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Administration of melatonin improves testicular blood flow, circulating hormones, and semen quality in Shiba goats

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ABSTRACT

Despite the role of melatonin in the regulation of the sleep-wake cycle and seasonal-reproduction, the present study investigated, for the first time, the potential role of melatonin on testicular blood flow (TBF) in goats. Twelve sexually mature male Shiba goats were exposed to a single s.c. injection of either melatonin suspended in one ml of corn oil (melatonin group; 36 mg/goat; n = 5) or one ml of corn oil (control group; n = 7). Monitoring the changes in TBF was done one week before (W-1), at the time of injection (W0), and once a week for 8 weeks after injection using color-pulsed Doppler ultrasonography. Concentrations of FSH, LH, inhibin, testosterone (T), estradiol (E2), and insulin-like growth factor-1 (IGF-1) in plasma were determined by radioimmunoassay. Melatonin and nitric oxide (NO) concentrations were measured using enzyme immunoassay kits. Moreover, semen collection and evaluation of some sperm parameters were performed once a week. Results revealed decreases ($P < 0.05$) in the Doppler indices (resistive index, pulsatility index) of the testicular arteries from W2 till W6 in the melatonin group. FSH, LH, and inhibin concentrations did not change between the two groups, while T, E2, IGF-1, NO, and melatonin concentrations increased ($P < 0.05$) in the melatonin group compared to the control. Estradiol and NO concentrations increased ($P < 0.05$), coinciding with decreases in the values of Doppler indices. Notable ($P < 0.05$) improvements in most parameters of semen quality were seen in the melatonin group. In conclusion, melatonin induced a stimulatory effect on TBF in Shiba goats and possibly, it could be a potential to improve male goats fertility.

1. Introduction

Reproduction is one of the most important economic factors in the livestock industry, based not only on the reproductive capacity of the female; but the male as well. Testicular hemodynamic is the main route for the transport of oxygen, nutrients, and other hormones to and from the testis. Due to the very low concentration of oxygen in the seminiferous tubules [1], control of blood flow is very critical in the testis than in other organs as this is necessary for the proper physiological function of the testis. In addition, exposure of the testis to acute hypoxic conditions (13% oxygen in inspired air) forces it to keep delivery of oxygen by increasing blood flow and oxygen uptake [2]. Since blood flow significantly impacts physiological functions of the testis such as spermatogenesis and hormone production, more studies need to be conducted on the various treatments that could enhance testicular blood flow (TBF) in farm animals [3].

The advent of ultrasonography enables researchers and veterinarians to assess and monitor tremendous reproductive events in

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goats' reproduction practice [4–6]. Color Doppler ultrasonography was developed to improve the accuracy of diagnoses in both research and clinical aspects because it depends on the extraction of the physiological function of the organ through evaluating its vascular hemodynamics [7,8]. Doppler ultrasonography has become an essential tool for assessment of male fertility based on the characterization of TBF in some animal species such as stallion [3], dogs [9], and goats [10,11].

Melatonin (N-acetyl-5-methoxytrypamine) is a secreted neurohormone of the pineal gland. It is synthesized and released into the circulation in circadian rhythm depending on the photoperiod in mammals [12–14] for the regulation of the sleep-wake cycle. In seasonally breeding animals such as certain breeds of sheep and goats, it has a great role to regulate reproductive functions in response to changes in daylight periods by a direct effect on the hypothalamic-pituitary-gonadal axis [15]. Melatonin is also considered as a powerful biological antioxidant inside the body, and functions to reduce the oxidative stress *in vivo* [16] through its potency as a free radicals scavenger [17], and up regulator of the expression and/or activity of antioxidant enzymes [18]. Many previous studies have illustrated the positive roles of melatonin on sexual activity, testicular function, and overall reproductive performance in goats with or without manipulation of the daylight period [19–22]. However, to date, the effect of melatonin on TBF in small ruminants has not been investigated.

Although Shiba goats are nonseasonal breeder animals [26], melatonin secretion by the pineal gland had marked diurnal changes depending on the daylight period [23]. Furthermore, our recent study in this breed [11] revealed a seasonal influence on testicular blood perfusion as assessed by the color-spectral Doppler ultrasonography. In the aforementioned study, maximum TBF was reported in the winter and autumn (short photoperiod) than in the summer and spring (long daylight) with concomitant seasonal changes in the gonadal activity; which was thought to be photoperiod sensitive: short day length is stimulatory whereas long day length negatively impacts the gonadal functions in Shiba goats [23]. Taking into consideration the already stated facts and the evidence of expression of melatonin receptors in testicular cells [24,25], our hypothesis is that melatonin has a role in TBF in goats. Moreover, studying the effect of melatonin on nitric oxide (NO), and circulating hormones; especially insulin-like growth factor-1 (IGF-1) is of great importance. Many studies showed the pivotal roles of NO as one of the major physiological regulators of basal blood vessel tone, and enhancement of TBF [26–28]. Also, IGF-1; a secreted hormone by Leydig-Sertoli cells; plays an important role in the regulation of testicular function [29]. There are strong correlations between concentrations of IGF-1 and some parameters of semen such as motility % of sperm [30]. In addition, plasma IGF-1 showed a seasonal pattern and was greatly influenced by photoperiod, and melatonin [31]. Therefore, the objective of the current study was to determine the effect of a single s.c. administration of melatonin in an oily base suspension on testicular hemodynamics as assessed by color spectral-Doppler ultrasonography in Shiba goats. In addition, it aimed at demonstrating whether or not there are changes in circulating hormones (FSH, LH, inhibin, testosterone, estradiol, and IGF-1), nitric oxide, and parameters of semen quality after administration of melatonin.

2. Material and methods

The present study was performed in male Shiba goats *(Capra hircus)*, a Japanese miniature nonseasonal breeder goat. It reaches puberty at 3.5 months and is considered a good model animal for studying the physiology of ruminants [32]. All procedures in the current study were carried out in accordance with ethical guidelines established by the Tokyo University of Agriculture and Technology, Japan, for the use of animals (Ethical approval # 30–78).

2.1. Animals and management

Twelve sexually mature male Shiba goats (*Capra hircus*), 22.5 ± 3.5 months of age, weighing 26.85 ± 3.25 kg, and housed in a paddock under natural daylight conditions were used in the current study. They were fed a diet of 400 gm of hay cubes per animal twice a day, while mineralized salt licks and clean tap water were available *ad libitum*. The goat paddock used belongs to the Laboratory of Veterinary Reproductive Physiology, Veterinary Medicine Department, Tokyo University of Agriculture and Technology, Japan (Latitude: 35° 40′8.31 "N; Longitude: 139° 28′39.58″ E; Decimal is 35.6667:139.483). Variations in daylight periods during the study (from March 2018 to June 2018) are ranged from 12.23 to 14.40 h. All goats were regularly exposed to immunoprophylaxis activities such as deworming using appropriate broad-spectrum anthelmintic medications, and none of them had any evidence of disease before the study. The animals were clinically healthy with good libido before the experiment. Moreover, each goat was exposed to a general examination including the testis and epididymis by ultrasonography to verify the absence of abnormalities in the reproductive tract before the start of the research study. For more guarantee, female goats were raised separately throughout the period of the study.

Goats were randomly allocated into 2 groups: (1) Melatonin group (n = 5), treated with a single s.c. dose (Fig. 1A) of melatonin powder (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) that was dissolved in one ml of corn oil as a suspension (36 mg/goat) and (2) control group (n = 7), which was treated with one ml of corn oil only without melatonin. The dose of melatonin was selected based on a previous report [33].

2.2. Ultrasonographic examinations

Monitoring of the changes in TBF was performed one week before injection (W-1), at the time of injection (W0), and once a week for 8 consecutive weeks after injection of the melatonin. The experiment was performed during spring and early summer 2018. Ultrasonographic assessments were performed throughout the period of the study by the same operator (the first author). All examinations were performed using an ultrasound scanner (EUB-7500, Hitachi Medical Corporation, Tokyo, Japan) equipped with a linear multi-frequency array transducer (6–14 MHz; Model EUP-L65; Hitachi Medical Corporation, Tokyo, Japan). Based on our previous studies [10,11], the bucks were simply secured without sedation to avoid its effect on TBF. Also, the hairs on both sides of the scrotum were removed by shaving, and a copious amount of ultrasonic gel was used on the transducer to facilitate the assessment by ultrasonography. In the present study, we monitored testicular hemodynamics in supratesticular arteries and marginal testicular arteries (Fig. 1B). The testicular artery in goats appeared convoluted; just before entry into the testis. This convoluted part is termed the supratesticular artery, STA: in this part, the testicular veins coalesce to surround the artery and form the testicular vascular cone. However, to differentiate between the testicular artery and vein by spectral Doppler assessment, the artery will typically have a spectral waveform representing the arterial pulse in each cardiac cycle (systole and diastole). In the vein, however, the flow has no pulse and is almost constant. After the vascular structures were identified and the largest longitudinal or oblique section of the STA, as well as the marginal testicular artery (MTA), was observed using B-mode ultrasonography, Doppler assessment of

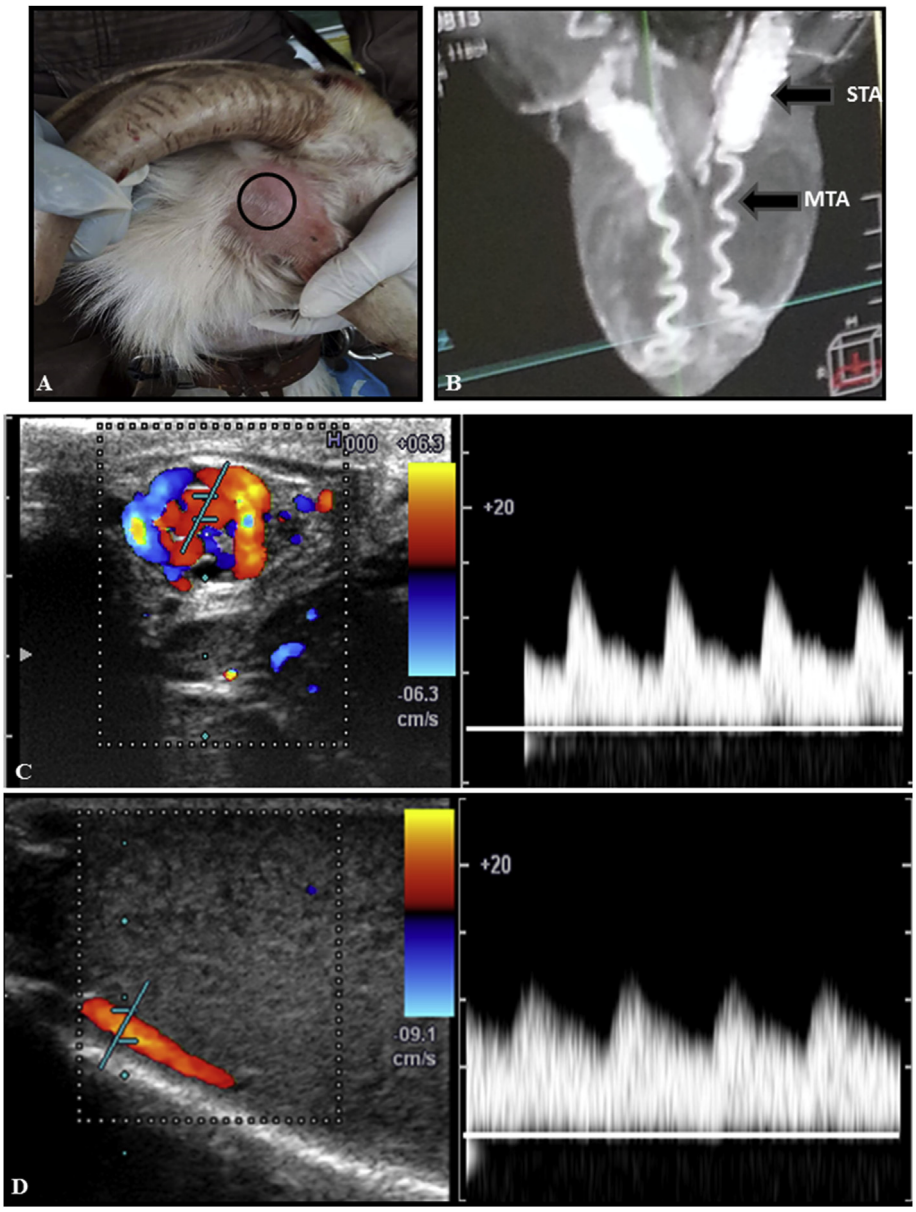

Fig. 1. (A) The site of s.c. injection of melatonin suspension at base of the ear; (B) the testis of a male Shiba goat imaged by computerized tomography to illustrate the locations of the supratesticular artery (STA) and marginal testicular artery (MTA). An assessment of the blood flow within the supratesticular artery (C) and marginal testicular artery (D) was performed by color-pulsed Doppler ultrasonography and the Blood flow within both arteries appeared as spectral-pattern with a wave-like display. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

TBF was carried out as described in stallions and goats [10,34]. After the appearance of the spectral pattern of the STA (Fig. 1C) as well as the MTA (Fig. 1D), appended parameters were assessed: peak systolic velocity (PSV, cm/sec), end diastolic velocity (EDV, cm/sec), and the time-averaged maximum velocity (TAMAX, cm/sec). Doppler indices studied were: resistive index (RI = (PSV- EDV)/PSV) and pulsatility index (PI = (PSV - EDV)/mean velocity). All spectral-Doppler examinations were carried out at certain times (9.00 A.M.), fixed, and standardized ultrasound setting (gains, focus, brightness, and contrast) by the same operator. The angle between the long axis of the examined vessel and the Doppler beam was less than 60° in the direction of blood flow. The high-pass filter and the Doppler gate were set constant at 50 Hz and 1.5 m, respectively.

2.3. Blood sampling and hormonal analysis

Blood samples (5 ml) were drawn, on the Day of ultrasonographic scanning, from the jugular vein into evacuated heparinized tubes (Venoject II, Terumo, Tokyo, Japan). Plasma was obtained after blood samples were centrifuged at 3200 rpm (600 *g*) for 15 min at 4 °C, then separated and stored at −20 °C till assessment of concentrations of circulating hormones.

Plasma concentrations of FSH, LH, and inhibin were determined by a double-antibody radioimmunoassay (RIA) system using ^{125}I-labeled radioligands as described in previous studies by Refs. [35,36], and [37]; respectively. Plasma concentrations of FSH (ng/ml) were measured using anti-ovine FSH, NIDDK-oFSH-RP-1 as a standard reference, and NIDDK–FSH–I-1 for radio-iodination,

Table 1
Changes in testicular blood flow as measured by color spectral Doppler ultrasonography of the supratesticular artery in male Shiba goats in the melatonin group (Mel; n = 5), and the control group (Con; n = 7) during different times (weeks) after administration of a single dose of melatonin (W0).

Time	PSV (cm/sec)		EDV (cm/sec)		TAMAX (cm/sec)		RI		PI	
	Mel	Con	Mel	Con	Mel	Con	Mel	Con	Mel	Con
W-1	15.44 ± 1.25	15.51 ± 0.38	7.65 ± 0.71	7.22 ± 0.31	10.5 ± 0.51	10.86 ± 0.30	0.495 ± 0.043	0.522 ± 0.016	0.704 ± 0.085	0.762 ± 0.035
W0	12.9 ± 0.98	16.11 ± 1.79	6.96 ± 0.90	7.97 ± 1.33	09.39 ± 0.95	11.43 ± 1.54	0.457 ± 0.050	0.520 ± 0.033	0.665 ± 0.117	0.761 ± 0.067
W1	14.82 ± 0.47	15.27 ± 1.19	9.04 ± 0.85	8.32 ± 0.77	11.58 ± 0.61	11.21 ± 0.86	0.393 ± 0.048	0.456 ± 0.030	0.519 ± 0.081	0.635 ± 0.058
W2	14.67 ± 0.94	15.71 ± 0.68	9.01 ± 0.75	8.24 ± 0.50	11.52 ± 0.83	11.24 ± 0.50	0.367 ± 0.027	0.473 ± 0.019	0.465 ± 0.043	0.666 ± 0.033
W3	12.93 ± 0.63*	18.18 ± 1.37	8.16 ± 0.41	8.51 ± 0.42	10.19 ± 0.52	12.50 ± 0.58	0.357 ± 0.023*	0.544 ± 0.027	0.455 ± 0.035*	0.812 ± 0.068
W4	14.12 ± 1.16	15.16 ± 1.05	8.16 ± 0.91	6.96 ± 0.45	10.79 ± 1.03	10.28 ± 0.49	0.408 ± 0.011*	0.561 ± 0.025	0.535 ± 0.017*	0.844 ± 0.053
W5	14.92 ± 1.49	16.41 ± 0.83	9.16 ± 1.04	7.62 ± 0.23	11.54 ± 1.21	11.38 ± 0.42	0.375 ± 0.021*	0.531 ± 0.017	0.486 ± 0.035*	0.771 ± 0.038
W6	13.41 ± 0.75	16.92 ± 1.10	7.38 ± 0.78	7.56 ± 0.60	10.02 ± 0.80	11.36 ± 0.76	0.401 ± 0.030*	0.550 ± 0.034	0.521 ± 0.050*	0.836 ± 0.077
W7	17.33 ± 0.96	16.07 ± 0.75	8.68 ± 0.93	6.47 ± 0.53	12.23 ± 0.73	10.08 ± 0.62	0.454 ± 0.015*	0.600 ± 0.021	0.633 ± 0.031*	0.973 ± 0.061
W8	17.43 ± 0.61	18.18 ± 0.99	7.74 ± 0.88	6.81 ± 0.56	11.86 ± 0.84	11.19 ± 0.46	0.561 ± 0.037	0.614 ± 0.043	0.851 ± 0.087	1.015 ± 0.103

Abbreviations: W: Week (as a time point); Values are means ± SEM. Generally, the treatment effect (Mel group versus Con group) showed significant difference in the values of PSV ($P < 0.001$), RI ($P < 0.0001$), and PI ($P < 0.0001$), while time effect showed significant differences ($P < 0.0001$) in the values of RI and PI. *Values in each parameter are significant different at least at $P < 0.05$ between the two groups. Parameters and indices of spectral Doppler ultrasonography that used were: PSV = Peak systolic velocity (cm/sec); EDV = End diastolic velocity (cm/sec); TAMAX = Time average maximum velocity (cm/sec); RI = Resistive index; PI = Pulsatility index.

while plasma LH (ng/ml) was measured using anti-ovine LH (YM 18), NIDDK-oLH-RP-24 as a reference standard, and NIDDK-oLH- I-3 for radio-iodination. Plasma concentration of inhibin (ng/ml) was measured using anti-bovine antiserum (TNDH-1) and bovine 32-kDa inhibin for radio-iodination. Concentrations of testosterone (T; ng/ml), and estradiol (E2; pg/ml) were measured in plasma as described by Taya et al. [38] using antisera against T (GDN 250), and E2 (GDN 244); respectively. Concentration of IGF-1 (ng/ml) was measured by RIA as described [39] using Anti-IGF-1(rabbit antiserum against human IGF-1; Lot: 150727 GW#14), and recombinant human IGF-1 for radioiodination and as the reference standard. The intra- and inter-assay coefficients of variation were 9.6 and 11.8% for FSH, 5.6 and 6.8% for LH, 4.2% and 12.3% for inhibin, 8.4 and 9.6% for T, 5.6 and 7.7% for E2, and 8.8 and 5.4% for IGF-1, respectively. All hormonal analyses were carried out in triplicate and run in the same laboratory at Tokyo University of Agriculture and Technology.

Concentrations of nitric oxide (NO; μM/ml) in the plasma were measured using enzyme immunoassay kits (Colorimetric Nitric Oxide Assay Kit, NB98, Oxford Biomedical Research, Inc., Funakoshi Co., Ltd, Tokyo, Japan) based on the protocol of endorsed instructions [40]. The sensitivity of the assay was 1 pmol/μl (~1 μM) NO produced in aqueous solutions. Concentrations of melatonin (ng/ml) in the plasma were measured using melatonin ELISA Kit (Enz-Kit150-0001; Enzo Life Sciences, Inc., USA) [41]. The sensitivity of melatonin assay is 0.162 ng/ml and the range of measurement is 0.08–50 ng/ml. Absorbance values for NO and melatonin were read in a microtiter plate reader at 540 nm, and 450 nm; respectively.

2.4. Semen collection and evaluation

Samples of goats' semen were collected once a week using an artificial vagina (44 °C). The ejaculates were protected from sunlight and transferred directly in a water bath (36 °C) to the laboratory for semen evaluation. To avoid a possible effect of sexual rest on semen quality, the data of semen quality that was obtained in the first collection (W-1) was not considered in the analysis [42].

The semen samples were subjectively analyzed on a pre-warmed slide using microscopic inspection (Olympus Optical Co., Ltd., Tokyo, Japan) according to the method described [43,44]. The assessed parameters were mass motility %, progressive motility %, viability % (live and dead sperm) and normal sperm morphology % (using the technique of eosin-nigrosin staining), and sperm concentration (using a hemocytometer).

For detection of the sperm acrosome integrity, the sperm cells were stained with Fluorescein Isothiocyanate conjugated to Peanut Agglutinin (FITC-PNA, Sigma) as described in a previous study [44]. Briefly, aliquots of 20 μl of semen (100 x 10^6) were placed on microscope slides, air-dried, and fixed in absolute methanol for 10 min to fix and permeabilize the sperm membranes for FITC-PNA analysis. Afterward, 30 μl of FITC-PNA (150 μg/ml in PBS) working

Table 2
Changes in testicular blood flow as measured by color spectral Doppler ultrasonography of the marginal testicular artery in male Shiba goats in the melatonin group (Mel; n = 5), and the control group (Con; n = 7) during different times (weeks) after administration of a single dose of melatonin (W0).

Time	PSV (cm/sec)		EDV (cm/sec)		TAMAX (cm/sec)		RI		PI	
	Mel	Con	Mel	Con	Mel	Con	Mel	Con	Mel	Con
W-1	10.81 ± 1.21	11.73 ± 0.90	6.96 ± 0.37	8.04 ± 0.73	8.70 ± 0.52	9.84 ± 0.78	0.319 ± 0.063	0.321 ± 0.026	0.432 ± 0.107	0.398 ± 0.038
W0	10.19 ± 0.81	10.72 ± 0.67	7.86 ± 0.77	7.54 ± 0.45	9.09 ± 0.81	8.83 ± 0.50	0.227 ± 0.015	0.273 ± 0.023	0.255 ± 0.019	0.327 ± 0.033
W1	10.21 ± 0.54	11.76 ± 0.65	7.85 ± 0.31	9.07 ± 0.39	8.94 ± 0.40	10.25 ± 0.49	0.225 ± 0.026	0.226 ± 0.015	0.260 ± 0.032	0.260 ± 0.020
W2	12.05 ± 0.81	11.05 ± 0.92	9.41 ± 0.61	8.29 ± 0.71	10.66 ± 0.72	9.55 ± 0.80	0.217 ± 0.016	0.265 ± 0.016	0.247 ± 0.020	0.311 ± 0.019
W3	10.74 ± 0.90	11.43 ± 0.78	8.09 ± 0.71	8.08 ± 0.54	9.28 ± 0.81	9.58 ± 0.59	0.246 ± 0.009	0.304 ± 0.029	0.286 ± 0.013	0.373 ± 0.045
W4	11.15 ± 0.67	12.14 ± 0.87	8.90 ± 0.58	8.57 ± 0.51	9.90 ± 0.62	10.18 ± 0.64	0.205 ± 0.017	0.302 ± 0.015	0.233 ± 0.023*	0.366 ± 0.028
W5	12.45 ± 0.62	13.05 ± 0.97	9.63 ± 0.43	9.29 ± 0.85	10.88 ± 0.46	11.05 ± 0.88	0.223 ± 0.014	0.321 ± 0.015	0.256 ± 0.019*	0.386 ± 0.019
W6	11.97 ± 0.64	13.36 ± 0.52	8.95 ± 0.43	9.57 ± 0.45	10.43 ± 0.55	11.35 ± 0.44	0.249 ± 0.009	0.290 ± 0.017	0.286 ± 0.011	0.344 ± 0.026
W7	12.13 ± 0.42	11.76 ± 1.21	8.86 ± 0.39	8.65 ± 0.97	10.44 ± 0.26	9.18 ± 0.82	0.236 ± 0.010	0.265 ± 0.014	0.270 ± 0.014	0.312 ± 0.019
W8	10.59 ± 0.96	13.72 ± 1.02	7.82 ± 0.75	9.67 ± 0.86	9.15 ± 0.87	11.43 ± 0.93	0.267 ± 0.014	0.298 ± 0.028	0.314 ± 0.021	0.365 ± 0.040

Abbreviations: W: Week (as a time point); Values are means ± SEM. Generally, the treatment effect (Mel group versus Con group) showed significant difference in the values of PSV ($P < 0.05$), RI ($P < 0.0001$), and PI ($P < 0.001$), while time effect showed significant differences ($P < 0.01$) in the values of RI and PI.*Values in each parameter are significant different at least at $P < 0.05$ between the two groups. Parameters and indices of spectral Doppler ultrasonography that used were: PSV = Peak systolic velocity (cm/sec); EDV = End diastolic velocity (cm/sec); TAMAX = Time average maximum velocity (cm/sec); RI = Resistive index; PI = Pulsatility index.

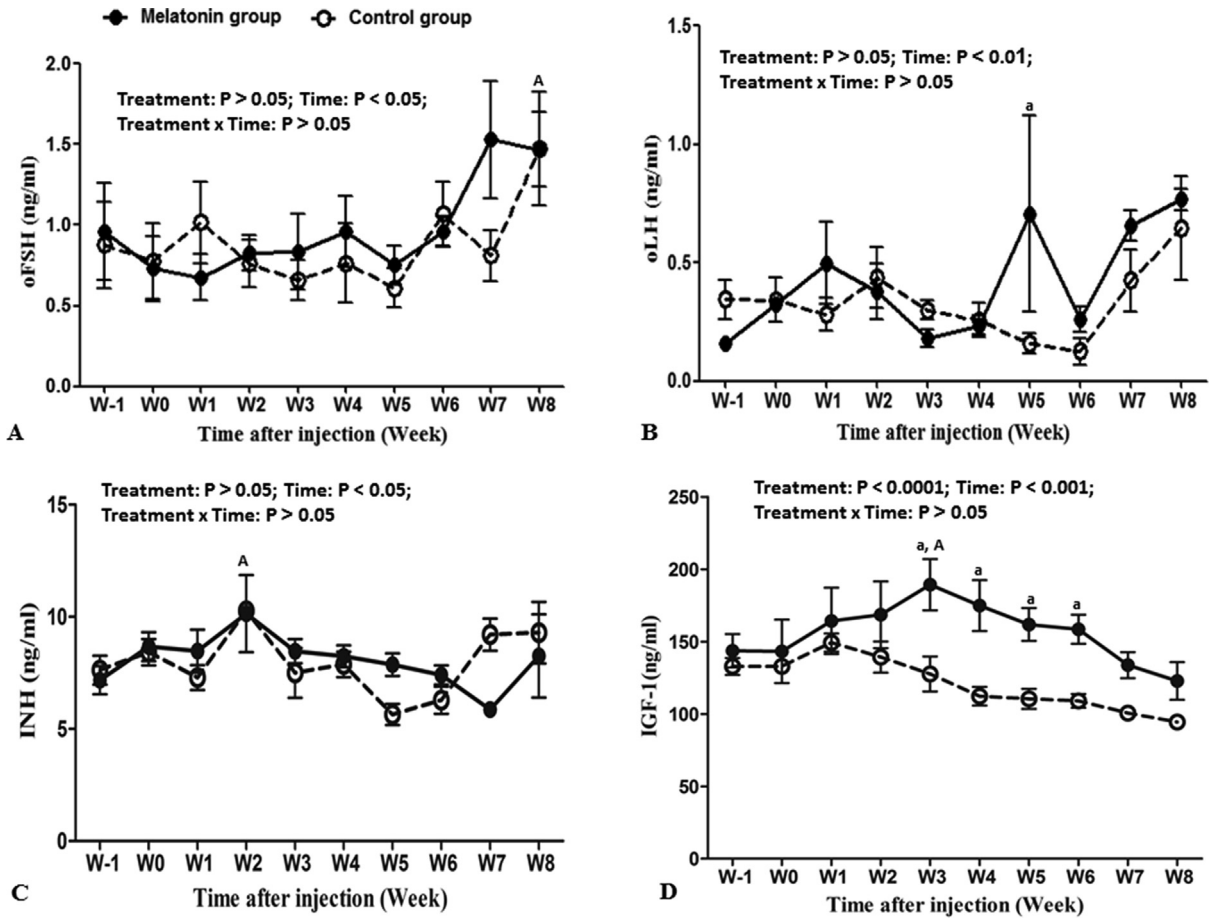

Fig. 2. Changes in plasma concentrations of FSH (oFSH, ng/ml; A), LH (oLH, ng/ml; B), inhibin (INH, ng/ml; C), and insulin-like growth factor-1 (IGF-1, ng/ml; D) in male goats that received (s.c.) one ml of melatonin suspension in corn oil (closed circles; melatonin group; n = 5) or one ml of only corn oil (open circles; control group; n = 7). Values are means ± SEM. [a]Values represent significant differences (P < 0.05) between both groups at the indicated times during the study. [A]Values represent significant (P < 0.05) time differences within treatment during the study.

solution was used over the slides for staining in a dark (absence of light) humidified box, followed by incubation for 30 min at room temperature. The slides were then rinsed twice (5 min each) by complete immersion in PBS with shacking and dried naturally in the absence of light. At assessment time, a drop of mounting medium was placed over the slide, and a coverslip was firmly mounted over (to avoid air bubbles). By using an epifluorescence analysis phase-contrast microscope (Olympus, Tokyo, Japan), 200 sperm were examined at a magnification of 1000× using an excitation wavelength of 480 nm and emissions of 530 nm. Sperm were classified into two groups: (1) normal intact acrosomal integrity displaying intensive and bright fluorescent green color of the acrosome cap; (2) abnormal acrosomal integrity displaying either a disruption in the acrosomal cap fluorescence (partially damaged) or absence of fluorescence in the acrosomal cap, that is, damaged acrosome or complete loss of the acrosomal cap.

2.5. Statistical analysis

Data were exposed to a normality test using the Kolmogorov-Smirnov test in GraphPad Prism to identify the homogeneity and the type of data. In this study, no significant differences were found among the studied goats between right and left testes; thus, the data for each buck was pooled and comparisons were done among the two groups. Data for Doppler parameters, hormonal results, and parameters of semen quality were presented as means ± standard error of the mean (SEM). The GraphPad prism5 software was used for all statistical analyses. Means were analyzed for the difference using repeated measures two-way ANOVA to study the effect of the treatment as a fixed factor and the time as a repeated factor. The effect of treatment (2 levels; melatonin versus control) on the changes in TBF in the STA as well as the MTA, on the concentrations of circulating hormones, and on parameters of semen quality was tested along with different time points followed by Bonferroni post hoc test. A value of P < 0.05 was considered significant.

3. Results

3.1. Testicular hemodynamics

The effect of melatonin administration on TBF in the present study is presented in Tables 1 and 2. In general, treatment effect (melatonin group versus control group) had notable differences in values of PSV, RI, and PI in the STA (P < 0.005, P < 0.0001, P < 0.0001; respectively) as well as in the MTA (P < 0.05, P < 0.0001, P < 0.0005; respectively). However, the time effect had differences only in the values of RI and PI of STA (P < 0.0001) and MTA (P < 0.005) between the two groups. Furthermore, the interaction (treatment x time) had non-significant differences in values of all studied Doppler parameters.

Decreases (P < 0.05) in the values of PSV (cm/sec) of the STA were observed in the melatonin group (12.93 ± 0.63 cm/s)

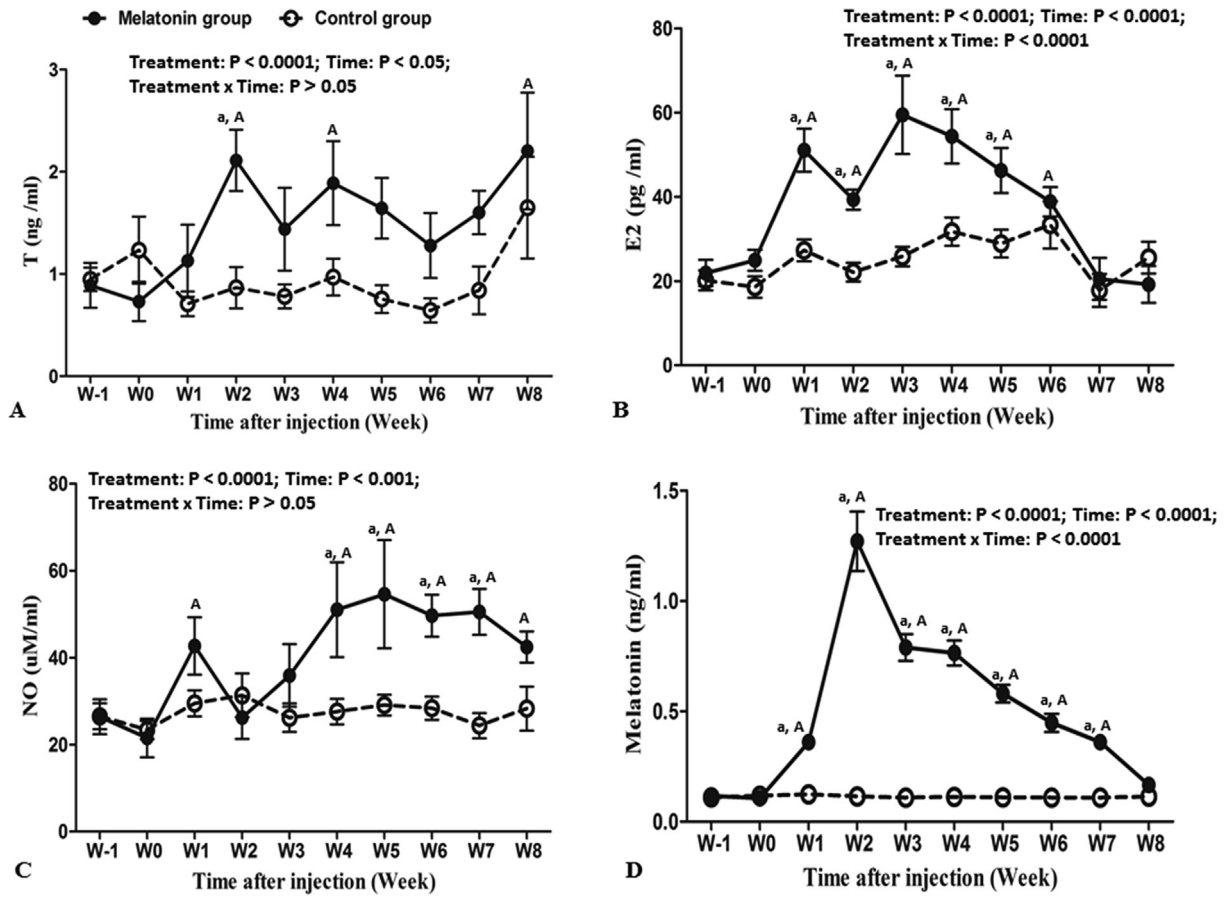

Fig. 3. Changes in plasma concentrations of testosterone (T, ng/ml; A), estradiol (E2, pg/ml; B), nitric oxide (NO, μM/ml; C), and melatonin (ng/ml; D) in male goats that received one ml of melatonin suspension in corn oil (closed circles; melatonin group; n = 5) or one ml of only corn oil (open circles; control group; n = 7). Values are means ± SEM. [a]Values represent significant differences (P < 0.05) between both groups at the indicated times during the study. [A]Values represent significant (P < 0.05) time differences within treatment during the study.

compared to that in the control group (18.18 ± 1.37 cm/s) at W3 post-injection. In the STA, animals in the melatonin group had lesser (P < 0.05) RI and PI values than those in the control group, and these values were lesser (P < 0.05) in the period of W3–W7 after treatment than at any other time. Also, values of RI of the MTA decreased (P < 0.05) in the melatonin administrated group between W3–W4 compared to its values in the control. Other parameters of pulsed Doppler ultrasonography of the testicular arteries showed non-significant changes between the two groups during the study.

3.2. Circulating hormones

Generally, the treatment effect (melatonin group versus control

Table 3
Semen characteristics (Mass motility %; Progressive motility %; Acrosome integrity %, Viability %, Sperm cell concentrations (10^9/ml), and Normal morphology of spermatozoa %) in male Shiba goats in the melatonin group (Mel; n = 5), and the control group (Con; n = 7) during different times (weeks) after administration of a single dose of melatonin (W0).

Time	Mass motility %		Progressive motility %		Acrosome integrity %		Viability %		Sperm cell Conc. (x10^9 sperm/ml)		Normal Morphology %	
	Mel	Con	Mel	Con	Mel	Con	Mel	Con	Mel	Con	Mel	Con
W0	79.0 ± 1.0	80.7 ± 1.7	72.0 ± 2.0	75.0 ± 1.1	82.6 ± 2.2	81.6 ± 1.2	82.8 ± 2.3	84.0 ± 1.2	2.80 ± 0.075	2.92 ± 0.044	90.8 ± 2.5	89.8 ± 1.4
W1	79.0 ± 1.0	79.3 ± 2.0	74.0 ± 1.9	72.9 ± 1.8	81.2 ± 2.2	79.8 ± 2.0	85.1 ± 2.2	83.2 ± 1.8	2.88 ± 0.078	2.83 ± 0.071	89.3 ± 2.4	87.8 ± 2.2
W2	83.0 ± 2.0	80.7 ± 2.8	76.0 ± 1.9	75.0 ± 1.5	84.1 ± 2.3	82.0 ± 2.4	85.8 ± 1.4	84.6 ± 1.6	2.98 ± 0.082	2.91 ± 0.085	92.4 ± 2.6	90.2 ± 2.6
W3	87.0 ± 2.0*	79.3 ± 1.7	79.4 ± 2.0	73.6 ± 1.4	84.0 ± 1.8	81.0 ± 1.6	87.3 ± 2.2	82.9 ± 1.3	2.98 ± 0.064	2.87 ± 0.072	88.2 ± 1.9	89.1 ± 2.1
W4	91.0 ± 1.9*	80.7 ± 1.7	84.0 ± 1.9*	75.7 ± 1.7	89.4 ± 2.4	82.8 ± 1.4	89.8 ± 1.5*	83.3 ± 1.9	3.01 ± 0.102	2.91 ± 0.056	89.4 ± 2.4	91.1 ± 1.6
W5	92.0 ± 1.2*	81.4 ± 1.4	87.0 ± 1.2*	76.4 ± 0.9	91.4 ± 1.3*	81.6 ± 1.7	90.3 ± 1.1*	83.9 ± 0.6	3.02 ± 0.034	2.91 ± 0.059	91.4 ± 1.3	89.7 ± 1.8
W6	91.0 ± 1.0*	80.7 ± 1.7	83.0 ± 2.0*	73.6 ± 1.4	92.3 ± 1.2*	80.9 ± 1.6	89.1 ± 1.1	83.8 ± 1.2	3.19 ± 0.044*	2.86 ± 0.063	92.4 ± 1.3	89.0 ± 1.7
W7	83.0 ± 1.2	79.3 ± 1.3	78.0 ± 1.2	73.6 ± 0.9	83.5 ± 1.3	80.0 ± 1.7	84.6 ± 1.2	81.4 ± 1.4	3.01 ± 0.044	2.79 ± 0.066	91.8 ± 1.4	87.9 ± 1.8
W8	81.0 ± 1.9	78.6 ± 1.4	75.6 ± 1.7	73.6 ± 1.4	83.2 ± 1.9	81.5 ± 2.1	84.9 ± 1.5	83.5 ± 1.1	2.92 ± 0.051	2.76 ± 0.069	91.5 ± 2.1	89.6 ± 2.2

Abbreviations: W: Week (as time point); Values are means ± SEM.*Values in each parameter are different at least at P < 0.05 among the two groups. Generally, the treatment effect (Mel group versus Con group) showed significant difference in mass motility % (P < 0.0001), progressive motility % (P < 0.0001), acrosome integrity % (P < 0.0001), viability % (P < 0.0001), and sperm cell concentration (P < 0.001), while time effect showed significant differences (P < 0.001) in mass motility %, progressive motility %, and acrosome integrity %.

group) had a non-significant difference in concentrations of oFSH, oLH, and inhibin. Concentrations of T, E2, IGF-I, NO, and melatonin; however, had differences ($P < 0.0001$) between the two groups. Regarding the time effect, significant differences were found in the concentrations of most of the studied hormones (oFSH; $P < 0.05$, oLH; $P < 0.01$, inhibin; $P < 0.05$, IGF-1; $P < 0.001$, T; $P < 0.05$, E2; $P < 0.0001$, NO; $P < 0.001$, and melatonin; $P < 0.0001$). Furthermore, the interaction factor (treatment x time) had non-significant differences in all assessed hormones except in E2 ($P < 0.0001$) and melatonin ($P < 0.0001$) concentrations.

Plasma concentrations of oFSH (Fig. 2A) and oLH (Fig. 2B) had non-significant differences between the two groups during the studied period. However, increases ($P < 0.05$) were noticed in the concentration of oLH at W5 (0.71 ± 0.41 ng/ml) in the melatonin group compared to the control (0.16 ± 0.04 ng/ml). Also, notable increases ($P < 0.05$) were found in oFSH at W8 (1.47 ± 0.23 ng/ml) after injection of melatonin compared to its values before injection (LH: 0.16 ± 0.01 ng/ml; FSH: 0.96 ± 0.30 ng/ml). The effect of melatonin administration on circulating inhibin had non-significant changes between the two groups during the study (Fig. 2C). However, increases ($P < 0.0001$) in the concentrations of IGF-1 were observed in the melatonin group from W3–W6 after injection as compared to the control group (Fig. 2D).

Increases in the concentrations of T (Fig. 3A) were observed in the melatonin group compared to that in the control group starting from W2 and onward, and its level was greater ($P < 0.0001$) at W2 (2.11 ± 0.30 ng/ml) post-injection compared to that in the control group (0.87 ± 0.20 ng/ml). Furthermore, increases ($P < 0.05$) were noticed in the concentration of T at W2 (2.11 ± 0.30 ng/ml), W4 (1.89 ± 0.41 ng/ml), and W8 (2.20 ± 0.57 ng/ml) after injection of the melatonin group compared to its values before injection (0.89 ± 0.22 ng/ml). Conversely, concentrations of E2 had increases ($P < 0.0001$) in the melatonin group compared to its values in the control group in the period from W1–W5 post-injection. Concentrations of E2 were high ($P < 0.0001$) in the melatonin group from W1 to W6 post-injection compared to its values before injection (Fig. 3B). Similarly, increases ($P < 0.0001$) in the concentrations of NO were observed in the melatonin group as compared to the control group from W4–W7 post-administration (Fig. 3C). Melatonin concentrations were also higher ($P < 0.0001$) in the melatonin group compared to that in the control one from W1–W7 post-administration (Fig. 3D).

3.3. Semen quality

The results of different parameters for assessment of goats' semen in the present study are summarized in Table 3. The positive impact of melatonin administration (about 10%; $P < 0.05$) was seen in mass motility % (W3–W6), individual progressive motility % (W4–W6), acrosome integrity % (W5–W6), and sperm viability % (W4–W5). Sperm cell concentration had non-significant differences during most periods of the study except at W6 in the melatonin group versus the control group. However, the percentage of normal morphology of sperm had non-significant differences between the two groups during the study.

4. Discussion

Although melatonin was discovered about 60 years ago, only recently have researchers claimed to investigate its various actions in peripheral tissues [45]. Till now and to the best of the authors' knowledge, the present study is the first to investigate the effect of melatonin administration; especially in the perspective of testicular hemodynamics in non-seasonal breeder animals such as Shiba goats. The results of the current study supported the hypothesis that a single dose of melatonin significantly influences testicular hemodynamics in Shiba goats, and also improved the semen quality. Providing such data is important for further enhancement of animal productivity and also as a tool for assisting in solving different problems associated with fertility in goats in the future.

In the present study, melatonin induced significant decreases in Doppler indices of testicular arteries (RI and PI) which have negative correlations with vascular perfusion of tissue downstream [7]. Decreased values for RI and PI indicate decreases in the blood flow resistance and in turn an increase in the testicular perfusion and a continuous supply of oxygen and nutrients to the testis [7,46]. The mechanism through which melatonin affects TBF was not certainly elucidated in the present study; however; three possible ways may interpret these changes. Firstly, melatonin may modulate TBF and semen quality as observed in the present study by regulating different levels of the hypothalamic-pituitary–gonadal axis, as observed in seasonally breeding mammals [15]. Exogenous melatonin implants result in direct effects on the hypothalamus-pituitary axis which induces an increase in GnRH pulsatile secretion [47], and increases in LH, FSH, and testosterone concentrations [48,49]. Although melatonin did not significantly alter gonadotropin hormones (FSH, and LH) in the present study, it cannot be ascertained that it did not act through the hypothalamus-pituitary axis based only on the results of weekly LH and FSH analyses. More importantly, the central effect of melatonin on the hypothalamus-pituitary axis in small ruminants is not solely dependent on the rising of basal LH and FSH levels, but, an increase of LH and FSH pulsatile secretion should also be considered, and that was not evaluated in this experiment. Therefore, the obtained data in this work must be interpreted with caution. Moreover, the significant increase of T in the melatonin treated bucks suggests that this hormone may act through the hypothalamus-pituitary axis in Shiba goats. However, the local effect of melatonin on Leydig cell steroidogenesis should be taken into consideration [50].

Secondly, the expression of melatonin receptors in the testicular cells [24,25] may operate its local effect on testicular function. Melatonin has a crucial role in the conversion of androgen into estrogen by regulating aromatase transcription [51]. The significant increases in the concentrations of E2 concomitantly with the decreases in the values of RI and PI of testicular arteries that were noticed in the current study might highlight its potential role for regulating TBF because of the marked vasodilatory effect of estrogen and its role in testicular perfusion [52,53]. Similar studies also found strong correlations between Doppler indices of TBF (RI and PI) and E2 concentrations in goats [10,11] and stallion [52].

Thirdly, the results of the present study may be attributable to the effect of melatonin as a strong antioxidant agent and free radical scavenger on the cardiovascular system [13], since receptors of melatonin were identified throughout the cardiovascular system [54] including endothelial vascular cells [55]. In light of some studies in sheep and cows, supplementation of melatonin during pregnancy; especially in compromised conditions or during the second half of gestation; resulted in an improvement of uterine blood flow at least 25% increases [56–58]. These studies found melatonin increases blood flow by regulating the local tone of uterine vasculature [56] either by direct way through binding to melatonin receptors or indirectly by attenuating the oxidative stress in the vascular system [57]. In the current study, melatonin administration significantly increased the concentrations of NO in the melatonin group than in the control. This finding may contradict the notion that melatonin in the treated animals acts as a strong antioxidant agent or free radical scavenger. In fact, the crucial role of melatonin as an antioxidant may be potential in pathophysiological conditions exposed to oxidative stress [59]. In the present study, animals were not under stress conditions to

evoke this pivotal role of melatonin. Also, it is worthy to notice that NO produced in the vascular endothelial cells is rapidly inactivated by ROS such as superoxide anion radical (O_2^-) to produce peroxynitrite [60]. Exogenous administration of antioxidants, such as melatonin, can effectively scavenge ROS from the vasculature before its reacting with NO resulting in increases in NO bioavailability [56]. Authors think the stimulatory effect of melatonin on TBF that was observed in the present experiment may be attributed in part to increased levels of NO which is considered one of the powerful vasodilators [26,55]. Nitric oxide is synthesized from L-arginine methyl ester amino acid by NO synthase enzyme in various peripheral tissues, including the testicular vasculature and seminiferous tubules [27]. Some studies proposed the local effects of NO to regulate the distribution of oxygen, nutrients, and hormones by the testicular vessels [26,55]. Also, previous studies reported crucial roles of NO in the regulation of basal blood vessel tone, and testicular hemodynamics [26–28]. However, how exactly the mechanism of *in vivo* exposure to melatonin would impact local vascular tone is not investigated in the current study and further research is needed.

In the present study, concentrations of T and IGF-1 increased in the melatonin group compared to that in the control group. Our results were in agreement with those reported in other previous studies in goats [20,42], and sheep [50]. The mechanisms by which melatonin upregulates T secretion are very complex and may include both indirect pathways through its central effect on the hypothalamic-pituitary–gonadal axis and also direct effects on the Leydig cells of the testis [14]. Recently, it was reported that the stimulatory effect of melatonin on testosterone production by sheep Leydig cells was through enhancing the IGF-1 receptors [50]. Melatonin took two weeks in the current study to peak in the plasma because of slow absorption due to the administration of melatonin in an oil vehicle s.c. at the base of the ear (an area with very low blood supply). Also, it persisted so long in the plasma due to its slow clearance associated with its sustained release as explained by Li et al. [61]. Similar results were recorded in buffaloes [62], where the s.c administration of melatonin showed slow absorption and clearance.

In the current study, significant increases in some parameters related to semen quality such as acrosome integrity %, live/dead ratio, motility, and normal sperm morphology were observed in the melatonin group compared to the control. These results were in agreement with those found in other studies in goats [21,42], rams [24], and buffalo bull [63]. These improvements may be reflections of the pivotal systemic or local roles of melatonin in the testis, increases in TBF, and increases in the concentrations of steroids (T and E2) and IGF-1 following administration of melatonin. Insulin-like growth factor-1 plays an important paracrine or autocrine role in the regulation of testicular function [29,30]. Previous studies indicated that serum IGF-1 concentrations are genetically correlated with the reproductive traits of males [30]. Some studies also reported the localization of IGF-1 receptors on cattle and buffalo sperm, suggesting the importance of this factor in regulating sperm fertilizing capacity [64]. Authors think increases in IGF-1 production may be incorporated to improve the quality of semen as observed in the melatonin group compared to the control group. These findings were in agreement with those reported in buffalo [65]. The protective effect of IGF-1 may be attributable to its antioxidant properties [64] or its autocrine or paracrine roles on androgen production [29,50].

5. Conclusion

Collectively, slow-release melatonin treatments in Shiba goats increased TBF as measured by color pulsed Doppler ultrasonography (through decreasing values of RI and PI of testicular arteries). Concomitantly, there were improvements in some parameters of semen quality and increases in the concentrations of E2, NO, and IGF-1. Explaining the actual mechanism for blood flow changes, however, was beyond the scope of the current study and needs to be investigated in future studies.

Declaration of competing interest

The authors state that there is no conflict of interest.

CRediT authorship contribution statement

Haney Samir: Conceptualization, Project administration, Methodology, Investigation, Visualization, Data curation, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Paul Nyametease:** Investigation, Validation, Writing - review & editing. **Mohamed Elbadawy:** Investigation, Validation, Writing - review & editing. **Kentaro Nagaoka:** Visualization, Validation, Writing - review & editing. **Kazuaki Sasaki:** Visualization, Validation, Resources. **Gen Watanabe:** Supervision, Visualization, Validation, Funding acquisition, Resources, Writing - review & editing.

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