Animal Reproduction Science 205 (2019) 18–26

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

# Effect of dietary grape marc on fresh and refrigerated boar semen

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ARTICLE INFO

*Keywords:*
Boar
Grape marc
Antioxidant
Spermatozoa

ABSTRACT

In several studies there has been evaluation of the dietary addition of antioxidants to improve the quality of fresh and stored semen in domestic animals. Grape marc (GM), as the residue of vinification, contains large amounts of polyphenols with antioxidant, anti-inflammatory, antimicrobial, and antiaging effects. In this study, two regimens of dietary GM supplementation (2% and 4%) were tested regarding effects on the characteristics and lipid peroxidation of fresh and stored semen. The dietary supplementation of GM improved sperm characteristics in fresh semen, especially at 4% of GM. There were greater values for all kinetic variables and membrane integrity, and lesser values for sperm abnormalities and lipid peroxidation with 2% and 4% GM supplementation. Lipid peroxidation of the pellet was less with both 2% and 4% GM supplementation. During sperm storage, the dietary supplementation of GM improved the quality of sperm, with greater values for kinetic variables and membrane integrity at day 15 of storage. In the present study, supplementation of GM in boar diets improved fresh semen characteristics and reduced the lipid peroxidation of ejaculated spermatozoa, possibly due to the effect of polyphenols present in the GM. Consequently, this likely resulted in improved sperm quality during storage.

## 1. Introduction

The boar sperm membrane contains large amounts of unsaturated fatty acids which make these cells highly susceptible to the damaging effects of reactive oxygen species (ROS). Lipid peroxidation leads to a loss of numerous structures and functions such as membrane integrity (MI), inactivation of cellular enzymes (White, 1993), DNA fragmentation (Baumber et al., 2003), and cell apoptosis with consequent reduction of sperm quality (Sanocka–Maciejewska et al., 2005). Furthermore, Kobayashi and Suda (2012) reported a significant positive correlation between concentrations of ROS and the percentage of spermatozoa with morphological abnormalities such as acrosome, head and mid piece anomalies, tail defects and cytoplasmic droplets. In human reproduction, there have been various reports that teratozoospermic men with fertility problems have relatively greater concentrations of ROS than men without fertility problems (Agarwal and Said, 2005; Rato et al., 2012).

Physiologically, the ROS concentrations are maintained optimal as a result of the presence of antioxidants in seminal plasma as well as β-mercaptoethanol, vitamin E and C, cysteamine, cysteine, taurin and hypotaurin (Holmes et al., 1992; Chen et al., 1993; Rolf et al., 1999; Kitagawa et al., 2004; Bucak et al., 2007). To keep ROS concentrations optimal for sustaining high sperm quality, numerous studies were conducted where there was addition of antioxidants to the cooling or freezing medium. In these experiments,

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https://doi.org/10.1016/j.anireprosci.2019.03.016
Received 29 October 2018; Received in revised form 11 March 2019; Accepted 26 March 2019
Available online 27 March 2019
0378-4320/ 

there has been supplementation of crocin in bull sperm (Sapanidou et al., 2015), astaxanthin for boar sperm storage (Basioura et al., 2018), alpha-tocopherol, cystein, and rosemary for boar semen cryopreservation (Jeong et al., 2009; Malo et al., 2010), vitamin C, taurine, catalase, vitamin E, and vitamin B16 in cryopreserved dog semen (Michael et al., 2007), and hypotaurine in human semen (Brugnon et al., 2013). It has been proposed that dietary supplementation of antioxidants is a potential way to improve male reproductive outcomes in subfertile men (Wong et al., 2000; Eskenazi et al., 2005), or to increase semen quality in animals (Marin-Guzman et al., 1997; Castellini et al., 2002; Deichsel et al., 2008; Contri et al., 2011).

The residues of wine processing, such as grape marc (GM), were found to be alternative sources of antioxidants (Beres et al., 2017). The GM is made from the skins, seeds, and stems that remain after grapes (*Vitis vinifera*) have been pressed to produce wine. The GM contains large concentrations of both fat and tannin (Spanghero et al., 2009), significant amounts of anthocyanins, such as cyanidine 3-glucoside, malividin 3- glucoside, cyaniding, and peonidin, belonging to the family of flavonoids, with a large amount of antioxidant activity (Auger et al., 2004). The dietary supplementation with GM increased sperm quality in epididymal ram spermatozoa (Zhao et al., 2017) and ejaculated rabbit spermatozoa (Eid, 2008). There has been use of GM in feeding piglets for its anti-inflammatory action (Gessner et al., 2013; Fiesel et al., 2014), but to the best of our knowledge, there are no studies that have evaluated the effects of GM on the quality of boar semen.

Thus, the aim of the present study was to characterize the effects of GM dietary supplementation on fresh and stored boar semen characteristics and lipid peroxidation.

## 2. Material and methods

### 2.1. Animals

The study was conducted with ten crossbred boars aged 2 or 3 years following semen quality assessments of each boar for a 10-month period. The boars were housed on a farm for the production of semen for commercial artificial insemination and maintained in individual stalls (3.0 m x 3.5 m) in a ventilated building with a controlled temperature of less than 25 °C. Animals were fed 2.5 kg of a basal diet consisting of digestible energy 3.06 Mcal/kg total protein 16.11%, fat 4.47%, crude fibre 5.49%, lysine 0.81%, methionine 0.25%, threonine 0.6%, tryptophan 0.2%, valine 0.77%, calcium 1%, total phosphorus 0.83%. The nutrient composition of the GM is reported in the Table 1. Water was also provided *ad libitum* from a nipple drinker system. After a period of 4 weeks there was supplementation in the boar diets of 2% of GM for 3 months. The animals were subsequently fed for 3 months the basal diet (wash out, WO), followed by dietary supplementation of 4% of GM for another 3 months.

The animals were cared for using management methods approved by the Italian legislation on animal care (DL No. 116, 27/01/1992). The owner of the animals gave informed consent for the procedures performed in the present study. Aliquots for semen evaluations were part of the ejaculates collected for artificial insemination by farm personnel, and no collections were performed specifically for the present study.

### 2.2. Experimental design

Due to the individual effect on semen variables, in this study each boar served as a self-control for the effect of the GM dietary supplementation. Semen collections occurred weekly for all animals. To define basal variables, there were assessments of semen from four collections before dietary supplementation was initiated. Each boar was subsequently supplemented with 2% GM in the diet for 3 months, and after month 2 from the beginning of the dietary supplementation, there were four semen collections on which sperm variable assessments occurred. Each boar was subsequently fed the basal diet (WO), without any dietary supplementation for 3 months, and after month 2, there were four collections in which sperm variable assessments occurred. Each boar was subsequently fed a diet with supplementation of 4% GM for 3 months, and after month 2, sperm variables were assessed in four collections (Fig. 1).

### 2.3. Semen collection and storage

Semen was collected using the gloved hand technique (Hancock and Hovell, 1959) which was conducted by the same person. The

**Table 1**
Nutrient composition of grape marc used in the study.

| | Value |
|---|---|
| Dry matter (%) | 89.7 |
| Crude protein (%) | 12.1 |
| Starch (%) | 1.6 |
| Total sugars (%) | 0.8 |
| Crude fat (%) | 5.1 |
| Crude fibre (%) | 23.1 |
| Ash (%) | 7.2 |
| Lignin (%) | 29.8 |
| Gross energy (kcal/Kg) | 4036 |

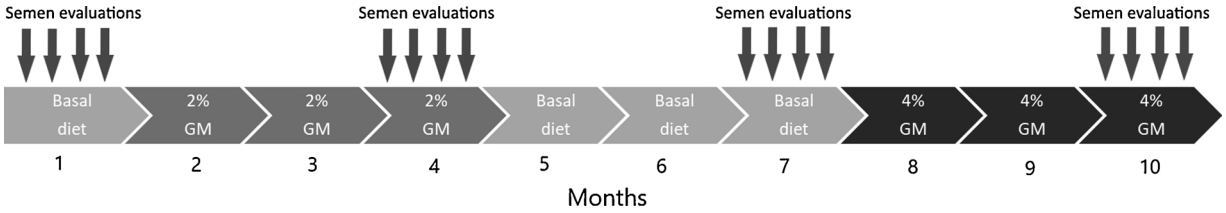


**Fig. 1.** Time schedule of the semen collections during supplementation of grape marc in boar diets.

boars started the trial in different months (three boars in February, three in April, two in June, and two in October). The total volume of the sperm-rich fraction was recorded then the semen was filtered through gauze. The semen was transported to the laboratory at 38 °C for evaluation. To evaluate the lipid peroxidation of spermatozoa at ejaculation, an aliquot of 10 mL fresh semen was centrifuged at 700 *g* for 10 min at 4 °C for separation of the pellet and plasma and washed twice (300 *g* for 10 min, at 4 °C) in phosphate buffered saline (PBS). The pellet was subsequently stored at −80 °C until lipid peroxidation (LPO) evaluation.

After motility and concentration evaluations, estimated as subsequently described, part of the ejaculate was diluted in Beltsville Thawing Solution (BTS – dextrose 3.7 g, sodium citrate dehydrate 0.6 g, sodium bicarbonate 0.125 g, disodium ethylenediamine tetraacetate 0.125 g, potassium chloride 0.075 g, gentamycin 0.06 g, in 100 ml) to a final sperm number of $3 \times 10^9$ spermatozoa and a volume of 35 mL in 50-mL sterile plastic tubes, and stored in ventilated air-conditioned boxes at 17 °C for 15 days. Sperm kinetic variables, membrane integrity and acrosome integrity, as subsequently described, were performed after collection of fresh semen (T0), and after 3 (T3), 5 (T5), 9 (T9), and 15 (T15) days of refrigeration at 17 °C. Because the morphology was similar during refrigeration, sperm abnormalities were assessed at all time-points, but only values recorded at T0 were reported. To assess the amount of peroxidation of the diluted semen during refrigeration, aliquots of diluted semen (10 ml) were removed from the storage boxes at T0, T3, and T5 and also stored at −80 °C until LPO analysis.

### *2.4. Semen evaluation*

The sperm concentration was estimated using a hemocytometer (Bürker chamber; Merck, Leuven, Belgium) using the standard procedure.

Sperm motility and kinetics were evaluated objectively using a computer-assisted sperm motion analyzer (CASA) system IVOS 12.3 (Hamilton-Thorne Bioscience, Beverly, MA, USA). For the analysis, an aliquot of raw semen was diluted at $30 \times 10^6$ sperm/mL with Beltsville Thawing Solution (BTS), and incubated for 10 min at 39 °C (Broekhuijse et al., 2011). A 2-μL aliquot of semen was subsequently loaded into a four-chamber 20-μm slide (Leja, Nieuw-Vennep, the Netherlands) and analyzed. Kinetic variables were collected and recorded by the analysis of 12 non-consecutive fields. The anti-collision algorithm was activated. The variables considered in this study were total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, as VSL/VAP, %) and linearity (LIN, as VSL/VCL, %). The percentage of rapid sperm (%), as the cells with a VAP > 45 μm/s, was also recorded. Cells with VAP > 45 μm/s and STR > 45% were considered progressive (Broekhuijse et al., 2011).

Sperm MI was evaluated using a propidium iodide (PI) and SYBR-14 fluorescent stain (Live/dead sperm viability kit®; Molecular Probes Inc., Eugene, OR, USA) as previously described (Garner and Johnson, 1995) with modifications. Briefly, an aliquot (200 μL) of diluted semen was incubated with 2.4 μM of PI and 20 nM of SYBR-14 (final concentration) at 37 °C in the dark. After 10 min, spermatozoa were fixed with 1 μL of 3% glutaraldehyde and 6 μL of this solution were placed on a slide. A coverslip was applied, and stained spermatozoa were examined using an Olympus BX51 epifluorescent microscope (Olympus Italy, Milan, Italy) at 400 x magnification. Spermatozoa with bright green fluorescence (SYBR-14) were considered to have membrane integrity, whereas those partially or totally red (PI) were considered to have membrane damage. Membrane integrity was calculated on at least 200 spermatozoa for each sample.

Acrosome integrity was evaluated using lectin from Arachis hypogaea (PNA) conjugated with Alexa Fluor 488 (L21409, Thermo Fisher Scientific, Molecular Probes Europe, Leiden, The Netherlands), using an epifluorescence microscope, as previously described (Standerholen et al., 2014) with some modifications. In brief, the sample was diluted to the concentration of $10 \times 10^6$ sperm/mL with BTS and stain was added at the final concentration of 50 μg/mL. Semen was incubated in the dark at room temperature for 15 min A 10-μL drop was transferred onto a slide, covered with a 22 x 22 mm coverslip and evaluated using an epifluorescent microscope (Olympus BX51) at 400 x magnification. A bright green fluorescence of the head of the sperm was interpreted as a cell in which there had been an acrosome reaction, and spermatozoa without fluorescence were considered to have acrosome integrity. The acrosome integrity was calculated as percentage on at least 200 spermatozoa.

For morphology evaluation, aliquots of semen were fixed in 2% formol-buffered saline solution (Hancock, 1957) and were examined using a phase-contrast microscope at 1000 x magnification. The morphological abnormalities were estimated on at least 200 spermatozoa (Malmgren, 1997).

The lipid peroxidation was estimated by the measurement of the malondialdehyde (MDA) of spermatozoa in fresh semen to estimate the extent of lipid peroxidation soon after ejaculation. During storage, the lipid peroxidation was measured on aliquots of 3 mL of diluted semen with MDA being the marker compound for extent of semen lipid peroxidation. For assessment of the extent of

lipid peroxidation, an analysis was performed as previously described with modifications (Grotta et al., 2017). After thawing the sample, 500 µl of a pellet from fresh or 3 mL of stored boar semen was homogenized (Ultra-Turrax T-25) with 2.5 mL of 10% trichloroacetic acid and centrifuged for 10 min at 3000 x *g* at room temperature. Subsequently, 1.5 mL of the sample was mixed with 1.5 mL of 2-thiobarbituric acid (TBA) in a glass tube and placed into a boiling water bath at 95 °C for 45 min and cooled in an ice bath. The absorbance was read at 534 nm with a Jenway 6305 UV/vis Spectrophotometer (Barloworld Scientific, Milano, Italy). The standard calibration curve was developed using a stock solution (5 µg/mL) of the standard solution (STD) of MDA in methanol, taking 0.25, 0.5, 0.75, 1, 2 and 4 mL of stock solution that corresponded to 1.25, 2.5, 3.75, 5, 10 and 20 µg, respectively. The assay was conducted in duplicate, and reported as µmol/L.

### 2.5. Statistical analysis

Data are presented as mean ± standard deviation (SD). The normal distribution of the data recorded in this study was tested using the Shapiro-Wilk test. Only the kinetic variables (VAP, VSL, VCL, ALH, BCF, STR, and LIN) were not normally distributed, and required a log-transformation of the data for the statistical analysis.

The effect of the percentage GM dietary supplementation on semen quality was evaluated using a repeated measure General Linear Model (rGLM), based on the analysis of variance (ANOVA). In the rGLM model, the GM dietary supplementation percentage (before supplementation, WO, 2% GM, and 4% GM), and the time of liquid preservation (T0, T3, T5, T9, and T15), were used as fixed factors. Possible correlations between the values for these variables at the same time interval were estimated by calculation of the Pearson's correlation coefficient.

For all the analyses, the level of significance was set at $P < 0.05$. The statistical evaluations were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Effects of the GM dietary integration on fresh boar semen characteristics

The mean semen volume and concentration in the boars were similar before the supplementation (228 ± 84 mL; 329 ± 84 × $10^6$ sperm/mL), at 2% GM (269 ± 86 mL; 270 ± 57 × $10^6$ sperm/mL), during the WO (232 ± 78 mL; 321 ± 79 × $10^6$ sperm/mL), and at 4% GM (230 ± 74 mL; 331 ± 93 × $10^6$ sperm/mL), ($P > 0.05$). As a consequence, the total number of sperm/ejaculate was similar before and after GM dietary supplementation.

Sperm variables before and after dietary supplementation of GM are summarized in Table 2. Sperm TM and PM were greater after GM dietary supplementation at both 2% and 4% ($P < 0.05$). Sperm velocity variables (VAP, VSL, and VCL), such as ALH, were greater when there was 4% GM dietary supplementation ($P < 0.05$). Beat cross frequency and STR were greater when there were 2% and 4% dietary supplementations ($P < 0.05$), compared with the values before the supplementation and the WO ($P > 0.05$). Sperm MI in fresh semen increased after GM dietary supplementation, but there were only significant differences between the pre-supplementation values and those recorded in the WO, compared with 4% GM ($P < 0.05$). Acrosome integrity was, however, similar before supplementation, WO, and after dietary supplementation of GM (2%, and 4%). The percentage of normal sperm morphology

**Table 2**
Seminal variables of fresh semen before the dietary supplementation (Pre-supplementation) with 2% GM, during the wash out, and with dietary supplementation at 4% GM.

| | Pre-supplementation Mean ± SD | 2% GM Mean ± SD | Wash out Mean ± SD | 4% GM Mean ± SD |
|---|---|---|---|---|
| TM (%) | 78.3 ± 7.3$^{a}$ | 91.1 ± 8.2$^{b}$ | 80.2 ± 6.4$^{a}$ | 96.1 ± 4.2$^{b}$ |
| PM (%) | 42.6 ± 18.4$^{a}$ | 54.2 ± 16.7$^{ab}$ | 45.7 ± 15.6$^{a}$ | 64.4 ± 14.2$^{b}$ |
| VAP (µm/s) | 60.6 ± 11.2$^{a}$ | 61.7 ± 10.3$^{ab}$ | 61.9 ± 9.2$^{a}$ | 72.3 ± 10.7$^{b}$ |
| VSL (µm/s) | 34.9 ± 7.8$^{a}$ | 44 ± 7$^{ab}$ | 37.9 ± 6.9$^{a}$ | 51 ± 7.6$^{b}$ |
| VCL (µm/s) | 99.3 ± 20.3$^{a}$ | 119.3 ± 15.5$^{ab}$ | 102.8 ± 18.6$^{a}$ | 138.5 ± 17.9$^{b}$ |
| ALH (µm) | 6 ± 1.1$^{a}$ | 6.7 ± .6$^{ab}$ | 5.6 ± .8$^{a}$ | 7.4 ± .6$^{b}$ |
| BCF (Hz) | 32.8 ± 4.8$^{a}$ | 41.2 ± 1.6$^{b}$ | 34 ± 2.9$^{a}$ | 37.4 ± 1.4$^{b}$ |
| STR (%) | 60.4 ± 7.9$^{a}$ | 70.8 ± 3.6$^{b}$ | 59.7 ± 6.4$^{a}$ | 69.6 ± 3.3$^{b}$ |
| LIN (%) | 34.7 ± 4.6$^{a}$ | 38.4 ± 3.8$^{a}$ | 34.4 ± 2.9$^{a}$ | 38.1 ± 3.3$^{a}$ |
| Rapid sperm (%) | 54.9 ± 11.2$^{a}$ | 70.8 ± 9.2$^{b}$ | 58.2 ± 10.3$^{a}$ | 78.1 ± 9.8$^{b}$ |
| MI (%) | 80.1 ± 8.8$^{a}$ | 88.1 ± 6.8$^{ab}$ | 81.3 ± 5.8$^{a}$ | 93.2 ± 2.7$^{b}$ |
| AI (%) | 92.6 ± 6.7$^{a}$ | 94.1 ± 5.9$^{ab}$ | 90.3 ± 6.9$^{a}$ | 95.8 ± 3.6$^{b}$ |
| Abn (%) | 20.8 ± 20.2$^{a}$ | 16 ± 17.8$^{ab}$ | 21.7 ± 15.4$^{a}$ | 5.4 ± 5.3$^{b}$ |
| MDA (µmol/L) | 45.6 ± 8.2$^{A}$ | 35.7 ± 3.7$^{B}$ | 42.1 ± 5.1$^{A}$ | 33.3 ± 3.1$^{B}$ |

In the same row, the differences between values with capital letters in superscript (A/B) are highly significant ($P < 0.01$), those with lowercase letters in superscript (a/b) are significant ($P < 0.05$).
Total motility (TM); progressive motility (PM); average path velocity (VAP); straight line velocity (VSL); curvilinear velocity (VCL); amplitude of lateral head displacement (ALH); beat cross frequency (BCF); straightness (STR); linearity (LIN); membrane integrity (MI); acrosome integrity (AI); abnormal sperm (Abn), malondialdehyde (MDA).

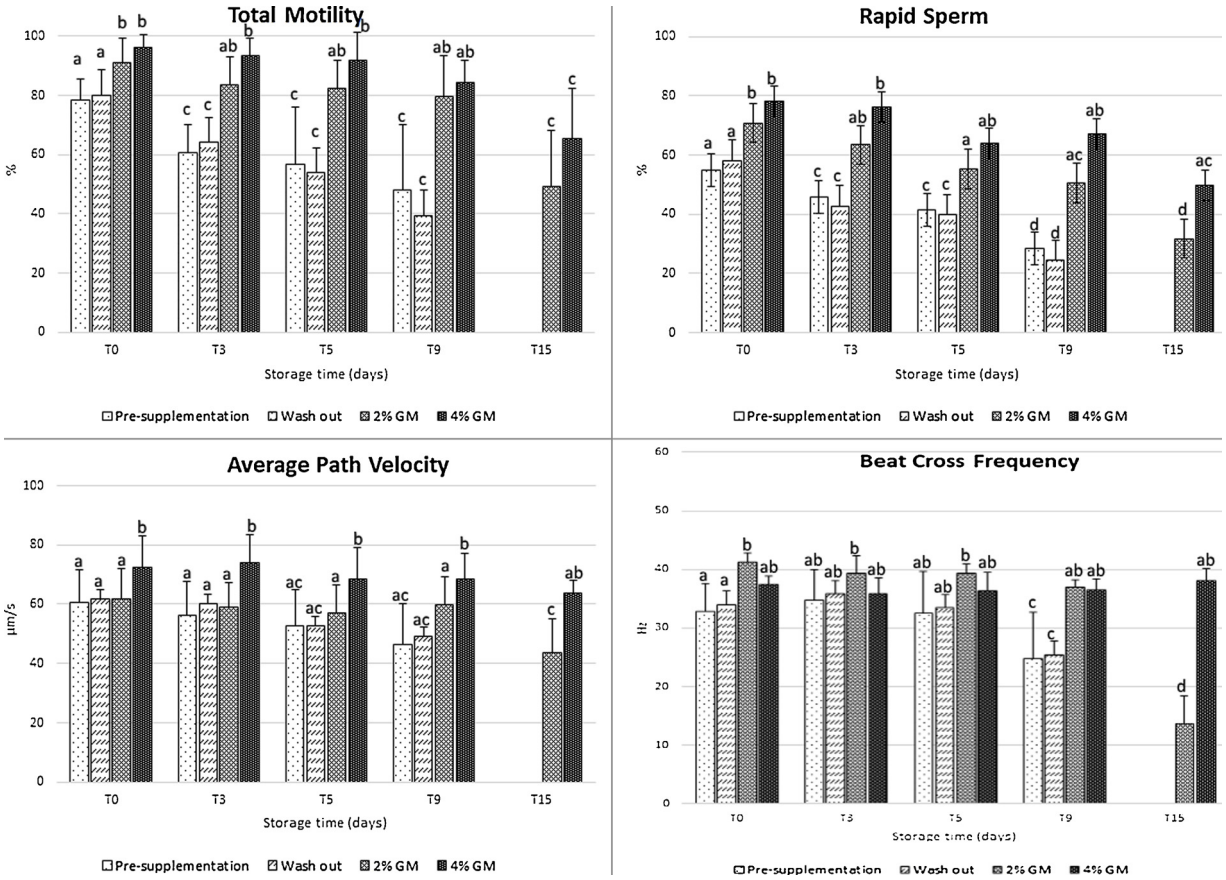


**Fig. 2.** Histograms of the total motility, percentage of rapid sperm, average path velocity, beat cross frequency during storage at 17 °C in boars before the supplementation (pre−supplementation), during the wash out, and after dietary supplementation of 2% and 4% grape marc (GM); Different letters in superscript corresponded to differences (P < 0.05).

was affected by GM dietary supplementation, with a significant reduction of sperm abnormalities after supplementation of 4% GM ($P < 0.05$), but not after 2% GM supplementation ($P > 0.05$).

Lipid peroxidation, measured using MDA as the marker molecule in the pellet, was less in samples after GM supplementation, independent of percentage GM supplementation in the diet ($P < 0.01$).

In samples evaluated at T0, there were positive and significant correlations between TM and membrane integrity ($R = 0.851$; $P < 0.01$), and values for all the kinetic variables ($P < 0.01$). Percentage of abnormal sperm morphologies and the values for amount of lipid peroxidation were also significantly correlated ($R = 0.533$; $P < 0.05$). There were negative correlations between the percentages of abnormal morphologies and values for TM ($R = 0.570$; $P < 0.05$), and MI ($R = 0.600$; $P < 0.05$).

### *3.2. Effects of the GM dietary supplementation on boar semen characteristics during liquid storage*

During liquid storage, sperm motility and kinetic variables were markedly affected by the dietary supplementation of GM. Without supplementation, diluted semen had a progressive decrease in TM and PM, with a significant difference between values at T0 and T3 ($P < 0.05$). For the Rapid sperm variable, there was a trend with a significant difference between T0 and T3, T5, and T9 ($P < 0.05$; Fig. 2). For most of the kinetic variables, the decrease was significant at T5 (VAP, VSL, VCL, ALH, STR, LIN), or T9 (BCF). After dietary supplementation of 2% and 4% GM, TM, such as the values for the Rapid sperm variable there were similar at the different time points. Progressive motility was, however, less at T3 compared to T0 with 2% GM dietary supplementation ($P < 0.05$), but not in samples when there was 4% GM dietary supplementation, where values were comparable until T9. The percentage of dietary supplementation of GM had an effect because at different time-points the TM (Fig. 2), PM, and Rapid sperm (Fig. 2) were greater in samples with 4% GM dietary supplementation ($P < 0.05$). In samples from animals where there was dietary supplementation of GM, values for VAP (Fig. 2), VSL, and VCL were greater in samples with 4% compared to 2% supplementation at the different time points ($P < 0.05$), while values for ALH, BCF (Fig. 2), STR, and LIN in stored boar semen were similar between T0 and T9, without there being differences when there was supplementation of 2% and 4% GM ($P > 0.05$). In pre-supplementation and wash out samples, there was no spermatozoa motility at T15.

During liquid storage, the values for MI were of the same trend as the values for TM. The percentage of MI was greater in the

samples after 2% and 4% GM dietary supplementation compared to values before the supplementation or during the WO at T0, T3, T5, and T9 ($P < 0.05$). The sperm acrosome integrity appeared to be less sensitive to the GM supplementation and to the effect of liquid storage. The values were similar for all treatment groups until T5. A small but significant increase in acrosome integrity was detected at T9 and T15 independent of GM treatment ($P < 0.05$; data not shown).

There were differences in the amount of MDA in the refrigerated samples before and after dietary supplementation of GM, but these were not significant ($P > 0.05$).

## 4. Discussion

### 4.1. Effects of GM dietary supplementation on fresh boar semen characteristics

In the present study the dietary supplementation with grape marc had negligible effects on sperm volume, concentration, and total sperm/ejaculate. In a previous study with rabbits (Eid, 2008) the dietary supplementation with 10% and 20% GM there was a greater sperm concentration and semen volume. These differences in the effect of the supplementation with GM may be related to the amount of GM supplementation in diets because these were greater in the previous study. Zhao et al. (2017) reported that there was an increase in the spermatozoa recovered from the ram epididymis when GM was supplemented at 10%, but no differences were reported regarding this variable with 5% GM supplementation in the diet.

Data from the present study were, however, consistent with those in previous studies in which antioxidants such as selenium was supplemented in the diet of boars (Lovercamp et al., 2013; Petrujkic et al., 2014), but are not consistent with the findings in another study, in which a selenium dietary supplementation improved sperm concentration (Lopez et al., 2010). Strzezek et al. (2004) reported an increase in the volume and total sperm/ejaculate after boar supplementation with polyunsatured fatty acids and antioxidants. These differences among studies could be related to the different substances supplemented in the diet, which could have a different effect on testicular function or accessory sexual gland secretions.

Although GM supplementation had a negligible effect on sperm quantity, the results reported in the present study indicate the supplementation of GM in the diet improved boar sperm quality. Although sperm characteristics without GM supplementation were comparable with those previously reported (Lovercamp et al., 2013; Schulze et al., 2013), the supplementation of GM in the diet in the present appeared to result in a dose dependent response for most of the sperm variables assessed. There was an obvious effect of GM with 4% dietary supplementation on membrane integrity, abnormal morphology, and kinetic variables which were greater for the semen of all the boars. When there was GM dietary supplementation at 2%, there was a trend in the improvement of the seminal variables, but this improvement was not the same for all boars. These findings indicate that there is an individual difference between testicular status, or a different sensitivity of the boar to the polyphenol dietary supplementation.

Different from the sperm characteristics, the lipid peroxidation, measured on the basis of the MDA concentration, was markedly reduced after dietary supplementation of GM, independent of percentage dietary supplementation. These data confirmed the findings previously reported in the rabbit, in which there was a reduction in the MDA concentration in seminal plasma after there was supplementation of grape pomace in the diet (Eid, 2008). The reduction of the MDA concentration, as a result of decreased ROS production, appeared parallel to the decrease in sperm abnormalities, as corroborated by the significant correlations between the values for these variables. These data are consistent with those of a previous study where it was reported that there was a significant positive correlation between sperm abnormalities and ROS concentrations (Kobayashi and Suda, 2012).

The effect of the GM on improvement of seminal characteristics could be related to the large content of polyphenols. Phenolic compounds are widely present in the whole residue of vinification because only 30%–40% of these components are extracted (Ky et al., 2014). The main polyphenolic components present in the grape seeds and skins are flavonoids, such as anthocyanins or quercitin, stilbenes, such as resveratrol, and tannins (Beres et al., 2017). Polyphenols have antioxidant, anti-inflammation, antiaging, and antimicrobial properties (Xia et al., 2010). Quercetin was found to protect human spermatozoa against lipid peroxidation damage (Moretti et al., 2012), and to reduce the hydrogen peroxide in rabbit sperm stored at 15 °C (Johinke et al., 2014). In mice, resveratrol was found to have a protective effect on sperm motility, viability, and mitochondrial membrane potential, with a lesser amount of ROS and lipid peroxidation after ferrous iron/ascorbate-induced oxidative damage (Mojica-Villegas et al., 2014). Consistent with these findings, the data reported in the present study corroborate the role of different polyphenols on testicular function, suggesting that an increase in polyphenol intake in the diet could lead to improved spermatogenesis and functions of ejaculated spermatozoa. In addition to the antioxidant effect of the polyphenols present in the GM, a further mechanism of action could be related to the anti-inflammatory effects of GM (Panico et al., 2006; Chacona et al., 2009; Terra et al., 2009).

The effects of dietary GM supplementation on health were studied in humans and rodents (Martin-Carron et al., 2000; Auger et al., 2004; Xia et al., 2010; Landete, 2012), in the pig intestinal tract (Gessner et al., 2013; Fiesel et al., 2014), and in avian species (Viveros et al., 2011; Chamorro et al., 2013; Starcevic et al., 2015; Yang et al., 2017). The dietary supplementation with GM received limited attention in animal reproduction. In a previous study with rabbits, the dietary supplementation of GM at 10% and 20% resulted in an increased seminal volume and the sperm concentration, with an increase in sperm membrane integrity and motility (Eid, 2008). These data are consistent with findings in the present study regarding the quality of spermatozoa. Zhao et al. (2017) reported an increase of the sperm epididymal total motility and normal spermatozoa with GM dietary supplementation at 5% and 10% in lambs.

### 4.2. Effects of GM dietary supplementation on boar semen characteristics during liquid storage

In the present study, the supplementation of GM in boar diets resulted in effects on sperm characteristics soon after semen dilution. Johnson et al. (2000) suggested a threshold of 60% motility to achieve an optimal fertility with boars. In the present study, the values for this variable were greater than the threshold value until T3 for 0% GM, until T9 for the 2% GM and until T15 for the 4% GM dietary supplementation. In a previous study comparing different extenders for boar semen storage, TM of samples extended with BTS was less at 72 h of storage, similar to when semen was stored in similar to conditions as those imposed in the present study without dietary GM supplementation. The sperm velocities in 0% GM samples diluted with BTS were also consistent with those reported in a previous study (De Ambrogi et al., 2006), in which semen was evaluated for kinetic variables until 96 h of storage. Sperm TM and velocities were, however, greater in samples collected after 2% and 4% GM dietary supplementation, indicating the increase in the concentrations of polyphenols had a positive effect on the function and possibly metabolism of spermatozoa.

During storage, there is a progressive reduction in boar semen quality, mainly due to the changes in the microenvironment, sperm ageing, and effects of the reduced temperature on the sperm's outer and inner membranes (Waberski et al., 2011). There are previous reports that when there is the combination of large amounts of ROS, and limited numbers of antioxidant molecules there is lesser sperm motility (Baumber et al., 2003; Kumaresan et al., 2009). In several studies, there was supplementation of extenders with antioxidants with variable results. Cerolini et al. reported that the supplementation of α-tocopherol (0.2 mg/ml) in the BTS medium improved sperm viability and reduced lipid peroxidation of boar semen stored for 5 days (Cerolini et al., 2001). Basioura et al. reported a significant protective effect of astaxanthin (0.5 μmol/L), a potent natural antioxidant, on boar semen stored for 48 h (Basioura et al., 2018). The addition of taurine (5 mmol/L) in Modena extender had protective effects during storage of boar spermatozoa at 17 °C for 6 days (Li et al., 2017). In all these studies, the molecules added to the extenders could only protect spermatozoa after ejaculation, without effects on testicular and epididymal tissues. Dietary supplementation was proposed as a different way to increase semen quality during boar liquid storage (Strzezek et al., 2004; Lopez et al., 2010; Speight et al., 2012; Lovercamp et al., 2013). Part of this improvement in boar semen quality in the present study could be related to the activity of GM, possibly as a result of its polyphenolic components, on the quality of ejaculated spermatozoa, as indicated by the values for fresh samples. The effects during storage could also be related, however, to an increase in the antioxidant capacity of the seminal plasma, as was previously reported (Eid, 2008). This improvement of the seminal plasma antioxidant capacity could be the result of the presence of polyphenols in the sexual accessory gland secretions, even when there is dilution of the raw semen during storage that would result in lesser concentrations of the bio-active components. Although a possible role of the antioxidant properties of the polyphenols from the GM, the amounts of MDA in the diluted samples, measured until day 5 of storage were not significantly different. This finding could be explained by the reduction in the metabolic capacity of the spermatozoa in the samples from boars when there was not dietary GM supplementation as evidenced by the reduced motility detected at days 3 and 5 in samples before the GM treatment or during the wash out. It was proposed that the major source of peroxidation is the mitochondrial activity (Koppers et al., 2008), thus it is likely that the reduction of the sperm with active metabolism resulted in reduced ROS generation in the system which would negate the effects of the decreased antioxidant capacity in the same samples.

In addition to the antioxidant capacity of the polyphenols, the long-term effects on semen could be related to the antibacterial effects of these substances (Papadopoulou et al., 2005; Baydar et al., 2006), but this hypothesized activity would have to be ascertained in specific studies designed to evaluate antibacterial effects.

## 5. Conclusions

The results of the present study indicate that the supplementation of GM in boar diets improved fresh semen characteristics, as evidenced by the greater values for the kinetic variables and the membrane integrity, and by the reduced amount of sperm abnormalities and reduced lipid peroxidation. This outcome is likely due to both the effect of the polyphenolic content on spermatogenesis and epididymal maturation, and on seminal plasma composition with the result being spermatozoa with greater integrity and function, with a reduced sensitivity of spermatozoa to the detrimental effects of storage. Further studies are required to clarify the specific action mechanisms of the polyphenols present in the GM.

## Funding

GA is a postdoctoral fellow supported by a grant from Rural Development Plan 2007–2013 – MISURA 1.2.4 - Regione Abruzzo - Italy. This work is part of the Project "Miglioramento delle carni bovine, suine e avicole attraverso l'utilizzo di sottoprodotti della filiera enologica per fini mangimistici", supported by a grant from Rural Development Plan 2007–2013 – MISURA 1.2.4 - Regione Abruzzo - Italy.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgments

The authors are grateful to the Azienda Agricola Di Mascio Pierluigi for their cooperation. The authors also acknowledge Dr.

Abigail Rose Trachtman for the kind language revision of the manuscript.

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