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Abstract

The purpose of this study was to investigate the effects of ram melatonin treatment on the sperm quality and metabolite composition of the seminal plasma in the non-breeding season. Four mature rams were treated with 54 mg melatonin in March subcutaneous implants and four untreated rams served as control. At 0, 30, 90 and 120 days semen samples were collected and sperm, separated from seminal plasma, was evaluated for its capacity to fertilize and produce embryos in vitro. Seminal plasma me- tabolites were extracted and analyzed by capillary electrophoresis/mass spectroscopy. In the resulting electropherograms, the area corresponding to selected metabolites was extracted and quantified. Ram melatonin treatment affected the in vitro fertilization competence of sperm. Blastocyst output increased until 90 days after treatment (27.20 ± 7.35 vs 54.7 ± 4.4% at 0 and 90 days respectively; $p < 0.05$) while the untreated group did not show statistical differences.

In treated rams, the concentration of melatonin in seminal plasma increased from 3.34 ± 1.70 at day 0 e9.65 ± 2.89 AU (Arbitrary Units) after 90 days, then decreased to reach the level of the untreated ram after 120 days ($p < 0.05$). During 90 days after melatonin treatment, an increase ($p < 0.05$) in seminal plasma concentrations of glutamic acid (6.28 ± 1.53 vs 14.93 ± 1.53 AU at 0 and 90 days respectively), glutamine (16.89 ± 4.65 vs 54.51 ± 4.65 AU), carnitine (22.97 ± 9.81 vs 104.30 ± 9.81 AU), acetyl-carnitine (48.15 ± 17.32 vs 217.69 ± 17.32 AU), choline (1.82 ± 1.55 vs 14.16 ± 1.55 AU) and arginine (1.31 ± 1.08 vs 14.25 ± 1.08 AU) was detected. Tyrosine concentration increased during 30 days from melatonin treat- ment (12.79 ± 3.93 vs 27.08 ± 3.04 AU) but at 90 days its levels were similar to the untreated group. In conclusion, melatonin treatment during the non-breeding season improves the concentration of several metabolites in seminal plasma and sperm fertilization competence in Sarda breed ram.

1. Introduction

Melatonin, an indoleamine synthesized by the pineal gland, plays important roles in several fields of physiology, e.g. in the nervous system, antioxidant defense mechanism, immune system, gastrointestinal tract and reproductive system, as has been largely reviewed [1,2]. A positive relationship between the daily light cycle and the characteristics of the sperm and its fertility potential [3,4] was highlighted. In contrast, no correlation has been found be- tween sperm concentration, motility or morphology, and mela- tonin, testosterone and estradiol levels in men's blood or seminal plasma [5]. Addition of 2 mM melatonin to human sperm signifi- cantly improved the percentage of motile, progressive motile and rapid sperm, decreasing the intracellular nitric oxide concentration but not reactive oxygen

species (ROS) [6]. Previous findings have reported that the administration of melatonin to Wistar rats negatively influences motility, morphology, and epididymal sperm concentration [7]. On the contrary, it has been recently showed that melatonin treatment does not affect the motility of Wistar rat sperm [8].

Melatonin implants significantly increased the activity of anti- oxidant enzymes in seminal plasma during the non-reproductive season [9] and positively influenced the percentage of progressive motile sperm in Rasa Aragonesa breed ram. In vitro treatment with melatonin did not affect the kinematic parameters of sperm but increased the fertilization rate of oocytes following IVF.

Treatment with melatonin has been shown to increase the viability, motility, intracellular ATP concentrations and DNA integ- rity of Sarda breed sperm during the non-reproductive season [10]. This contradictory effect of melatonin could be determined by the difference in sensitivity to seasonality, the duration of treatment and the concentration of hormones [3]. To improve performance during the non-breeding season, slow-release melatonin implants have been widely used to control the reproduction of small rumi- nants for over thirty years both in highly seasonal [11] and Medi- terranean [12] ewes, including the Sarda breed [13]. Commercial melatonin implants have been designed to maintain high levels of the pineal hormone for 40e70 days [14], although they may release melatonin for more than 100 days [15]. However, several issues related to their mode of action are not yet clear [12].

In the ram, although sperm production continues throughout the year, during the non-breeding season the quality of the sperm is low [16] and is associated with a reduction in the diameter of the testis [17] and of the hormonal profiles [12]. It has been reported that melatonin treatment of rams of different breeds during the non-breeding season increases sperm quality and its fertility po- tential, modulate hormonal interactions and modifies the biochemical composition of the seminal plasma [16e19]. This indolamine interacts with the hypothalamus-pituitary axis which induces an increase in the pulsatile secretion of gonadotropin- releasing hormone (GnRH), low levels of prolactin and increases in luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone secretion [20] and the improvement of the quality of fresh and frozen semen in different breeds during the non- breeding season [10,18,19,21].

Melatonin implants influence sperm capacitation by modifying the activity of the plasminogen activator (PA) and acrosin [22,23] in ram sperm and seminal plasma. These enzymes are essential for sperm capacitation [24] and, whereas PA seems related to testos- terone concentration [23], acrosin activity is independent of the fluctuations of this hormone and may be the result of a direct action of melatonin on sperm cells.

The seminal plasma is a complex fluid secreted from rete testis, epididymis, seminal vesicles, prostate and bulb-urethral glands [25]. It provides metabolic support, as an energy source for the sperm cells, and influences the functionality of the sperm [26]. Seminal plasma metabolites are involved in different sperm fea- tures related to sperm function, fertilization and embryo develop- ment in the female reproductive tract [27e29]. Mammalian testis secretes considerable amounts of amino acids (200 mmoles/day) into the seminal plasma [30] that play a key role in defining the sperm quality. Free metabolites of seminal plasma, including choline, carnitine, acetyl-carnitine, arginine, tyrosine, are involved in several metabolic ways increasing sperm fertility potential [31]. The concentration of seminal plasma metabolites gives several positive actions on the sperm developing the prevention of lipid peroxidation [31], supporting the transport of fatty acids into the mitochondria for b-oxidation [32], promoting ATP synthesis [33], providing ready energy [34], protecting against oxidative damage [35] and repairing ROS insults [35].

Treatment with melatonin has been shown to modify the composition of seminal plasma [5,9,18,36,37]. However, studies evaluating the effect of melatonin administration in the non- breeding season on sperm quality and on the composition of seminal plasma metabolites are scarce.

In the present study, the impact of melatonin implantation in ram during the non-breeding season on the sperm fertilization capacity as index of fertility potential of ram sperm and on the composition of the seminal plasma metabolites season were investigated.

2. Materials and methods

2.1. Reagents and animals

All reagents and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

The experimental procedures with Sarda breed ram (*Ovis aries*) were approved by the Animal Care and Use

Committee of the University of Sassari, Italy. All procedures were carried out at the experimental facilities of the Regional Research Centre (AGRIS) of Sassari, Italy (latitude $40^0 40^0$ N) from March to June during the non- breeding season, described for this breed at this latitude from late January to late March [13]. These facilities meet the requirements of the European Union for Scientific Procedure Establishments. This study followed ethical guidelines for care and use of agricultural animals for research (EC Directive 86/609/EEC for animal experiments).

2.2. *Experimental design*

Melatonin implants maintain high serum levels of melatonin for 40e70 days [11] but release the hormone for about 100 days [15]. Four times were selected for semen collection to assess whether melatonin treatment affects seminal plasma composition or sperm fertilization competence, and how long its effect is detectable. We chose 30 days after treatment to assess whether this affects the concentration of melatonin and metabolites studied in the seminal plasma and the fertility potential of sperm in the short time, while 90 and 120 days were chosen to assess whether the effect on the analyzed factors lasts when the release of melatonin is minimal. The semen of melatonin-treated ram trained to artificial vagina was collected after 0, 30, 90 and 120 days from treatment.

To evaluate whether melatonin affects metabolite seminal plasma composition and sperm fertility competence, seminal plasma was separated from sperm and treated for analysis. Choline, glutamine, glutamic acid, histidine, carnitine, arginine, tyrosine, acetyl-carnitine and melatonin were extracted from seminal plasma and quantified by Capillary electrophoresis/mass spectrometry.

To estimate sperm fertilization capacity, in vitro matured sheep oocytes were fertilized with separated sperm and development to the 2-cell at 30 h and blastocyst stages at 7 days were detected.

2.3. *Semen collection*

Eight adult Sarda breed rams aged 3e5 years-were maintained in an outdoor environment and received 400 g of commercial concentrate feed per head (crude protein 20.4% and 12.5 MJ ME/kg DM), divided into two times of the day (morning and evening), water and hay (crude protein 11.1% and 7.2 MJ ME/kg DM) ad libi- tum. Rams, randomly divided into two groups, were kept in sepa- rated pens, in visual contact with each other. The treated group, consisting of 4 rams which received three contemporary subcu- taneous melatonin implants (Melovine, CEVA VETEM S.P.A., Milano, Italy: 18 mg melatonin each implant, 54mg/treatment) at the base of the ear [21,36]; four rams were left untreated as a control group. Ejaculates were obtained from each ram by a 34 ^{0}C thermo- stated artificial vagina on days 0, 30, 90 and 120 following treat- ment. Ejaculates were transported to the laboratory at a temperature between 13 and 18°C, within 5 min after collection and immediately treated. The seminal plasma was separated from the sperm by centrifugation (Microfuge, Eppendorf, Hamburg,Germany) at 10 000 g for 10 min at 4° C. The supernatant was again centrifuged under the same conditions and the seminalplasma was recovered, immediately aliquoted and kept at 80°C until analysis.

2.4. *Assessment of seminal plasma metabolites*

To determine differences in seminal plasma composition be- tween treated and untreated rams, metabolites were extracted from seminal plasma according to the method described by Cap- piello et al. [38] and were analyzed by capillary electrophoresis/ mass spectroscopy (CE/MS). CE apparatus from Agilent Technolo- gies (Waldbronn, Germany), equipped with a diode array UV de- tector and external nitrogen pressure. The CE apparatus was coupled with the Agilent mass spectrometer (Agilent Technologies, Waldbronn, Germany) via a coaxial sheath liquid ESI interface (Agilent Technologies, Waldbronn, Germany). The sheath liquid was delivered by an external syringe pump (Agilent Technologies, Waldbronn, Germany) at a constant flow rate of 180 mL/h. Nebu- lizing and drying gas (nitrogen) were set at 8.0 psi and 5.0 L/min, respectively. The dry gas temperature was 250 ^{0}C. The MS capillary voltage was 4500 V. Separations were performed in 50 mm id, 375 mm od fused silica uncoated capillaries (Composite Metal Services, Hallow, Worcester, UK) of a total length of 79.5 cm. The effective length was 21.5 cm for UV detection and 79.5 cm for MS detection. The acquisition of the Tandem mass spectrometry (MS^2- ms/ms) extracted ion current (EIC) signals was made in 50e220 *m/z* mass range using a maximum accumulation time of 200 ms and a set target value of 20 000 by activating the ion charge control function. The temperature of the CE assembly cartridge was set at 25 ^{0}C. The CE running voltage was 23 kV

(positive polarity). Sam- ples were injected at the anodic end at 50 mbar-10 or 20 s followed by the background electrolyte (BGE) injection at 50 mbar-15 s. Formic acid at 1.0 or 1.5 M concentration was used as BGE. After CE separation, metabolites were ionized in the ESI sprayer of the mass spectrometer in positive mode. Ionization occurs via protonation and the detected ionic species are metabolite protonated molecules (M H)þ [39]. The ion molecular masses corresponding to each molecular species are reported in Table 1. Each metabolite was validated by fragmentation (ms/ms) where a transition between the molecular ion and analyte-specific *m/z* fragments can be observed (Table 1). Fragmentation data were searched and vali- dated in a public ms/ms metabolite database (Metlin, https:// metlin.scripps.edu).

Water/methanol 30:70 v/v mixtures containing formic acid in the range of 0.1e3% v/v final content were used as sheath liquid solutions. The BGE and sheath liquid solutions were prepared daily. New capillaries were conditioned using the following procedure: (i) water (5 bar-2 min); (ii) 0.1 M sodium hydroxide (5 bar-20 min);

(iii) water (5 bar-5 min). At the beginning of each working day, the capillary was rinsed with water (6 bar-1 min), 0.1 M sodium hy- droxide (6 bar-2 min) and water (6 bar-2 min). For every four runs, the capillary was rinsed with a shorter procedure: water (6 bar- 0.7min), 0.1 M sodium hydroxide (6 bar-0.7 min) and water (6 bar-1.0 min). To validate the assessed metabolites we performed a ms/ms fragmentation of picks corresponding to the molecular weight of each of them (Table 1). Electropherograms corresponding to the molecular mass of choline, glutamine, glutamic acid, histi- dine, carnitine, arginine, tyrosine, acetyl-carnitine and melatonin were extracted and peak areas were quantified.

2.5. *Assessment of fertility potential*

The sperm fertility potential was assessed by determining the development of fertilized oocytes at the cleavage and blastocyst stages. Cleavage rate was calculated on the fertilized oocytes and blastocyst rate on cleaved oocytes.

The ovaries were recovered at the local slaughterhouse, placed in Dulbecco's PBS at a temperature between 25 and 35 ^{0}C, and used for in vitro production as routinely made in our laboratory. The antral follicles in the ovarian surface were sliced in TCM199 (with Earle's salts and bicarbonate) supplemented with 25 mM Hepes, penicillin and streptomycin and 0.1% (w/v) of PVA. The cumulus- oocyte complexes (COCs) consisting of 4e10 layers of granulosa cells and oocytes with a uniform cytoplasm, homogenous distri- bution of lipid droplets in the cytoplasm and with an outer diam- eter of about 90 mm (mean) were selected for the experimental procedure. The selected COCs, after three washes in the same fresh medium, were matured in TCM199, supplemented with a 10% heat- treated estrous sheep serum, 10 m$props_value{literPattern}/mL of FSH/LH (Pergonal, Serono, Rome, Italy), 100 mM of cysteamine and 8 mg/mL of pyruvate. The COCs were placed in groups of 30e35, in 600 mL of the maturation medium in a four-well Petri dish (Nun- clon, Nalgene Nunc International, Denmark), layered with 300 mL mineral oil and cultured for 24 h in 5% CO2 in air at 39 ^{0}C [40]. After maturation, the COCs were partially stripped of the granulosa cells, divided into six random groups (40e45 oocytes per group; 4 rep- licates) and fertilized in vitro. The fertilization medium consisted of synthetic oviduct fluid (SOF) supplemented with 2% of heat-treated estrous sheep serum (v/v), 50 mg/mL streptomycin and 50 IU/mL penicillin. For in vitro fertilization, aliquots of sperm obtained from each ejaculate were pooled and diluted in equilibrated SOF at a 10^6 sperm/mL final concentration and co-incubated with matured oocytes, in mineral oil in four-well Petri dishes, in 5% CO2, 5% O2 and 90% N2 atmosphere at 39 ^{0}C [40]. After 22 h, presump- tive zygotes were washed and cultured at 39 ^{0}C in four-well Petri dishes in 400 mL SOF, containing essential and nonessential amino acids at oviduct concentration and 0.4% BSA in mineral oil, in a maximum humidified atmosphere with 5% CO2, 5% O2, and 90% N2 up to expanded blastocyst stage [41].

Table 1

MS/MS data used to identify metabolites assayed in the seminal plasma of rams treated with melatonin and control. The metabolites separated by CE have been identified in relation to the molecular mass and to the characteristic transition ions(*m/z*) obtained by fragmentation (MS/MS).

Compounds	Molecular Mass (M + H)þ	MS/MS Transitions (*m/z*)
Arginine	175	175 -> 70.4 þ 116.0 þ157.0
Carnitine	162	162 -> 60.4 þ 85.2 þ 103

Acetyl-carnitine	204	204 -> 60.4 þ 85.1 þ 145
Melatonin	233	233 -> 174
Tyrosine	182	182 ->163
Glutamine	147	147 -> 130
Glutamic Acid	148	148 -> 130
Histidine	156	156 -> 110
Choline	104	104 -> 60

2.6. *Statistical analyses*

Assessment of plasma metabolites and sperm fertility potential from each ejaculate were made in triplicate. Data were analyzed by an Analysis of variance (ANOVA) test after analysis for homogeneity

of variance by Levene's test. Statistical analysis was performed using the statistical software program Statgraphic Centurion XV (version15.2.06 for Windows; StatPoint, Inc., Herndon, VA, USA) and a probability of P 0.05 was considered the minimum level of significance. All results are expressed as mean ± S.E.M.

3. **Results**

After CE-MS all the metabolites evaluated in the seminal plasma of treated and untreated Sarda breed ram have shown different retention times (Supplementary File 1).

Table 1 summarizes the MS/MS data used to identify the quantified metabolites.

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Histidine	156	156 -> 110
Choline	104	104 -> 60

Melatonin treatment influenced melatonin concentration in seminal plasma (Table 2; $p < 0.05$). The level of this indolamine, expressed as Arbitrary Units (AU), was found practically constant during the 120 experimental days in the control group (1.32 ± 0.89 AU, 2.31 ± 0.83 AU, 3.28 ± 0.77 AU and 2.71 ± 0.77 AU respectively at 0, 30, 90 and 120 days). In the treated ram, melatonin increased up to day 90. Subsequently, at day 120, it decreased to the control levels (3.34 ± 0.89 AU, 6.51 ± 0.69 AU, 9.65 ± 0.89 AU, 2.07 ± 0.77 AU respectively at 0, 30, 90, 120 days from melatonin treatment).

Table 2
Area of melatonin peaks in electropherograms obtained from CE/MS of seminal plasma of melatonin-treated and untreated rams during the non-breeding season. Values are expressed in arbitrary units as mean ± SEM. Different superscripts indi- cate statistical difference (ANOVA: $p < 0.05$).

Days from melatonin treatment	Treated rams	Untreated rams
0	3.34 ± 1.70^{a}	1.96 ± 0.13^{a}
30	6.51 ± 1.69^{b}	2.30 ± 1.08^{a}
90	9.65 ± 2.89^{c}	3.28 ± 1.57^{a}
120	2.07 ± 0.87^{a}	2.71 ± 1.77^{a}

The quantification of metabolite data, summarized in Fig. 1, showed that the concentration of 7 out of 8 analyzed metabolites was affected by melatonin treatment. In the control group both molecules carnitine and glutamine were statistically constant during 120 days of melatonin treatment, but in treated rams, their levels increased till day 90 (carnitine 104.3 ± 9.8 AU; glutamine 54.5 ± 4.6 AU) and decreased to control levels at 120 days from the insertion of melatonin device ($p < 0.05$). Glutamic acid, acetyl- carnitine, arginine and choline concentration levels were statisti- cally higher at 90 days ($p < 0.05$) in treated animals compared to that of the control (glutamic acid: 54.51 ± 4.64 AU vs 16.97 ± 4.02 AU; acetyl-carnitine: 217.69 ± 17.32 AU vs 53.28 ± 15.00 AU; argi- nine: 14.24 ± 1.08 AU vs 6.95 ± 0.934 AU; choline 14.16 ± 1.55 AU vs 8.08 ± 1.34 AU respectively). Tyrosine concentration was found different between the two experimental groups ($p < 0.05$) being higher at 30 days in seminal plasma of treated animals (27.08 ± 3.04 AU) compared to the control (19.26 ± 2.57 AU). At 90 and 120 days, the levels of tyrosine were superimposable to the untreated. His- tidine is the only analyzed metabolite whose concentration did not change between treated and control ram.

Fig. 2A shows the data on cleavage rates obtained after oocyte in vitro fertilization using sperm from males treated with mela- tonin versus controls. Mean values do not differ statistically be- tween treated and controls at each experimental time nor between different interval times after treatment. Mean cleavage rates during 120 days were 70.3 ± 3.6% in the treated group and 70.3 ± 2.6% in the untreated. Fig. 2B summarizes the data obtained from the embryonic cul- ture of zygotes in vitro until the blastocyst stage. Blastocyst output is different between the treated and control groups ($p < 0.05$). The blastocysts rate did not show differences after 30 days from treatment (37.7 ± 5.2% in treated and 36.5 ± 3.5% in control) while after 90 days blastocyst rate in the treated group (54.7 ± 4.37%) was higher than the untreated (39.14 ± 6.6%).

4. Discussion

It has been shown that melatonin affects the fertility features of ram sperm and is present in seminal plasma where its concentra- tion reflects the fluctuation of serum in the blood during the year [21,36,42]. Fertility traits of semen can be categorized as compen- sable or uncompensable [43]. The compensable attributes are those that influence the accessibility of a population of viable sperm to the ovum at the time of ovulation and can be improved by increasing the number of sperm per insemination. It is generally believed that compensable attributes are associated with measures of sperm viability (ie, motility, acrosomal, cell membrane integrity, and the like), however, these cannot predict fertility definitively. Uncompensable traits appear to be closely related to abnormal sperm morphology, DNA integrity, and the ability of the male to sustain normal embryonic development following fertilization and do not respond to an increase in the numbers of sperm per dose with improved fertility. The evaluation of the distribution of em- bryo quality following in-vivo or in-vitro fertilization is a useful bioassay of sperm competency (for review see Ref. [44]).

Differences in male fertility in vitro are not simply due to the failure of sperm to penetrate the oocyte but also when compen- sable attributes are adjusted to equalize fertilization rates among different males, differences in embryonic development to the morula and blastocyst stage persist [45]. With this in mind, we assessed uncompensable attributes, such as cleavage rates and blastocyst output in vitro after fertilization of in vitro matured oocytes using sperm from melatonin-treated animals. Our results have shown that melatonin treatment does not affect the rate of cleavage but increases blastocyst rates. Our data agree with those reported by Pang et al. [46] which demonstrated a beneficial effect of melatonin treatment in vitro on bull sperm, in blastocyst pro-

duction but not on cleavage rates. The development of fertilized oocyte depends upon maternally provided factors until zygotic genome activation (ZGA) that occurs in the mammals between 2 and 4 cell stage. The mRNA synthesized by the activated embryo genome replaces the maternal mRNA and guides the subsequent development [47]; it is at this time that the sperm genome can begin to affect the development of the embryo.

Embryos produced in vitro with melatonin-treated sperms have been shown to have reduced abundance of pro-apoptotic CASP3 and BAX mRNAs and increased relative mRNA abundance of the anti-apoptotic genes BCL2 and XIAP and of the antioxidant gene CAT that are consistent with the reduction of apoptosis observed in blastocysts. Therefore, the anti-apoptotic and anti-oxidative effects of melatonin can act by reducing oxidative stress on the sperm, thereby improving the quality of the resulting bovine embryo [46]. It has been observed in several species that sperm quality affects the rate of blastocyst formation in vitro [48,49]. We have shown an increase in the blastocyst rate for up to 90 days, due to melatonin treatment, which indicates that sperm competence is increasing during this period.

Our data highlighted the increase in melatonin concentration in seminal plasma of melatonin-treated rams, with a 90-day peak from implantation of the device confirming other works which demonstrated the release of melatonin from these devices for more than 100 days [15]. These results suggest that the increase in melatonin in the seminal plasma may directly influence the uncompensable parameters of the sperm through its MT1 and MT2 receptors [50], which justify the higher production of embryos after treatment of rams with melatonin.

The molecular events/mechanisms that determine the fertilizing potential of a semen sample are not yet well understood. Seminal plasma, a complex mixture of secretions originating from the testis, epididymis and male accessory glands, plays a funda- mental role in the fertilizing capacity of the sperm [48,49]. Mela- tonin receptors have been evidenced in epididymis, prostate and testis [51,52], suggesting these cells as targets of the pineal hor- mone. The concentration of molecules related to different path- ways involved in the acquisition of sperm quality is altered in the seminal plasma of rams treated with melatonin. We have found that melatonin treatment induces an increase in the levels of some amino acids (glutamic acid, glutamine, arginine, tyrosine) and molecules involved in lipid catabolism and in mitochondrial bio- energetics (carnitine, acetyl-carnitine and choline) regulating lipid and glucose metabolism [53] in the seminal plasma of the ram. The fluctuation of these molecules during the 120 days following the treatment with melatonin shows a similar trend to that of mela-tonin, suggesting a stimulatory action of this indolamine on the secretory activity of testis cells [30]. These molecules are involved in determining in the seminal plasma a favorable environment for sperm that can play a fundamental role in determining the quality of the ejaculate.

The high concentration of these metabolites in conjunction with the best sperm competence suggest that these molecules, acting in synergy to melatonin, promote energy metabolism and antioxidant defense, improving the quality of the sperm. Therefore, they can be involved in determining the fertilization potential of the sperm during the non-breeding season, acting in different ways, mainly in the antioxidant defense.

These results confirmed previous data obtained by NMR in humans [54] and bovines [55] where a panel of metabolites has been related to the quality of the sperm. The ability of CE-MS analysis to quantify differences in seminal plasma metabolomics profiles in subfertile male subjects, therefore, could be proven as a diagnostic tool to evaluate fertilizing potential and semen quality and to investigate male infertility.

In conclusion, the results of our study suggest that the exoge- nous melatonin administration to Sarda breed ram during the non- reproductive season influences the seminal plasma composition modifying the levels of both melatonin and metabolites involved in the antioxidant defense and lipid metabolism of sperm. Sperm collected from ram treated with this indoleamine gain the high capacity to sustaining embryonic development up to the blastocyst stage after in vitro fertilization. In addition, our work introduces CE-MS as a valid tool to assess the fertility potential of ejaculate semen and adds a panel of metabolites to the list of ejaculate quality markers. These metabolites associated with sperm fertility potential should be further investigated to assess their validation as markers of sperm quality in a broad range of fertility grades. Therefore, in the view of the development of new approaches for a correct diagnosis of male infertility, overall understanding of the biochemical and molecular composition and its role in the regu- lation of sperm quality is highly desirable.

Conflicts of interest

The authors declare no conflict of interest.

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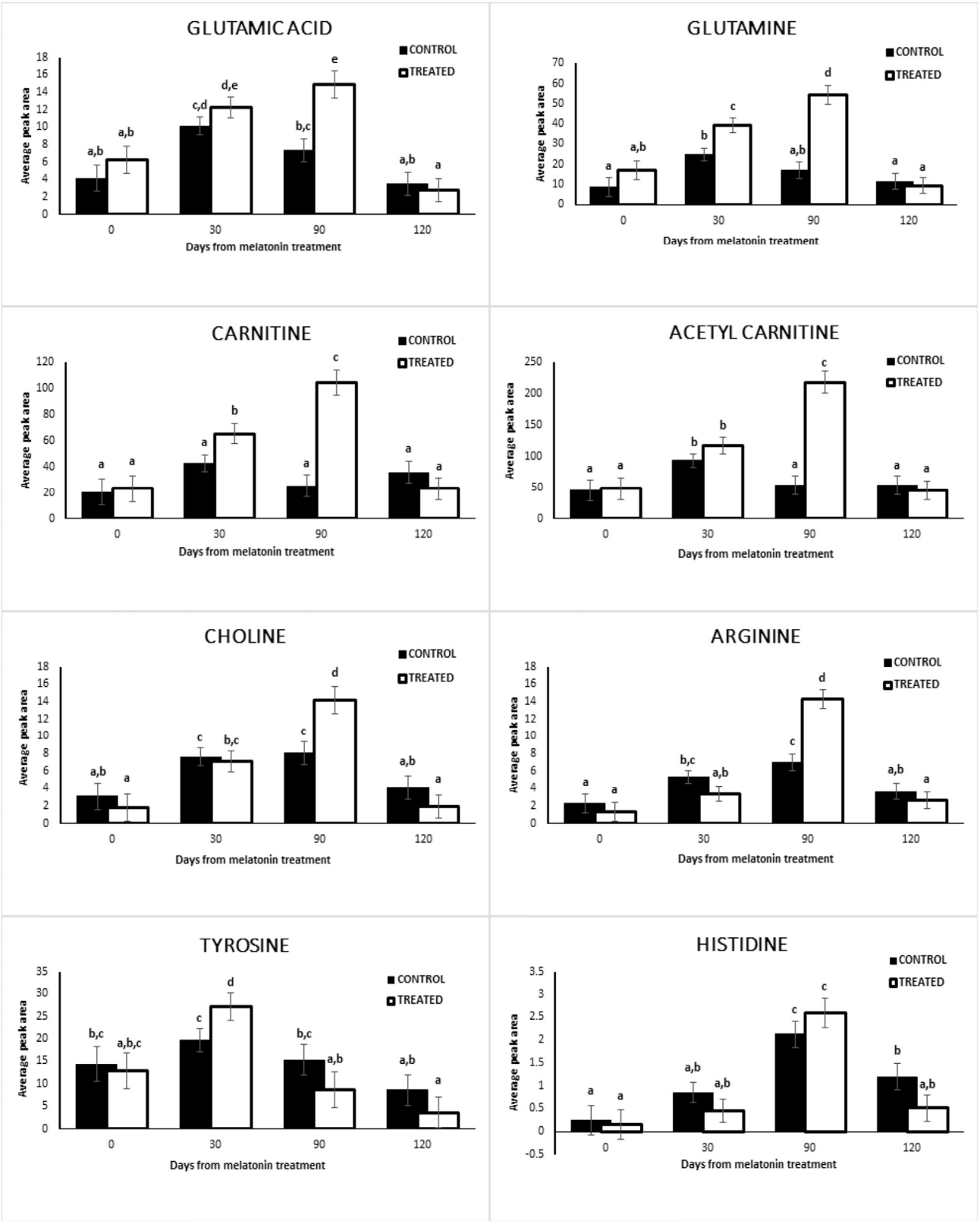

Fig. 1. Peaks area of the selected metabolites in electropherograms obtained from CE/MS analyses of the seminal plasma of melatonin-treated and untreated (control) rams during the non-breeding season. Values are expressed in arbitrary units as mean ± SEM.). Different superscripts indicate statistical difference (ANOVA: $p < 0.05$).

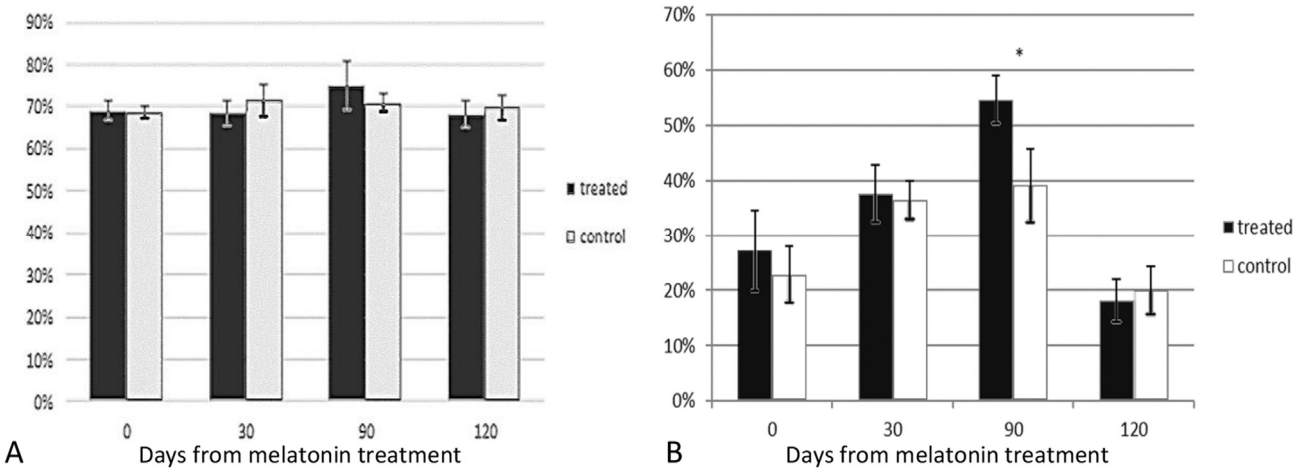

Fig. 2. Cleavage (A) and blastocyst rates (B) obtained after in vitro culture of zygotes produced using spermatozoa from melatonin-treated (n ¼ 1735) and untreated (control; n ¼ 1648) rams at different days after treatment. * indicate statistical difference (ANOVA: $p < 0.05$).

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