



THE BENEFICIAL EFFECTS OF ENRICHED DIET ON TESTICULAR BLOOD FLOW AND SEMINAL PARAMETERS USING COLOUR AND PULSED DOPPLER ULTRASOUND IN DOGS

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Summary

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Maintenance of the reproductive fitness quality in dogs is a field of interest in animal practice. Therefore, this study was conducted for the first time to predict the response of sub-fertile dogs to vitamin and mineral supplementation by evaluating testicular haemodynamics and measuring the changes in peak systolic velocity (PSV), end-diastolic velocity (EDV), resistance index (RI), and pulsatility index (PI). Dogs were categorised into three groups (15 in each group): control, vehicle group and supplemented group. Semen evaluation, blood sampling and Doppler were performed on days (D) 0, 30, 60, and 90. In the sub-fertile dogs, semen volume increased from D0 to D90, and Doppler indices correlated negatively ($P \leq 0.05$) with testicular blood velocities, nitric oxide metabolites (NO), testosterone levels, scrotal circumference, testicular volume, and testicular coloured area. Doppler indices decreased ($P \leq 0.05$) in the hypospermic males from D0 to D90 compared to the normal values. The supplementation improved significantly blood flow by elevating the testicular colouration and decreasing both Doppler indices as the increase in testicular coloured pixels in the supplemented males may be accompanied by an increase in testicular volume, testosterone, and nitric oxide levels.

Key words: canine, distal testicular artery, nitric oxide, pampiniform

INTRODUCTION

Almost about 15–20% of dogs used for breeding are affected by subfertility (Domosławska *et al.*, 2019). Dogs are suffering from sub-fertility due to hypospermia especially when linked with altered semen analysis parameters, as hypospermic dogs

may be sub-fertile. This problem could be due to nutritional deficiency besides the bad lifestyle (De Celis *et al.*, 1996; Agarwal & Prabakaran, 2005). The major problems in dogs suffering from sub-fertility are related to the poor semen

quality; however, several reports investigated many protocols to improve the semen quality in both humans and animals by supplementation of micronutrients (Rooke *et al.*, 2001). Besides nutritional factors, the n-3 poly fatty acids (PUFA) derived from fish could help in the improvement of sperm motility as reported in human (Agarwal & Prabakaran, 2005) and veterinary medicine (Liu *et al.*, 2016) studies in addition to vitamin E and zinc oxide that play an important role in organ protection against oxidative stress with an improvement of the sperm quality in humans (Raederstorff *et al.*, 2015), sheep (Yue *et al.*, 2010) and rabbits (Yousef *et al.*, 2003).

The application of colour-spectral wave Doppler is extensively used in human medicine (Sriprasad *et al.*, 2001). Doppler could be used in the detection of degree of spermatogenesis (Biagiotti *et al.*, 2002; Pinggera *et al.*, 2008), in addition many authors reported the importance of testicular Doppler velocities for prediction of fertility (Gloria *et al.*, 2018) as well as for determination of testicular pathology (Ortiz-Rodriguez *et al.*, 2017). However, few reports have studied the colour Doppler effect in dog testes (Carrillo *et al.*, 2011; Souza *et al.*, 2014) and penis (Abdelnaby *et al.*, unpublished data). Also, few literature reports evaluating the normal (Gumbsch *et al.*, 2002) and abnormal testicular vascularity in a dog (Günzel-Apel *et al.*, 2001) are available. To our knowledge, no paper has reported the influence of daily dietary supplementation on the distal suprastesticular vascularisation status in male dogs. Therefore, the aims of this study were to determine for the first time the beneficial effect of the enriched diet on the canine distal portion of suprastesticular arteries monophasic flow pattern in relation to testicular

volume, testosterone, nitric oxide levels, and seminal parameters using Doppler ultrasound.

MATERIALS AND METHODS

Ethical approval

This study was conducted at the Faculty of Veterinary Medicine, Cairo University between 1 September and 30 November 2019. The study protocol has been reviewed and approved by the Ethical Committee of Cairo University (Approval No Vet CU24112020251)

Complex supplementation preparation

The supplementation of a micronutrient complex was hypothesized to expand the effect on motility and testicular vascularity parameters. Dogs with sub-fertility (prolonged time of non-conception) associated with hypospermia in the form of low semen production (n=15) were supplemented daily for 60 days with a composition of vitamins and minerals. Such period is associated with the accurate critical time required to achieve the spermatogenesis process in canine (Soares *et al.*, 2009). This daily administration, abbreviated as ZFO3-VB6-B12-E, was composed of zinc oxide (10 mg/25 kg b.w.), folic acid (0.02 mg/kg b.w.), a packet of Omega-3 (fish oil 25% DHA, 10% EPA) + Vitamin B6 (pyridoxine 0.15 g) + Vitamin B12 (0.5 mg) and Vitamin E (5 mg/kg b.w.). The daily supplement must be freshly prepared before administration. This supplement in humans is applied at about 3500 mg/day, so the male dog dose was calculated by converting the dose based on human surface area and body weight 60 kg, therefore the obtained equivalent dose was $3500/60=58.55 \times 1.8=105$ mg/day (Nair & Jacob, 2016).

Animals and experimental design

German Shepherd dogs (n=15) with normospermia (body weight: 25–35 kg, age: 4–6 years) were selected for the study among those presented as usual semen donors for natural and artificial insemination and served as control group that received no treatment. All normal dogs underwent two-three monthly semen collections in the first four months before starting the study, with normal semen results (Tesi *et al.*, 2018). Hypospermic German Shepherd dogs (n=30) (body weight: 25–35 kg, age: 4–6 years) were simultaneously selected to avoid any possible seasonal effect on sperm parameters and categorised into two groups: one serving as a vehicle (n=15) that received the equivalent volume of supplement based on individual body weight (2 µL stock solution DMSO) and another group of supplemented hypospermic males (n=15). All animals were housed indoors, without any remarkable difference in the environmental condition. They were fed commercial food 3–4 cups/day with energy of 4000 kcal/kg and free access to water.

Each male was subjected by the operator to a general examination including history evaluation, clinical and ultrasonographic exam of all male genital organs including testes and penis (Alonge *et al.*, 2018a,b; Davidson *et al.*, 2009). At D0 in the early morning, all dogs were subjected to semen collection, blood sampling, testicular ultrasonographic B-mode and Doppler ultrasound examination of both distal suprastesticular arteries, then all parameters were assessed on monthly basis (D30, D60 and D90) to test the effects of food supplementation on seminal parameters, and testicular Doppler parameters compared to D0. Starting from D0, food enrichment was added to the diet for 60 days, while normal dogs did not receive

any type of supplement, only dry commercial diet.

Inclusion and exclusion criteria

Dogs suffering from subfertility in the form of hypospermia (n=30) were previously judged to be sub-fertile if their whelping rate was $\leq 75\%$ when bred appropriately to normal bitches. The inclusion criteria were dogs presented with primary infertility that received no treatment; while the exclusion criteria comprised previous hernia repair or another pelvic surgery.

Collection and evaluation of semen samples

Semen was collected in all males by digital manipulation; dogs came close to a bitch in estrus. Semen was collected in Falcon tubes 50 in three separated fractions: first pre-spermic, second spermatic (sperm rich), and third prostatic, then collected semen was transferred immediately to the laboratory for seminal analysis. Seminal quantitative factors (volume, number of sperm/ejaculation), as well as qualitative parameters (progressive motility, morphology, and head abnormalities %) were evaluated. Dog semen was evaluated manually subjectively, the second sperm-rich fraction only was evaluated for morphology, hundred sperm cells were evaluated for normality (normal shape and structure), while the abnormal sperm samples were categorised into one of the following groups: acrosome defects, mid-piece, tail and head defects as the most common abnormalities in dogs were mainly head anomalies.

Blood collection and assays

Before each ultrasound examination, blood samples were collected from the jugular vein and then centrifuged at

2000×g for 10 min. Plasma and serum samples were harvested and then stored at -20° C until assayed. Testosterone was analysed using ELISA commercial kits (DRG, Germany; Abdelnaby *et al.*, 2021b). For measuring nitric oxide in the form of its metabolites, 100 µL serum sample was mixed with equal volume Griess reagent and incubated for 15 min at room temperature. The intra- and inter-assay coefficients of variations were 5.3% and 6.9% respectively, the test total sensitivity was 0.227 µmol/L nitrites as previously reported (Abdelnaby *et al.*, 2018; Abdelnaby, 2020a).

Ultrasound evaluation

Before each ultrasound examination a scrotal circumference was measured by tape in both control and dogs with hypospermic sub-fertility. All ultrasonographic investigations were carried out by the same operator just after blood collection and before semen collection. All Doppler ultrasound examinations were performed with 7.5 MHz linear-array transducer with power: max (100%), pulse repetition frequency: 5 kHz (Abdelnaby, 2020b; Maher *et al.*, 2020a; El-Sherbiny *et al.*, 2020), optimisation of colour and pulsed wave Doppler parameters to detect very slow flows, Doppler angle: 50°–60°, size of the gate sample 1 mm, 2 colour maps, and wall filter =100 (EXAGO, France) (Abdelnaby & Abo El-Maaty, 2020; 2021).

- Doppler application and measurements

All dogs were simply restrained without analgesia or sedating agents. To eliminate the presence of air spacing, the hair on the scrotum was shaved well, and the amount of gel covered the transducer as previously performed in cows (Abdelnaby *et al.*, 2020a). After locating the testicular artery by the specific spectral

pattern graph, the three best successive waves with complete systolic and diastolic of a cardiac cycle were measured to evaluate the following Doppler velocimetry parameters: peak systolic velocity (PSV), Doppler indices expressed by resistance index (RI) as well as pulsatility index (PI) (Maher *et al.*, 2020b).

- Evaluation of pampiniform coloured area and testicular volume

Testicular length (L), height (H) and width (W) were measured using calipers, to estimate the testicular volume using the ellipsoid shape formula: $L \times W \times H \times 0.5236$ (Gouletsou *et al.*, 2008), while pampiniform coloured area percentage was measured by dividing pampiniform coloured area in pixels by area of the region in pixels. In addition, testicular coloured area in pixels was evaluated.

- Image analysis

Grey normal B mode/coloured Doppler images with two main colours were stored in flash memory after export from the ultrasound device for blood flow analysis. The coloured area was determined and counted in pixels using Adobe Photoshop CC, then each measurement was used to count the selected areas in pixels by magnetic lasso tool (Moxon *et al.*, 2015).

Statistical analyses

Data were processed using SPSS software. All data are presented as the mean ± standard error of the mean (SEM) and were first checked for normality. The seminal concentration was analysed in sub-fertile hypospermic compared to control at D0. Differences in head abnormalities, motility percentage, as well as volume and total count of sperms per ejaculation were analysed using repeated-measures ANOVA. Correlation coefficients among blood flow and semen pa-

rameters were calculated. Duncan multiple range test was used to differentiate significant means.

RESULTS

There was no significant difference between the right and left testes of the normospermic, vehicle and hypospermic groups of dogs with respect to testicular volume and Doppler measures. Fig. 1 presents the spectral Doppler differences between the right and left testes of the sub-fertile group at D0 (1A, 2A), D30 (1B, 2B), D60 (1C, 2C) and D90 (1D, 2D).

Testicular coloured area and percentage of coloured areas in pampiniform plexuses

The testicular blood flow and pampiniform plexus expressed by amount of blood supply were evaluated to measure the percentage of coloured area in this region. The colour area of supplemented group increased at the end of supplementation (Day 90, Fig.2A) compared to the start of supplementation (Day 0, Fig. 2B).

Semen characteristics

Semen volume, head anomalies, motility and morphology percentages in addition to the total count of sperms per ejaculation at D0, D30, D60 and D90 are shown on Fig. 3. Interestingly, the semen volume

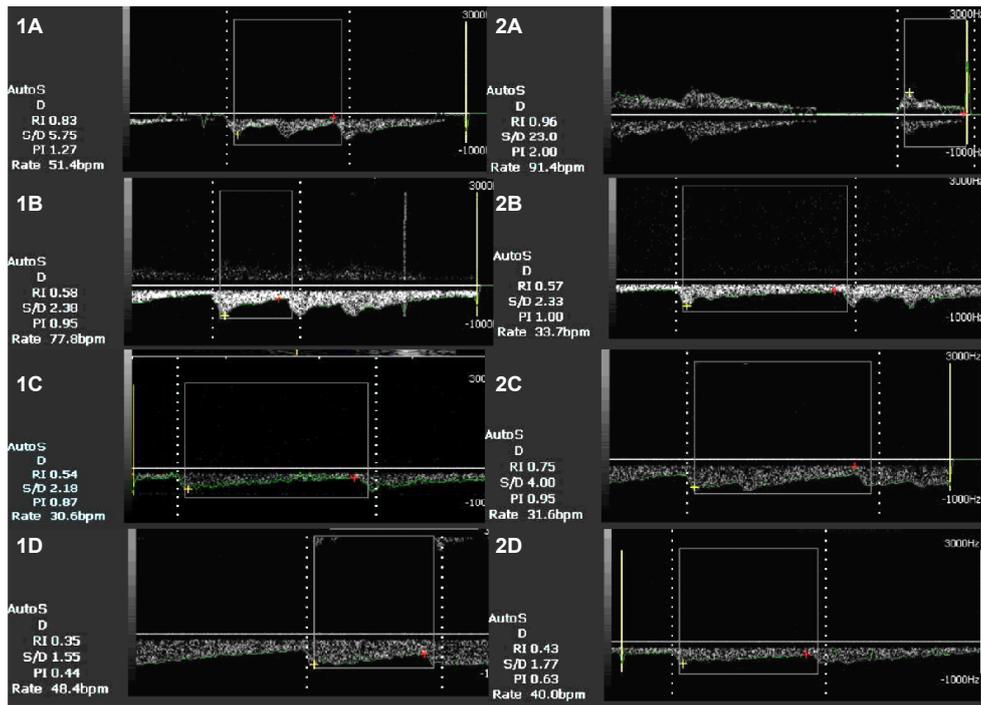


Fig. 1. Pulsed wave Doppler spectral graph of peak systolic velocity and end diastolic velocity of both testicular arteries in the right (1A-D) and left testes (2A-D) at day zero (1A, 2A), day 30 (1B, 2B), day 60 (1C, 2C) and day 90 (1D, 2D) in the sub-fertile supplemented group (n=15).

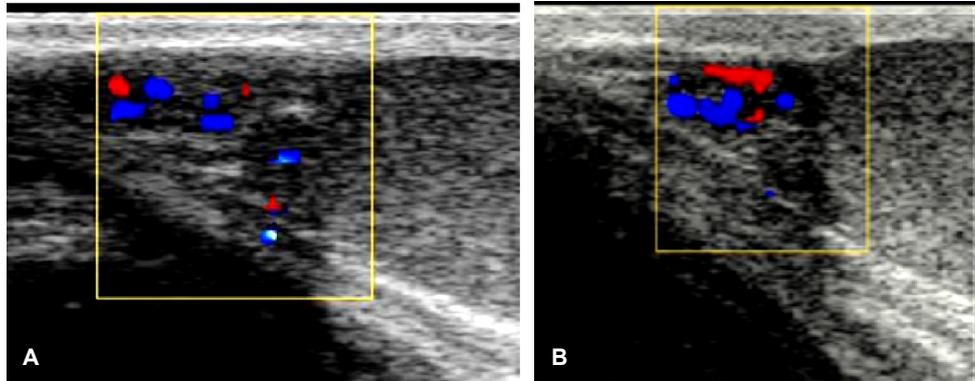


Fig. 2. Colour mode Doppler ultrasound showing pampiniform coloured area/pixels at day 30 (A) and day 90 (B) in the sub-fertile supplemented group (n=15).

increased from D0 value of 2.55 ± 0.01 mL to 4.51 ± 0.11 mL reaching a maximum at D 90 in the supplemented dogs, while the volume in control group ranged from 4.5 ± 0.32 to 6.12 ± 0.12 mL.

Semen concentration showed a significant ($P \leq 0.05$) increase from $80.56 \pm 15.32 \times 10^6/\text{mL}$ at D0 to $230.25 \pm 58.21 \times 10^6/\text{mL}$ at D90 in sub-fertile hypospermic treated and vehicle groups, while in the normal dogs the semen concentration ranged from 130.45 ± 41.21 to $280.27 \pm 31.25 \times 10^6/\text{mL}$. Further, the total number of sperms per ejaculation increased ($P \leq 0.05$) from $450.36 \pm 32.65 \times 10^6/\text{total ejaculated}$ at D0 to $500.02 \pm 36.25 \times 10^6/\text{total ejaculated}$ at D30 and from $600.32 \pm 37.25 \times 10^6/\text{total ejaculated}$ (D60) to $703.85 \pm 61.25 \times 10^6/\text{total}$ (D90) after the end of supplementation in subfertile treated group, but these values were low in comparison to normal dogs where they ranged from 800.34 ± 64.31 to $900.76 \pm 25.47 \times 10^6/\text{total ejaculated}$. The percentage of motility ($P \leq 0.05$) increased from D0 value of $60.51 \pm 4.55\%$ to $80.09 \pm 6.12\%$ at D90 and was similar to the control values that ranged from 75.11 to 85.06% . The morphology and head abnormalities percentages The percentage of spermatozoa with

normal morphology increased from D0 value of 55.05 ± 3.62 to 85.32 ± 8.44 at D90 in the supplemented group ($P \leq 0.05$) and was similar to that in control dogs (from 80.32 ± 6.32 to $85.05 \pm 3.62\%$). Finally, the head abnormalities in the subfertile hypospermic dogs decreased significantly ($P \leq 0.05$) from $20.12 \pm 1.32\%$ (D0) to $10.08 \pm 2.01\%$ (D90).

Distal suprastesticular arterial Doppler measurements

The distal supra-testicular arteries pulsatility index (PI) mean values were affected by the day of supplementation. As shown on Fig 4, the PI of both testicular arteries demonstrated a decrease ($P \leq 0.05$) from D0 value of 1.22 ± 0.02 to 0.45 ± 0.01 that reached a minimum value at D90 in the supplemented group when compared to the normal dogs' values from D0 till D90 (1.15 ± 0.02 – 1.02 ± 0.03), while in the vehicle group values ranged from 1.18 ± 0.01 to 0.84 ± 0.01 . The resistance index (RI) showed a decline from a D0 value of 0.95 ± 0.02 to 0.43 ± 0.02 ($P \leq 0.05$) with minimum value at D90 in the supplemented group vs normal dogs (0.88 ± 0.03 – 0.81 ± 0.01) and vehicle group (0.99 ± 0.01 – 0.69 ± 0.01).

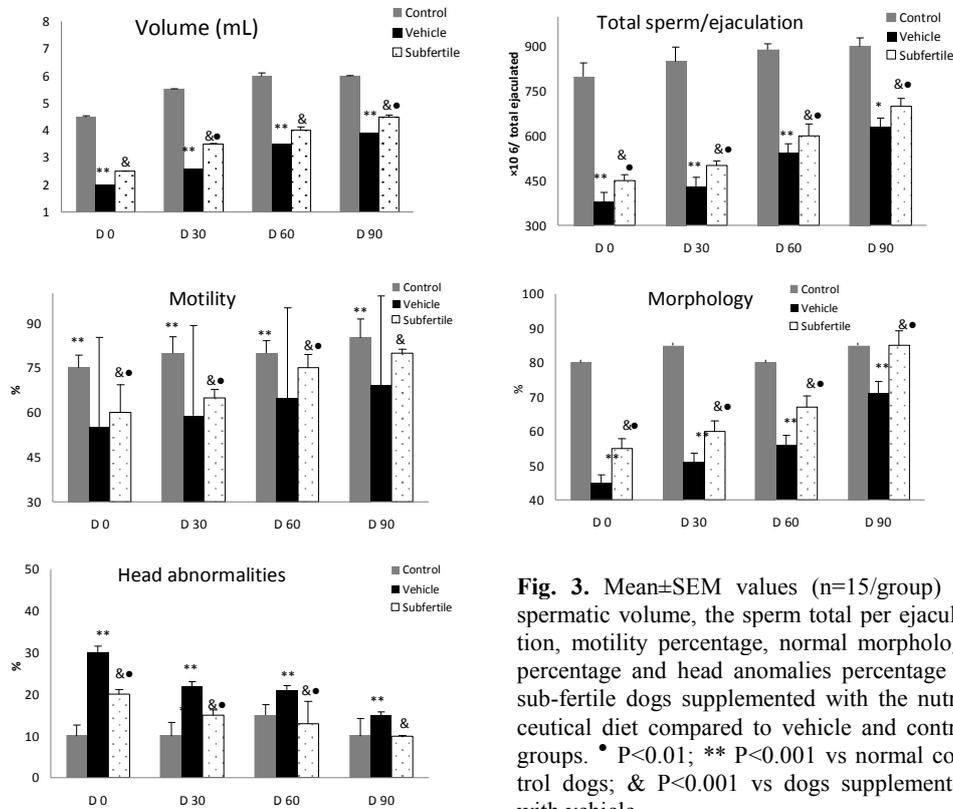


Fig. 3. Mean±SEM values (n=15/group) of spermatic volume, the sperm total per ejaculation, motility percentage, normal morphology percentage and head anomalies percentage in sub-fertile dogs supplemented with the nutraceutical diet compared to vehicle and control groups. • P<0.01; ** P<0.001 vs normal control dogs; & P<0.001 vs dogs supplemented with vehicle.

The testicular volume measured by ultrasound increased ($P \leq 0.05$; Fig. 4) from a D0 value of $9.32 \pm 0.11 \text{ cm}^3$ to $11.66 \pm 0.12 \text{ cm}^3$ at D90 and was higher than control value range (from 10.14 ± 1.21 to 10.19 ± 0.65). The percentage of pampiniform coloured area/pixels measured by Adobe Photoshop by freezing the image (Fig. 3 and Fig. 4), increased from 40.21 ± 6.22 (D0) to 70.94 ± 5.12 (D90) while in the vehicle group it ranged from 44.5 ± 7.32 to 51.5 ± 2.84 . As shown in Table 1, dogs were distributed on the basis of their seminal concentrations immediately after the semen collection by masturbation. The seminal concentration in normal dogs ranged from $130\text{--}280 \times 10^6/\text{mL}$, while in subfertile dogs semen concentration

ranged within $80\text{--}230 \times 10^6/\text{mL}$. Testicular echotexture circumference, Doppler measurements and hormonal levels were measured at the start of supplementation (D0) until the end of treatment (D90) (Table 2). Scrotal circumference, PSV, EDV and testicular coloured area increased ($P \leq 0.05$) on day 90 (14.99 ± 3.25 , 16.25 ± 1.62 , 4.57 ± 0.02 and 5689 ± 11.21 respectively) after the end of mixture supplementation, in addition to testosterone and nitric oxide levels that showed a pattern of increase at day 90 vs day 0, but in the control dogs there was not any significant difference between testicular echotexture circumference, Doppler measurements, and hormonal levels (Table 2).

The two distal supra-testicular blood flow Doppler indices (RI and PI) values were negatively correlated with Doppler testicular blood velocities (PSV and EDV), nitric oxide, plasma testosterone, scrotal circumference, testicular volume, and testicular coloured area (Table 3).

DISCUSSION

Sperm motility is considered one of the most critical male fertility factors (Knox *et al.*, 2008), and in this study, the motility and morphology % were significantly influenced by zinc, considered as important dietary factor (Hussain *et al.*, 2011). The scrotal circumference increased in subfer-

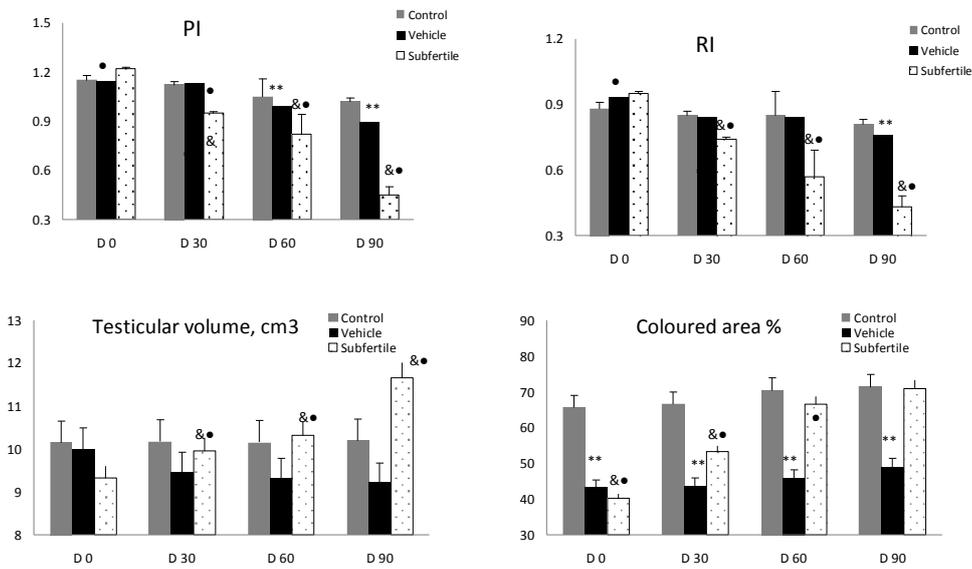


Fig. 4. Mean±SEM values (n=15/group) of Doppler parameters measured in the distal suprastesticular arteries: pulsatility index (PI), resistance index (RI), testicular volume and percentage of pampini-form coloured area/pixels in sub-fertile dogs supplemented with the nutraceutical diet compared to vehicle and control groups. • P<0.01; ** P<0.001 vs normal control dogs; & P<0.001 vs dogs supplemented with vehicle.

Table 1. Dog distribution on the basis of seminal concentration (m/ml) obtained after semen collection in sub-fertile hypospermic treated and vehicle groups compared to control at D0 before any treatment

Sub-fertile hypospermic treated and vehicle groups (n=30)			Normal control (n=15)		
Concentration	N	%	Concentration	n	%
≤80	15	50%	≤130	2	13.33%
>80≤130	7	23.33%	>130≤180	4	26.66%
>130≤180	6	20%	>180≤230	3	20%
>180≤230	2	6.66%	>230≤280	6	40%
Total	30	100	Total	15	100

Table 2. Testicular echo-texture circumference, Doppler measurements and hormonal levels (mean±SEM) in sub-fertile and normal control dogs

Parameter	Subfertile hypo-spermic (n=30)		Normal controls (n=15)	
	D0	D90	D0	D90
Scrotal circumference(cm)	11.21 ^a ±2.36	14.99 ^b ±3.25	14.55±3.21	14.98±1.58
Peak systolic velocity (cm/s)	14.32 ^a ±2.55	16.25 ^b ±1.62	16.99±2.15	17.01±3.01
End diastolic velocity (cm/s)	4.01±0.84	4.57±0.02	4.86±0.01	4.92±0.03
Testicular coloured area (px)	5021 ^a ±21.21	5689 ^b ±11.21	5790±22.61	5798±21.25
Testosterone (ng/mL)	5.02 ^a ±0.01	6.12 ^b ±0.66	6.54±0.06	6.64±0.02
Nitric oxide (µmol/L)	39.14 ^a ±3.25	55.26 ^b ±6.32	64.85±14.21	65.01±11.34

Means with different superscripts within rows are significantly different at P<0.05 in subfertile hypo-spermic group.

Table 3. Correlation coefficients between distal suprastesticular blood flow measurements and semen parameters in dogs (n=30)

Paired measures	Correlation coefficients
PI vs. scrotal circumference	-0.618*
PI vs. testicular volume	-0.564**
PI vs. testicular coloured area	-0.759*
PI vs. testosterone	-0.874**
PI vs. nitric oxide	-0.793*
PI vs. peak systolic velocity	-0.753**
PI vs. end diastolic velocity	-0.766*
RI vs. scrotal circumference	-0.732
RI vs. testicular volume	-0.845*
RI vs. testicular coloured area	-0.705*
RI vs. testosterone	-0.682**
RI vs. nitric oxide	-0.491*
RI vs. peak systolic velocity	-0.832*
RI vs. end diastolic velocity	-0.901*

* significant at P<0.05; ** means significant at P<0.01.

tile group at D90 compared to D0. The pampiniform plexus colouration also increased in the present study, as colour Doppler can be used to measure blood flow parameters of the spermatic vein and testicular arteries related to the quality of blood flow in a region (Kim *et al.*, 2015). In this study, the distal suprastesticular arteries were evaluated in both groups as

these arteries are the most prominent in the spermatic cord, so the use of Doppler was very critical to determine the special distal testicular artery located in the spermatic cord at pampiniform plexus as reported by other studies (Gumbsch *et al.*, 2002). In this study, the distal suprastesticular arterial Doppler indices (PI and RI) reported a significant decrease from a D0 value that reaches a minimum value at D90, affecting positively the testicular blood flow pattern when compared to the values in control males. Our study revealed that the RI and PI were negatively correlated with Doppler testicular blood velocities (PSV and EDV), nitric oxide, plasma testosterone level, testicular volume, and testicular coloured area. Similarly, a study in patients with varicocele (Rehman *et al.*, 2019), revealed a negative correlation only between progressive motility and circumference and RI of sub-capsular ($r=-0.236$; $P=0.07$) and intra-parenchymal arteries ($r=-0.28$; $P=0.02$). In agreement with our results, Semiz *et al.* (2014) reported a negative correlation between vascularity dynamics pattern and seminal parameters, except for a relationship between testicular arteries peak systolic velocity and semen count in humans with clinical varicocele. Another human

study found out that testosterone was the only known hormone correlating with the Doppler flow indices and analysed Doppler velocities, concluding that there were no differences between flow parameters in both testes (Wielgoś *et al.*, 2000). The evaluation for a breeding examination soundness of the normal animals showed that all control dogs were healthy and able to participate and complete the study. Testicular volume measurement in this study by ultrasonographic scanning was done to evaluate the clear echogenic parenchyma with a thin clear white line called mediastinum testis as previously shown in studies in dogs (Nyland & Matton, 2004).

Accurate determination of testicular volume is probably of great benefit to evaluate the reproductive development of the animal, and it also serves as a tool for diagnosing diseases that may lead to testicular dysfunctionality. The testicular volume determination showed no significant difference between both testes in all dogs, in contrast with a study reported in Tori and Estonian breed stallions (Kavac *et al.*, 2003) and dogs (Souza *et al.*, 2014). Dogs suffering from sub-fertility problems were considered common challenges in small animal management. Breeders usually seek accurate specific treatments to solve this huge problem. Several studies reported many different procedures to mend and enhance sperm quality (Kirchhoff *et al.*, 2017), but this study is the first to report the monthly changes of daily dietary supplementation correlated with blood flow and the pampiniform plexus region colouration and scrotal circumference. The major constraining factors of breeding programmes are represented by poor sperm function that affects the semen quality, so supplementation with a certain daily intake of micronutrients is very important to im-

prove the semen quality. The results in this investigation demonstrated that a healthy diet, enriched with vitamin E, zinc oxide, n-3 polyunsaturated fatty acids with vitamin B6 and B12, and folic acid mixture increased significantly the number of spermatozoa, enhanced motility and morphology percentage, and improved the total number of sperm/ejaculate in hypospemic sub-fertile dogs. Similar results were measured in 15 dogs with low fertility using selenium and vitamin E only (Domosławska *et al.*, 2015), in four dogs with poor semen quality by vitamin E (Kawakami *et al.*, 2015), and in five healthy dogs by n-3 PUFA (Risso *et al.*, 2016), but few reports are available on using the combination of all of the mentioned supplements. Vitamin E, another micronutrient, is one of the major antioxidants in the body that could play a major role in protecting different organs against oxidative stress and stabilising the sperm membranes by complex formation. In contrast to our findings, Kirchhoff *et al.* (2017) reported no effect on seminal parameters in healthy Cairn Terriers supplemented with a high dose of selenium and vitamin E, however it was mentioned before that if daily supplementation was useful for semen quality in humans (Gaskins & Chavarro, 2018), the excess of usage or excessive dose may lead to sperm damage (Kumalic & Pinter, 2014). It was in fact demonstrated that any excess in vitamin E with folic acid can adversely affect sperm motility by decreasing semen quality in men (Danikowski *et al.*, 2002). The present results showed that the repeated monthly semen collection did not improve seminal parameters, but in the hypospemic dogs, the mixture supplementation with the diet improved the semen quality and decreased the percentage of the static sperm at D30 vs D0. This

result is in agreement with a previous report, in which authors have supplemented vitamin E and selenium only, and demonstrated a significant improvement at the same period – D30 after one month of supplementation (Domosławska *et al.*, 2015). In this current study, there was an improvement in morphology and total sperm count/ejaculation after daily supplementation of the mixture in dogs with hypospermia (treated group), similar to another study (Wong *et al.*, 2002) that reported the same increase in total sperm count after adding the supplement to the diet. Vascular perfusion activation with enhancement of the immune system responding to supplementation was associated with increased production of nitric oxide as previously discussed (Hussein *et al.*, 2012; Abdelnaby *et al.*, 2020b). The elevation of plasma nitric oxide in the form of nitrites in the supplemented group enhanced the testicular vascularisation as NO plays an important role in the vascular organ function (Bucala *et al.*, 1991).

In this study, the measurement of the distal testicular artery was performed to get a constant wave at each examination. In agreement with our data, other authors reported that the testes showed a pattern of monophasic spectral waves as they are parenchymal organs which required a constant perfusion (Carvalho *et al.*, 2008) which is similar to the monophasic wave in testicular arteries in humans (Tanriverdi *et al.*, 2006) and dogs (Gumbsch *et al.*, 2002); however another study reported that there a biphasic wave pattern in the spermatic cord in dogs (Carrillo *et al.*, 2011), similar to that obtained in the horse (Pozor & McDonnell 2004). Finally, the wave characteristic parameters were similar between groups in this study. These results could help the veterinarians to determine the normal testicular Doppler

parameters in healthy adult dogs (Zelli *et al.*, 2013).

CONCLUSION

The resulting relevant data related to normal group testicular volume, blood flow and pampiniform colouration throughout three months could help in predicting canine testicular function. The daily supplementation significantly improved testicular haemodynamics by a marked increase in testicular colouration, flow volume and amount of a coloured area. The daily use of tested supplementation for 60 days is recommended as both Doppler indices with total number of sperm per ejaculation could predict the supplementation effect at day 60.

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