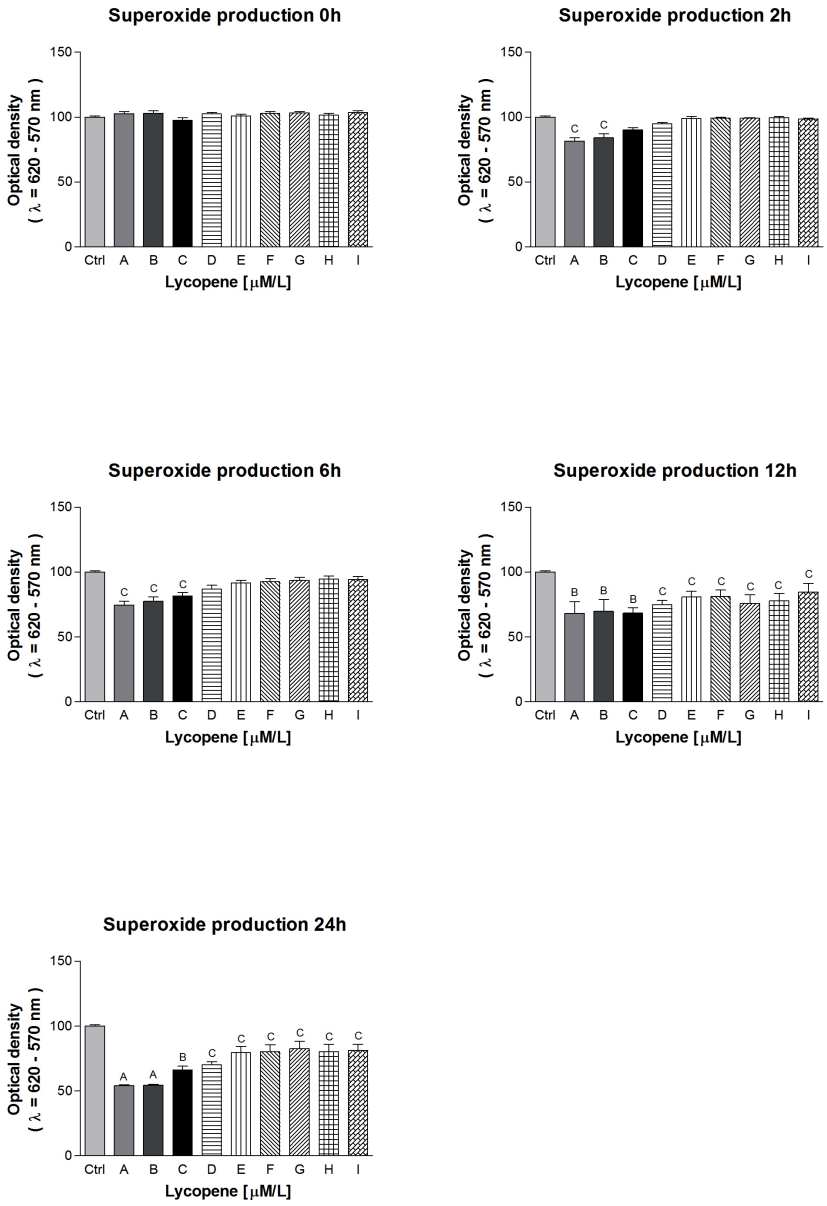

Fig. 2. The effect of various doses of lycopene on superoxide production of spermatozoa (n=20) at 0h, 2h, 6h, 12h and 24h. Each bar represents mean (±SEM) optical density as the percentage of controls, which symbolise 100%. The data were obtained from five independent experiments. The level of significance was set at ^{A}(P<0.001); ^{B}(P<0.01); ^{C}(P<0.05). Ctrl – 0; A – 2000; B – 1000; C – 500; D – 200; E – 100; F – 50; G – 10; H – 5; I – 1 μM/L LYC.

those authors noted that LYC supplementation to a commercial extender significantly increased sperm viability after *in vitro* storage, as a result of specific protective effects of this antioxidant against cell damage, probably through its ROS-quenching abilities and protective effects against lipid peroxidation (LPO). Using a broader array of LYC concentrations Uysal and Bucak [2007] reported that similarly to our observations, 800 µg/mL (approximately 1.5 µM/L) LYC supplemented to a culture medium for ram semen prevented typical deleterious effects on spermatological indicators including a decreased motion, increased sperm abnormalities, acrosome damage and dead sperm. Although the highest concentration of LYC applied (3200 µg/mL, equal to approximately 6 µM/L LYC) was revealed to inhibit sperm vitality, the carotenoid had no principal toxic effects on the motility, membrane integrity, viability or morphology of ram spermatozoa.

Although LYC had no instant effects on the superoxide production (Time 0h), the NBT assessment at 2h revealed that the administration of 1000 and 2000 µM LYC led to a significant decline of the superoxide formation in comparison to the control (Fig. 2). At 6h a significantly improved *in vitro* oxidative balance was detected in experimental groups A, B and C (Fig. 2). Starting at Time 12h and extending throughout the remaining *in vitro* culture, all LYC concentrations exhibited protective effects on the sperm cell structures vulnerable to the escalating superoxide production, which became statistically significant, correspondingly to the growing timeframe of spermatozoa cultivation (Fig. 2).

Our observation that LYC exhibits an ability to diminish the superoxide production by spermatozoa complements earlier studies reporting on the antioxidant properties of this carotenoid and its biological role on sperm structures by means of measuring diverse oxidative stress biomarkers, including the amount of LPO or oxidative DNA fragmentation [Atessahin 2006a,b; Zini *et al.* 2010]. Sarkar et al. [2012] and Aly et al. [2012] showed that LYC pretreatment provided protection to cellular and mitochondrial membranes against LPO - a typical consequence of superoxide overproduction and subsequent oxidative chain reactions leading to the breakdown of PUFAs. According to Aly *et al*. [2012], LYC administered before lipopolysaccharide treatment was accompanied by a decrease of malondialdehyde and H_2O_2 production through ROS scavenging, implicating antioxidant properties of this carotene. Furthermore, LYC may stabilise the activity of antioxidant enzymes, especially superoxide dismutase scavenging excessive superoxide radicals, as well as normalise the decrease of glutathione and vitamin C, detoxifying the remaining ROS.

Carotenoids are well known as highly efficient scavengers of singlet oxygen ($^1O_2$) and other ROS. During $^1O_2$ quenching it is hypothesised that energy is transferred from $^1O_2$ to the LYC molecule, converting it to an energy-rich triplet state. In contrast, trapping other ROS, including hydroxyl radical, nitric oxide or peroxynitrite, leads to oxidative breakdown of the molecule [Pavia and Russell 1999, Vardi *et al.* 2009]. Therefore, LYC may be a suitable substance effective against the first stages of oxidative insult to vulnerable cellular structures including the plasma membrane, nucleus and mitochondria [Gupta and Kumar 2002, Wertz *et al.* 2004].

In conclusion, our results suggest that lycopene supplementation may offer protection to sperm motility, mitochondrial activity and oxidative balance, adding more details on the behaviour of lycopene within an *in vitro* culture of mammalian spermatozoa. At the same time, more specific and complex experiments in enhancing male fertilization potential using lycopene are still necessary, as observational studies cannot establish the cause or effect with absolute confidence.

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