Superoxide dismutase mimics improves semen quality during chilled preservation of rabbit spermatozoa

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ABSTRACT

In rabbit farms, artificial insemination is usually undertaken using semen preserved around 18 °C. However, increased reactive oxygen species levels during preservation produce sperm dysfunction. Our aim was to evaluate the effect of adding superoxide dismutase (SOD) antioxidant mimics (Tempo and Tempol) to the extenders of rabbit semen on quality parameters, total lipid peroxidation (LPO) and SOD level. Ejaculates from 12 sexually mature males were diluted with an extender containing 0 (control), 0.5, 1 or 2 mM of Tempo or Tempol, respectively. Semen samples were cooled till 18 °C and stored for 0, 6 or 24 h. Sperm motility, velocity parameters, viability, plasma membrane integrity, SOD activity and level of thiobarbituric acid reactive substances (TBARS) were determined. Results showed that, in general, SOD antioxidant mimics significantly improved sperm motility compared to control after 6 and 24 h storage. Moreover, at 24 h, Tempo treatments showed motility rates higher than 80% whilst the control group showed the lowest motility rate among all treatments (58.5%, $P < 0.05$). Sperm viability showed no significant differences between treatments at 6 h and 24 h of storage. At 24 h, most SOD antioxidant mimics treatments significantly improved both curvilinear and mean velocity parameters, but only Tempo treatments improved linear velocity parameter. Also, SOD mimics protected sperm cells decreasing TBARS concentration at 6 and 24 h compared to 0 h. In conclusion, the addition of SOD antioxidant mimics during conservation at 18 °C and storage of semen until 24 h reduces lipid peroxidation and preserves rabbit semen quality.

1. Introduction

In rabbit farms, artificial insemination (AI) is usually undertaken using fresh or cooled semen due to the low fertility rates achieved with cryopreserved semen (Rosato and Iaffaldano, 2011a). The AI facilitates efficient breeding programs, stable production cycles, easy management control and reduces the number of males required at the farm (Carluccio et al., 2010). Several studies have reported that cryopreserved rabbit sperm results in lower fertility rates compared to other species that makes them not useful on the commercial level (reviewed by Mocé and Vicente, 2009). Therefore, storage of fresh semen without affecting the fertilizing capability is a high demand in rabbit commercial farms. The semen doses are conserved at a temperature of 15–18 °C, which proves to be more efficient than storage at 5 °C for short times (Johinke et al., 2014). The ability to extend semen storage time of chilled semen without reducing fertility parameters is a need in order to facilitate the transport and widespread application of semen from

outstanding genetically superior bucks for AI (Gogol, 2013; Rosato and Iaffaldano, 2011b). During the cooling process, high levels of reactive oxygen species (ROS) have been observed in mammalian semen (Chatterjee and Gagnon, 2001; Saleh and Agarwal, 2002). These levels of ROS lead to oxidative stress which has been identified as one of the factors affecting male fertility by reducing sperm motility, antioxidant enzyme activity, peroxidation of unsaturated fatty acids in sperm membranes and sperm DNA fragmentation (Maia et al., 2010; Peris et al., 2007). Under normal conditions, ROS is created by sperm cells. However, the imbalance between ROS generation and scavenging induce the oxidative stress state (Agarwal et al., 2007). The anti-oxidant mechanism in seminal plasma, including enzymatic and non-enzymatic antioxidants, plays an important role to keep the ROS at its physiological level (Sikka, 2001). Several antioxidants have been reported to protect sperm cells against the excess level of ROS during cryopreservation or cooling processes in different mammalian species including rabbits (Yousef et al., 2003), bovine (Tuncer et al., 2010), ovine

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(Santiani et al., 2014a), equine (Baumber et al., 2005) and swine (Gadani et al., 2017). Superoxide dismutase (SOD) is one of the enzymatic antioxidants present in the seminal plasma that protects spermatozoa by catalysing the dismutation of superoxide anions to hydrogen peroxide and oxygen, thereby protecting mature spermatozoa against excessive superoxide anion accumulation (Aitken et al., 1996). Tempo and Tempol are nitroxide compounds, which have similar activity to SOD, and, compared with SOD, have low molecular weight, high solubility, and penetrate cell membranes easily (Mitchell et al., 1990). These SOD mimics (Tempo and Tempol) have been added to semen extenders at different concentrations, under different temperatures in different species with varying results. It has been reported that addition of SOD mimics to spermatozoa at higher temperatures (beginning of the cooling process) has a negative effect on the sperm characteristics and decreases the sperm motility (Mata-Campuzano et al., 2012). However, using Tempol during the storage of ram spermatozoa at 15 °C improved semen motility and in vitro fertility (Mara et al., 2005a). Therefore, the objective of this study was to evaluate the effect of adding SOD antioxidant mimics (Tempo and Tempol) during the preservation process of semen on quality parameters, total lipid peroxidation (LPO) and SOD level in rabbits.

2. Materials and methods

All the experimental procedures used were performed according to the Spanish Policy for Animal Protection RD53/2013 (*Real Decreto 53/2013*, de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. (In Spanish), 2013), assessed and approved by the Animal Ethics Committee of the Community of Madrid (Ref. PROEX 302/15).

2.1. Animals, semen collection and processing

Twelve 8–10-month-old sexually mature New Zealand white male rabbits were used as semen donors. Males were housed in individual cages under standard ambient conditions (16 h/8 h light/dark cycle, 22–24 °C temperature and 55–60% relative humidity). A commercial pellet diet (NANTA S.L., Madrid, Spain) and fresh drinking water were provided ad libitum. Semen was collected from donor once weekly with an artificial vagina. Ejaculates with at least 80% progressive motility, a volume ≥ 0.5 mL and more than 400×10^6 spermatozoa/mL were processed. Collected ejaculates from each male were pooled to eliminate individual variations. Five pooled ejaculates with 4–5 mL each were included in this study. From pooled semen, seven samples were diluted to a final concentration of approximately 40×10^6 sperm/mL with a commercial Tris buffer as diluent (Inserbo S.L., Huesca, Spain) containing 0 (control), 0.5, 1 or 2 mM of Tempo or Tempol, respectively. Semen samples were introduced to a thermal bath at 18 °C where were stored for 0 (fresh), 6 or 24 h.

2.2. Sperm motility and velocity parameters

Motility and progressivity were analysed by computer-assisted sperm analysis (CASA; Integrated Sperm Analysis System, V.1.2., Proiser S.L., Valencia, Spain). Seven microliter aliquots of semen samples were placed on a slide and covered with a cover slip. The analysis was based upon the study of 25 consecutive, digitalized photographic images obtained from a single field at a magnification of $\times 100$ on a dark field. These 25 consecutive photographs were taken in a time lapse of 1 s, which implied a velocity of image-capturing of one photograph every 40 m/s. Five separate fields were taken for each sample with a minimum of 400 sperm evaluated. The motility descriptors obtained after CASA analyses were: Curvilinear velocity (VCL $\mu\text{m/s}$) that is the total distance between adjacent points, divided by the time elapsed; Straight-line velocity (VSL, $\mu\text{m/s}$) that is the straight-line distance

between the first and last tracked points, divided by the acquisition time; and Average path velocity (VAP, $\mu\text{m/s}$) that is a smoothed path constructed by averaging several neighbouring positions on the track and joining the averaged positions. Total motility was defined as the percentage of spermatozoa which showed a VAP above 10 $\mu\text{m/s}$, and progressivity as the percentage of spermatozoa which showed a VAP above 50 $\mu\text{m/s}$.

2.3. Sperm viability

Viable and nonviable spermatozoa were determined by eosin–nigrosine staining. Sperm suspension smears were prepared by mixing a drop of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide. Viability was assessed by counting 200 cells at 400 X (CKX41; Olympus Corporation, Tokyo, Japan). Sperm displaying partial or complete purple staining were considered nonviable; only sperm showing strict exclusion of stain were counted as viable.

2.4. Plasma membrane integrity

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm plasma membrane. HOST relies on the resistance of the membrane to loss of permeability barriers under stress condition of stretching in a hypoosmotic medium. Sperm cells with resistant membranes exhibited a swelling around the tail. The assay was performed by mixing 30 μL of semen with a 300 μL of 100 mOsm/kg hypoosmotic solution (9 g fructose plus 4.9 g sodium citrate per litre of distilled water). This mixture was incubated (37 °C) for 1 h, where 0.2 mL of the mixture was placed on a microscope slide and mounted with a cover slip and immediately evaluated at 400 X (CKX41; Olympus Corporation, Tokyo, Japan). A total of 200 spermatozoa were counted in at least five different microscopic fields. The percentages of sperm with swollen and curled tails were then recorded.

2.5. Biochemical assays

For biochemical assays, sperm samples were washed by centrifugation ($1200 \times g$ during 20 min), the supernatant was removed, and the pellet diluted to a volume of 1 mL with PBS. The washing step was repeated and samples were conserved at -80 °C until analysis.

Determination of SOD was done as described by Sun and Zigman (1978). A mixture of 0.4 mL sample, 0.1 mL of 0.9% NaCl and 0.4 mL of chloroform: ethanol (3:5) was centrifuged for 10 min at 2000 g. The reaction mixture contained 20 mL 3 mmol/L xanthine, 10 mL 0.6 mmol/L EDTA, 10 mL 0.15 mmol/L NBT, 6 mL 400 mmol/L sodium carbonate, and 3 mL 1 g/L BSA for 20 tests. An aliquot (50 μL) of xanthine oxidase (20 $\mu\text{mol}/2$ mL) was added to 2.45 mL of the reaction mixture and this mixture was incubated at 25 °C for 20 min. Subsequently, the reaction was stopped by adding 1 mL of 0.8 mmol/L CuCl_2 . Absorbance was recorded at 560 nm in a spectrophotometer, with SOD activity expressed as $\mu\text{kat/g}$ protein.

Lipid peroxidation was measured by using the thiobarbituric acid (TBA) reaction for malondialdehyde (MDA) (TBARS assay Kit, Cayman Chemical Company, Ann Arbor, MI, USA). 100 μL of seminal plasma from each sample was mixed with 2 mL of the TBA-trichloroacetic acid (TCA) reagent (15%, w/v TCA; 0.375%, w/v TBA and 0.25 N HCl) and incubated in a boiling water bath for 60 min and subsequently cooled in an ice bath for 10 min. After cooling, the suspension was centrifuged at $1600 \times g$ and 4 °C for 10 min. The supernatant was then separated, and absorbance was measured at 532 nm at room temperature over a period of 30 min. The assay was conducted in duplicate. The amount of TBARS produced ($\mu\text{mol/L}$) was quantified against a standard curve created using MDA as standard (Tecan, Safir 2, Tecan Group Ltd., Männedorf, Suiza).

Table 1
Effect of antioxidants (Tempo and Tempol) treatment with different concentrations (0.5, 1 and 2 mM) for different durations (0, 6 and 24 h) of rabbit semen preservation process (at 18 °C) on percentages of motility, viability and plasma membrane integrity (HOST).

Treatment	0 h			6 h			24 h		
	Motility %	Viability %	HOST %	Motility %	Viability %	HOST %	Motility %	Viability %	HOST %
Control	92.6 ± 1.62 ^{bc}	87.07 ± 1.41 ^{ab}	88.29 ± 1.38 ^{abc}	90.3 ± 2.26 ^{cd}	82.3 ± 1.53 ^{yz}	86.08 ± 2.18	58.5 ± 5.17 ^{cd,x}	79.72 ± 2.12 ^z	82.25 ± 3.27
Tempo 0.5	95.4 ± 0.59 ^{ab}	82.47 ± 1.92 ^{ab}	88.42 ± 1.75 ^{ab}	96.4 ± 0.53 ^{ab}	84.29 ± 2.36 ^y	85.74 ± 2.16 ^{yz}	80.05 ± 2.22 ^{bc,x}	78.27 ± 2.56 ^z	83.64 ± 3.49 ^z
Tempo 1	92.9 ± 1.6 ^{bc}	83.74 ± 1.89 ^{ab}	87.39 ± 2.11 ^{abc}	96.3 ± 0.65 ^{ab}	79.95 ± 2.51 ^z	84.81 ± 2.73	90.1 ± 1.67 ^{ab,x}	79.07 ± 2.28 ^z	80.66 ± 4.15
Tempo 2	95.45 ± 0.65 ^{ab}	81.07 ± 1.62 ^b	84.34 ± 1.90 ^{bc}	92.5 ± 1.05 ^{bc}	79.51 ± 2.35	78.71 ± 2.67	83.5 ± 2.8 ^{ab,x}	78.63 ± 2.82	78.72 ± 4.45
Tempol 0.5	96.15 ± 0.71 ^{ab}	83.05 ± 2.17 ^{ab}	90.61 ± 1.83 ^a	95.8 ± 0.37 ^{ab}	82.37 ± 2.47 ^y	86.83 ± 2.05	72.75 ± 3.55 ^{cd,x}	78.47 ± 2.61 ^z	81.17 ± 2.53
Tempol 1	92.35 ± 1.15 ^{bc}	80.82 ± 1.85 ^b	85.48 ± 1.91 ^{abc}	93.9 ± 1.14 ^{ab}	80.74 ± 2.22	80.7 ± 3.42	74 ± 2.65 ^{cd,x}	78.94 ± 2.26	81.15 ± 3.51
Tempol 2	93.65 ± 0.85 ^{ab}	79.85 ± 2.17 ^b	82.72 ± 1.44 ^c	96.3 ± 0.43 ^{ab}	78.89 ± 2.05	81.26 ± 2.16	69.85 ± 3.44 ^{dx}	77.76 ± 1.69	78.19 ± 4.06

Values are lsmean ± standard error

^{a,b,c,d,e} Significant differences ($P < 0.05$) between treatments at each time point (within columns).

^{y,z} Significant differences ($P < 0.05$) of each treatment along time (within rows).

2.6. Statistical analysis

The experimental unit was the pool. The effects of both type / antioxidant doses (0, Tempo 0.5, Tempo 1, Tempo 2, Tempol0.5, Tempol1, and Tempol2) and storage time (0, 6 h, and 24 h) on motility, velocity parameters, viability, HOST, SOD activity and Lipid peroxidation were analysed using a repeated measures analysis of variance using the PROC MIXED of SAS (SAS Inst. Inc., Cary, NC). All means were compared using a protected *t*-test. Differences were considered significant at $P < 0.05$. Results are presented as least squares mean ± standard error.

3. Results

3.1. Sperm motility

The results of motility, viability and plasma membrane integrity in the different experimental groups are showed in Table 1. Adding of SOD antioxidant mimics (Tempol or Tempo) at different concentrations to rabbit semen, cooling to 18 °C and storing for 0 h showed no significant change ($P > 0.05$) on sperm motility, except for Tempol 0.5 mM which improved the motility compared to the control group. After 6 and 24 h storage time, all SOD antioxidant mimics treatments (except Tempo 2 mM at 6 h) showed significant improvement of sperm motility compared to controls. Moreover, at 24 h storage time, Tempo treatments showed motility rates higher than 80% whilst the control group showed the lowest motility rate among all treatments. Over time and within each treatment, the results showed a reduction in the motility rates at 24 h compared to 0 and 6 h time points, except for Tempo 1 mM treatment which showed no significant differences between 24 and 0 h.

3.2. Sperm viability and plasma membrane integrity

Sperm viability and HOST (Table 1) showed no significant differences between treatments at 6 h and 24 h of storage. However, at 0 h sperm viability was significantly lower in Tempo 2 mM, Tempol 1, and 2 mM treatment groups compared to control group. HOST showed the lowest percentage in Tempol 2 mM group and the highest in Tempol 0.5 mM.

3.3. Velocity parameters

Sperm velocity parameters including were significantly decreased at 24 h compared to 0 and 6 h time points in all treatment groups (Fig. 1A–C). However, at 24 h, all SOD antioxidant mimics treatments (except Tempol 1 mM) significantly improved VCL and VAP (Fig. 1A and C), and only Tempo treatment groups improved VSL (Fig. 1B). At 0 h, there were no significant differences among the treatments. However, Tempo 0.5 mM treatment improved VSL and VAP and Tempo 0.5, 1 mM and Tempol 2 mM treatments improved VCL at 6 h time point compared to control group (Fig. 1).

3.4. Superoxide dismutase activity and lipid peroxidation

There were no significant differences between all groups at 0 h in SOD and LPO activity. SOD levels were significantly increased in Tempo and Tempol treatments with 1 mM each at 6 h and in Tempo 2 mM and Tempol 0.5 mM at 24 h (Table 2). LPO showed the lowest concentration of TBARS in Tempo 1 and 2 mM and in Tempol 0.5 and 2 mM groups at 6 h. However, at 24 h, the lowest concentration of TBARS has been found in Tempo 1 mM and in Tempol 0.5 and 1 mM groups (Table 2). Within each treatment group, there was a reduction in SOD activity across the time except for Tempo 2 and Tempol 0.5 mM groups. On the other hand, Tempol groups showed a reduction in TBARS concentrations by the time (Table 2).

(caption on next page)

Fig. 1. Effect of antioxidants (Tempo and TempoL) treatment with different concentrations (0.5, 1 and 2 mM) for different durations (0, 6 and 24 h) of rabbit semen preservation process (18 °C) on motility descriptors ($\mu\text{m/s}$) obtained after CASA analyses: A) curvilinear velocity (VCL), B) linear velocity (VSL) and C) mean velocity (VAP).

^{a,b,c} Significant differences ($P < 0.05$) between treatments within each time point.

^{x,y,z} Significant differences ($P < 0.05$) between time points within each treatment.

4. Discussion

We evaluated the effect of adding SOD antioxidant mimics (Tempo and TempoL) during the preservation process of rabbit semen on quality parameters, LPO and SOD activity. Our results showed that SOD antioxidant mimics can protect rabbit semen against oxidative stress during the preservation process. Sperm motility and velocity parameters showed a significant reduction after 24 h storage at 18 °C in all groups, including control group, compared to 0 or 6 h but this deteriorating situation was not similar in the six antioxidant treatments. A significant reduction in total sperm motility and viability due to storage time (48 h) and chilling temperature (5 and 15 °C) has been previously reported in rabbit semen (Rosato and Iaffaldano, 2011b). Several factors can play a role in sperm motility and viability reduction during storage. Oxidative stress is one of these factors that lead to sperm damage due to the high levels of ROS formation during storage with the limited endogenous antioxidant capacity of sperm (Aramli et al., 2013; Koppers et al., 2010). Moreover, sperm cells are highly susceptible to ROS stress due to the large amounts of unsaturated fatty acids allocated in the plasma membrane (Alvarez and Storey, 1984). ROS can directly attack sperm membranes, lipids, DNA and mitochondria results in lipid peroxidation, and reduce the efficiency of ATP production that lead to low motility and sperm cell death (Verma and Kanwar, 1998). Several studies reported the beneficial effect of exogenous antioxidant supplementation to semen extenders on semen quality as a way of defence against ROS. Antioxidants are compounds that can scavenge and quench ROS and neutralize their actions. Vitamins (E and C) have been used as non-enzymatic antioxidants to enhance sperm motility, viability and membrane integrity in several species including equine (Aurich et al., 1997), bovine (Zhao et al., 2015) and human (Moslemi and Tavanbakhsh, 2011). Enzymatic antioxidants like SOD, catalase, glutathione peroxidase, and glutathione play important roles in preserving and restoring cell redox homeostasis (Adewoyin et al., 2017; Sheweita et al., 2005). In the current study, we examined different concentration of TempoL and Tempo as SOD mimics during the storage process of rabbit semen. Although a general reduction in sperm motility was found after 24 h at 18 °C compared to 0 and 6 h, TempoL and Tempo treatments improved sperm motility compared to the control group. SOD mimics are redox cycling nitroxides that promotes the metabolism of many ROS and improves nitric oxide bioavailability that can protect cells from intracellular ROS (Wilcox, 2010). Adding of Tempo with a concentration of 1 mM showed the highest motility percentages (lowest reduction in motility across time points) among all

groups. In this treatment, there was a significant reduction in sperm motility at 24 h compared to 6 h but not with 0 h. This could be due to the changes in antioxidant enzyme activities during temperature transitions until reaching 18 °C at 0 h compared to the constant temperature at 6 h. Reduction in motility and viability have been observed when Tempo and TempoL were added to ram (Santiani et al., 2014b) and bovine (Foote et al., 2002) spermatozoa at the beginning of the cooling curve. Temperature transitions either from low to higher or from high to lower temperatures result in alterations of enzyme activities in different tissues (Bagnyukova et al., 2003; Lushchak and Bagnyukova, 2006). Among the different concentrations of Tempo, 1 mM showed a significant higher motility compared to 0.5 but 2 mM was not differed. This may indicate that moderate concentration of Tempo (1 mM) is optimum for rabbit semen. A significant decline in sperm motility has been observed when semen was treated with 150 U/mL SOD or without SOD compared to 100 U/mL (Perumal, 2014). These results could support the concept that SOD mimics protective effect does not increase with the increase of its concentration (Offer et al., 1998). In agreement with the current study, Santiani et al. (2013) indicated that loss of motility during cryopreservation of alpaca sperm could be partially prevented by supplementation of the semen extender with 1 mM Tempo or TempoL. In another study, a concentration of 5 μM TempoL significantly improved motility and viability post thawing and reduced DNA fragmented sperm in human (Bateni et al., 2014). Using TempoL with liquid semen stored under cooling conditions maintained high motility rates and resulted in good fertility in goat (Mara et al., 2007), turkey (Donoghue and Donoghue, 1997) and ram (Mara et al., 2005b). Velocity parameters obtained after CASA analyses showed the same pattern of motility reduction after 24 h compared to 0 and 6 h groups. However, Tempo treatment groups and TempoL 0.5 and 2 mM treatments showed increase in velocity parameters at 24 h than control group which showed the lowest values (Fig. 1). These results indicate the efficient protection mechanism of SOD mimics during storage against the deleterious effect of ROS on sperm motility and velocity. This mechanism could be through the maintenance of mitochondrial ATP production which determine sperm motility (Plaza Davila et al., 2015) and/or the role of TempoL and Tempo in preventing lipid peroxidation through the reduction of hydroxyl radicals formation (Santiani et al., 2013; Wilcox and Pearlman, 2008). Here we reported the lowest concentration of TBARS, an indicator of lipid peroxidation, in Tempo 1 mM group after 6 and 24 h storage time of cooled semen. In addition, TempoL groups (0.5 and 2 mM) showed significant decrease in TBARS concentration at 6 and 24 h compared to 0 h. In agreement

Table 2

Effect of antioxidants (Tempo and TempoL) treatment with different concentrations (0.5, 1 and 2 mM) for different durations (0, 6 and 24 h) of rabbit semen preservation process (at 18 °C) on SOD activity ($\mu\text{kat/g}$) and Lipid peroxidation (TBARS $\mu\text{mol/L}$).

Treatment	0 h SOD $\mu\text{kat/g}$	TBARS $\mu\text{mol/L}$	6 h SOD $\mu\text{kat/g}$	TBARS $\mu\text{mol/L}$	24 h SOD $\mu\text{kat/g}$	TBARS $\mu\text{mol/L}$
Control	24.23 $\pm$ 6.27	11.1 $\pm$ 4.51	12.35 $\pm$ 2.21 ^b	8.32 $\pm$ 1.77 ^a	20.89 $\pm$ 0.50 ^c	4.51 $\pm$ 1.07 ^a
Tempo 0.5	35.69 $\pm$ 3.29 ^y	7.93 $\pm$ 3.51 ^y	18.77 $\pm$ 2.86 ^{ab,x}	15.07 $\pm$ 4.51 ^{a,x}	19.74 $\pm$ 1.27 ^{c,x}	2.88 $\pm$ 0.92 ^{ab,y}
Tempo 1	35.76 $\pm$ 7.88 ^y	3.77 $\pm$ 1.35	26.73 $\pm$ 1.10 ^{a,y,z}	2.88 $\pm$ 0.99 ^b	18.75 $\pm$ 1.53 ^{c,x}	1.56 $\pm$ 0.36 ^b
Tempo 2	39.96 $\pm$ 6.68 ^y	6.22 $\pm$ 1.76	12.81 $\pm$ 0.42 ^{b,z}	4.05 $\pm$ 0.99 ^b	28.84 $\pm$ 0.83 ^{b,y}	5.91 $\pm$ 1.75 ^a
TempoL 0.5	38.42 $\pm$ 8.22 ^y	11.42 $\pm$ 2.64 ^y	20.23 $\pm$ 4.89 ^{ab,x}	2.24 $\pm$ 1.11 ^{b,z}	34.03 $\pm$ 0.87 ^{a,y}	2.10 $\pm$ 0.91 ^{b,z}
TempoL 1	37.87 $\pm$ 6.82 ^y	7.85 $\pm$ 2.82 ^{yz}	24.86 $\pm$ 6.30 ^{a,x}	8.86 $\pm$ 2.66 ^{a,y}	20.75 $\pm$ 1.02 ^{c,x}	1.91 $\pm$ 0.47 ^{b,z}
TempoL 2	41.19 $\pm$ 5.34 ^y	14.51 $\pm$ 5.23 ^y	17.63 $\pm$ 3.07 ^{ab,x}	4.36 $\pm$ 1.19 ^{b,z}	18.43 $\pm$ 2.51 ^{c,x}	3.27 $\pm$ 1.00 ^{ab,x}

Values are $\text{mean} \pm \text{standard error}$

^{a,b,c} Significant differences ($P < 0.05$) between treatments at each time point (within columns).

^{yz} Significant differences ($P < 0.05$) of each treatment along time (within rows).

with our results, it has been reported that SOD mimics can protect ram sperm during cooling process against lipid peroxidation induced by oxidative stress by decreasing the level of ROS production (Santiani et al., 2014c). A negative correlation between levels of TBARS and semen motility has been reported in stored boar semen with indication of higher oxidative stress (Zakošek Pipan et al., 2014). These results along with our findings confirmed that SOD mimics protect sperm cells by preventing lipid peroxidation which could explain the higher rates of sperm motility and velocity parameters in the treated groups. On the other hand, intracytoplasmic SOD activity levels were significantly higher in Tempo 1 mM and TempoL 1 mM groups at 6 h and in Tempo 2 mM and TempoL 0.5 mM at 24 h compared to control groups. It has been proved that SOD has a protective role against the negative effect of ROS by reducing the production of hydroxyl radicals during cooling process of ram spermatozoa (Kasimanickam et al., 2006; La Falci et al., 2011). However, cooling of semen has been reported to be highly correlated with the decreasing activity of natural SOD in sperm (Chatdarong et al., 2012). In our results, there was no clear correlation between SOD activity and lipid peroxidation among different treatments especially at 24 h. In addition, some treatments, as well as the control group, showed an increasing pattern of SOD activity at 24 h compared to 6 h. This could be due to the intracellular SOD leakage during semen storage. Several studies suggested that, storage of liquid semen samples for long time increase SOD activity in the seminal plasma because of the leakage of intracellular SOD from spermatozoa due to the damages occurred in sperm membranes by ROS (Nair et al., 2006; Partyka et al., 2012; Zakošek Pipan et al., 2014). Our results showed that viability and HOST, as indicator of plasma membrane integrity, for sperm stored for 6 or 24 h were not affected by SOD antioxidant mimics treatments but were affected by time of storage. The same results have been found in alpaca sperm with a beneficial effect of 1 mM Tempo or TempoL on sperm motility (Santiani et al., 2013). Other antioxidants including bovine serum albumin and foetal calf serum provided a protection for sperm plasma membrane when supplemented to liquid stored rabbit semen by their antioxidative properties (Sariözkan et al., 2013).

5. Conclusions

Preservation of rabbit semen for 24 h at 18 °C in the presence of SOD mimics (TempoL and Tempo) improved sperm quality in terms of motility and velocity parameters. SOD mimics protected rabbit sperm cells by reducing lipid peroxidation process which occurred due to oxidative stress and high level of ROS formation during liquid semen preservation. Tempo with a concentration of 1 mM showed better semen quality and lower lipid peroxidation.

Conflict of interest

The authors declare no conflicts of interest.

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