Received: 8 December 2021 | Accepted: 9 February 2022

DOI: 10.1111/rda.14096

Reproduction in Domestic Animals WILEY

ORIGINAL ARTICLE

Supplemental dietary zinc sulphate and folic acid combination improves testicular volume and haemodynamics, testosterone levels and semen quality in rams under heat stress conditions

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Abstract

This study was aimed to investigate the combined effect of zinc sulphate and folic acid (ZnF) dietary supplementation on testicular haemodynamics (TH), testicular volume (TV), plasma testosterone levels (T) and semen quality of rams under heat stress conditions. Fifteen Ossimi rams were allocated to three groups: (1) G0 ($n = 5$) received only basic diet; (2) G1 ($n = 5$) received basic diet +ZnF (Zn, 0.4 mg/kg bw; F, 0.02 mg/kg bw) and (3) G2 ($n = 5$) received basic diet +ZnF (Zn, 0.8 mg/kg bw; F, 0.04 mg/kg bw) daily for 60 days. TH was evaluated using colour (testicular coloration, TC) and spectral modes [resistive index (RI) and pulsatility index (PI)] Doppler of the supratesticular arteries (proximal and distal parts, STA). Semen traits including progressive motility (PM), alive sperm % (AS), sperm viability (SV), sperm abnormalities (SA) and acrosome integrity (AI) were also assessed. The examinations were carried out one month before (D-30), the beginning of ZnF inclusion in the diet (D 0) and continued for the successive two months (D 30 and D 60). TH was significantly ($p < .05$) improved at D 30 and D 60, evidenced by lowering both RI and PI and increasing of TC in G1 compared to G0 and G2. In addition, both TV and serum T levels were elevated ($p < .05$) at D 30 and D 60 in G1 compared to other groups. Semen quality parameters (PM, AS, SV and AI) were significantly ($p < .05$) augmented in the same trend as TH, TV and T in G1 versus G0 and G2. A marked decrease ($p < .05$) in SA % was noticed at Days 30 and 60 after ZnF inclusion in G1 compared to G0 and G2. In conclusion, supplementation of the summer diet with ZnF improved the whole reproductive functions such as testicular haemodynamics and semen quality of rams housed in heat stress conditions.

KEYWORDS
doppler, folic acid, ram semen, testicular artery, testosterone, zinc

1 | INTRODUCTION

Male fertility equally shares the pregnancy rate success in animal reproduction, which considered a substantial economic factor in the livestock industry. The testes are highly condensed reproductive organ, with low seminiferous tubules oxygen tension (Reyes et al., 2012). The only route to deliver oxygen and nutrients to the testes is the blood. Testicular physiological functions depend on the maintained and controlled testicular blood flow. Many reports have shown that any perturbations in the testicular haemodynamics could affect negatively the sperm production in rams (Batissaco et al., 2013), bulls (Claus et al., 2019) and stallions (Bollwein

et al., 2008). As testicular haemodynamics have a substantial impact on the sperm quality, more studies are needed for amelioration of the testicular blood flow (TBF) in farm animals.

Doppler ultrasound could be used in detection of the spermatogenesis potential (Biagiotti et al., 2002; Pinggera et al., 2008); in addition, many authors have reported the importance of testicular Doppler velocities in prediction of animal fertility (Gloria et al., 2018) and in determination of testicular pathology.

Oxidative stress (OS) is provoked by the heat stress, which in turn affects negatively the semen quality in terms of motility, viability and fertilizing capacity in rams (Cruz Júnior et al., 2015), bulls (Vince et al., 2018), buffalo bulls (Koonjaenak et al., 2007) and bucks (Wang et al., 2015). OS state occurs due to imbalance between the reactive oxygen species (ROS) generated and testicular antioxidant defence systems. ROS production increases dramatically during heat stress conditions through decline of superoxide dismutase (SOD) activity and SOD1 mRNA level that leads to increase in the mitochondrial superoxide anion (SOA) production. SOA has a substantial role in decrement of TBF through inactivation of nitric oxide in the peroxinitrite formation reaction, which alters endothelium-dependent vasodilation (Landmesser et al., 2003).

Zinc functions as a cofactor for approximately 80 metalloenzymes, integrated in DNA transcription, steroid receptors expression, testosterone production and function via Zn-dependent metalloenzyme 5α-reductase and synthesis of proteins (Prasad, 1991). Moreover, zinc plays a pivotal role in spermatogenesis, testicular development, sperm concentration (Wong et al., 2001) and sperm motility (Wong et al., 2002).

Folic acid, which delivers carbons for synthesis and methylation of DNA, is serious to spermatogenesis and acts as free radicals scavenger. Moreover, it hangs on zinc for better use, bioavailability and establishing the synergistic properties (Azizollahi et al., 2013; Favier et al., 1993). Synthetic combined forms of folic acid and zinc sulphate effectively act as antioxidants (Joshi et al., 2001), as the heat-stress mediated lower testicular blood flow is mainly caused by oxidative stress. Since zinc sulphate and folate intervention act as a potent antioxidant, we hypothesized that dietary zinc folate combination could ameliorate heat-stress mediated lower testicular blood flow and improve semen quality parameters. So, this study aimed, for the first time, to investigate the effects of daily dietary zinc sulphate and folic acid intervention on testicular haemodynamics, testosterone levels and semen quality parameters of rams housed under environmental heat stress.

2 | MATERIALS AND METHODS

This study was carried out during the period from May to July 2021. All experimental procedures were performed according to the international animal care committee in the Faculty of Veterinary Medicine, Cairo University, and conducted on the small ruminant farm belonging to the Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University.

2.1 | Animals housing and management

Fifteen sexually mature Ossimi rams, local Egyptian genotypic breed, aged 1–2 years old and weighing 40–50 kg have been used in the current study. All rams were considered to be apparently healthy in terms of cardiovascular, internal and reproductive diseases after physical, andrological and ultrasound examinations. They were housed and exposed to normal daylight, temperature and humidity. According to the NRC instructions, each ram was fed 400 g concentrate and 850 g green grass. Mineral supplements and fresh water were available ad libitum. Vaccination and deworming programmes were performed routinely. For estimating rams' heat stress, June, July and August were considered as the hottest months (Temperature >30°C; Relative humidity > 60%) in Egyptian climatic conditions (Hedia et al., 2020) based on the temperature humidity index (THI) calculation, following the equation presented by Papanastasiou et al. (2014) [THI = T − (0.31–0.31 RH)(T − 14.4)], where THI < 22.2 indicates no heat stress, and THI > 25.6 indicates severe heat stress. After calculation, the THI values during the experimentation were at least 28.06. Therefore, the animals used in the present study were exposed to environmental heat stress.

2.2 | Experimental design

Rams were randomly allotted to three groups as follow: (1) control group ($n = 5$) received basic diet without any additives (G0), treated groups ($n = 10$) received basic diet and oral administration of either zinc sulphate ($ZnSO_4$) and folic acid (F) formulation (0.4 mg and 0.02 mg/kg bw respectively; $n = 5$; G1) or (0.8 mg and 0.04 mg/kg bw respectively; $n = 5$; G2) as previously recorded (Abdelnaby et al.,2021; Alonge et al.,2019).

The treated groups received daily formulated supplement (zinc sulphate + folic acid) per-os for 60 days, as this period of examination is accompanied by the adequate time required to complete ram spermatogenesis process (Schanbacher et al., 1974). All rams (control and supplemented groups) were subjected to ultrasonographic assessment via Doppler scanning of the testes once monthly starting from 30 days before the treatment (D −30), day of starting administration (D 0) and through the study (D 30 and D 60) together with blood collection for testosterone assay and semen collection for evaluation of sperm individual motility, alive percentage, abnormalities, viability and acrosome integrity.

2.2.1 | Ultrasound assessment

Ultrasound examinations were performed by the same operator via Doppler scanner equipped with 5 MHz linear-array probe (EXAGO, France) with angle 45° and brightness 70% (Abdelnaby et al., 2018, 2021; Abouelela et al., 2021). Rams were restrained without sedation. Doppler colour and spectral parameters were optimized to measure the supra-testicular arteries (proximal and

distal) blood flow Doppler indices in the form of resistive index (RI) and pulsatility index (PI; Souza; Abdelkhalek et al., 2022; et al., 2014).

The testicular volume was measured using this equation: length (L) × width (W) × height (h) × 0.5236 (Abdelnaby, Emam, et al., 2021; Gouletsou et al., 2008) after obtaining transverse and longitudinal frozen images, while plexus coloration % was measured by pampiniform coloured area/pixels divided by area of the region /pixels as previously examined in the form of corpus luteum in cows (Abdelnaby et al., 2018). The coloured area was outlined by the lasso tool using adobe Photoshop software (Kutzler et al., 2011).

2.2.2 | Blood sampling and testosterone levels

On the day of Doppler inspection, blood samples were drained from the jugular vein into heparinized tube and centrifuged (1200 g for 15 min) for plasma retrieval (stored at −20°C). For assessment of plasma testosterone concentrations, Commercial ELISA kits (BioCheck, Inc.) were used. The intra- and inter-assay coefficients of variation were 3.3% and 4.8%, and assay sensitivity was 0.05 ng/ml.

2.2.3 | Semen collection and evaluations

Semen samples (n = 60; one sample per each animal in each time point 15 × 4) were collected at early morning using an artificial vagina (length 11 cm, diameter 6 cm; Minitube, Germany) adjusted at 42°C with lubrication. Immediately after collection, ejaculates were transferred to the laboratory and were placed in a warm water bath at 37°C. Semen samples were evaluated using the following standard laboratory techniques.

Individual motility

Individual motility was assessed in semen samples diluted with 2.9% sodium citrate dihydrate solution, spread almost evenly under a glass cover slide and examined microscopically (Olympus BH-2, Olympos Optical Co., Ltd.) using adjusted hot stage at 38–40°C. Individual sperm motility per cent was determined on a subjective scale of 0%–100% to the nearest 5% after viewing several microscopic fields.

Alive sperm percentage and sperm abnormalities

Alive sperm percentage or structural membrane integrity was evaluated using eosin-nigrosin stain. Duplicate smears from each sample were stained by mixing a drop of semen sample with two drops of the stain on a warm glass slide. A total of 200 sperm cells were examined randomly, 100 in each of the two smears. Normal alive spermatozoa exclude the eosin stain and appear white in colour, whereas dead spermatozoa (those with loss of membrane integrity) take up the eosin stain and appear pinkish in colour. At the same slide used for alive sperm assessment, total sperm abnormalities were evaluated (Campbell et al., 1956). A total of 200 sperm cells were examined randomly, and major spermatozoa abnormalities (proximal and distal cytoplasmic droplets, giant/pyriform heads, bent/coiled tails and deformed mid-piece) were recorded.

Functional membrane integrity

The hypo-osmotic swelling test (HOST) was used as a complementary test to the viability assessment protocol to evaluate the functional integrity of the sperm plasma membrane. Sperm cells with resistant membranes exhibited a swelling around the tail, such that the flagella became curled and the membrane maintained a swollen 'bubble' around the curled flagellum. The assay was performed by mixing 30 µl of semen with 300 µl of 100 mOsm/kg hypo-osmotic (9 g fructose plus 4.9 g sodium citrate per litre of distilled water) solution in a 1.5 ml micro-centrifuge tube (Revell & Mrode, 1994). This mixture was incubated (37°C) for one hour, and 20 µl of the mixture was placed on a microscope slide, mounted with a cover slip and immediately evaluated (400×) under the bright field microscope. A total of 200 spermatozoa were counted in at least five different microscopic fields, and percentages of sperm with swollen and curled tails were then recorded.

Acrosome integrity

Acrosome integrity was estimated using specific stain (Spermac stain, FertiPro N.V., Beernem, Belgium) according to Chan et al. (1999). A total of 200 sperm were counted in several microscopic fields using a bright field microscope under oil immersion lens (1000×), and the percentage of the intact acrosome (dark green acrosome with faint green head and tail) was recorded.

2.3 | Statistical analysis

In this study, all ultrasonographic data for each ram were pooled due to were no significant differences between the right and left testes. For data homogeneity and normality, Levene's test and Shapiro–Wilk test were used. Data for Doppler parameters, plasma testosterone concentrations and semen quality parameters were expressed as means ±standard error of the mean (*SEM*). Repeated measures two-way ANOVA was used for studying the effect of the treatment as a fixed factor and the time as a repeated factor followed by Bonferroni post hoc test. Means were considered significantly different at value of p <.05. The SPSS software (SPSS Inc.; Version 26.0) was used for all analyses.

3 | RESULTS

3.1 | Doppler examination

As shown in Figure 1, the proximal and distal supra-testicular arteries PI were affected by supplementation G1 at Day 30 (1.25 ± 0.01 for the proximal branch and 1.14 ± 0.01 for the distal branch) till Day 60 (1.18 ± 0.01 for the proximal branch and 0.98 ± 0.01 for the distal branch) (Figure 1a,b), while PI showed a slow pattern of

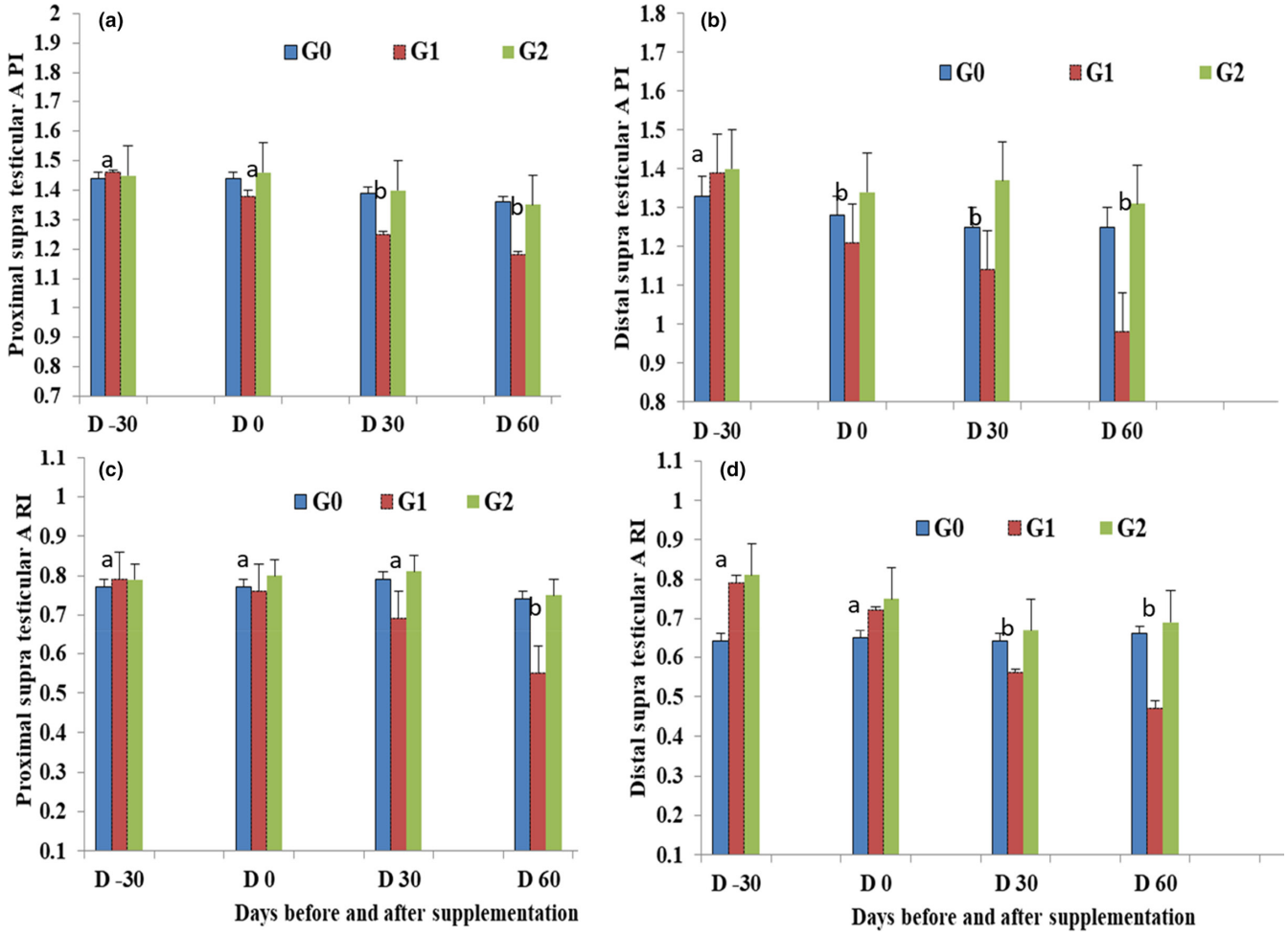

FIGURE 1 Mean value ±*SEM* of the proximal supra-testicular artery pulsatility index (PI, a), distal supra-testicular artery PI (b) and resistive index (RI) of both proximal (c) and distal supra-testicular artery (d) in both control and supplemented rams with zinc sulphate ($ZnSO_4$, 0.4 mg/kg bw), and folic acid (0.02 mg/kg bw) and rams supplemented (with $ZnSO_4$, 0.8 mg/kg bw), and folic acid (0.04 mg/kg bw) during days before and after supplementation

decline in the supplemented G2 group at Day 30 (1.41 ± 0.01 for the proximal branch and 1.37 ± 0.01 for the distal branch) till Day 60 (1.35 ± 0.01 for the proximal branch and 1.31 ± 0.01 for the distal branch) compared to other groups. RI values of the proximal supra-testicular artery were declined at Day 30 (0.69 ± 0.01) till Day 60 (0.55 ± 0.01) after supplementation by first dose (G1). In addition to the distal branch RI was also declined at Day 30 (0.56 ± 0.01) till Day 60 (0.47 ± 0.01) as shown in Figure 1c,d, while RI values were declined in the supplemented G2 group at Day 30 (0.81 ± 0.01 for the proximal branch and 0.67 ± 0.01 for the distal branch) till Day 60 (0.75 ± 0.01 for the proximal branch and 0.69 ± 0.01 for the distal branch) compared to other groups.

Both testicular volume and pampiniform coloration % results are presented in Figure 2a,b. The testicular volume was elevated by supplementation as the range of volume was calculated at Day 30 till Day 60 (86.25 ± 2.33 to 70.21 ± 2.58) in the supplemented G1 males, while in supplemented G2 group, the testicular volume was slowly elevated at Day 30 till Day 60 (61.25 ± 3.25 to 62.33 ± 6.32). While the ranges were observed in the normal males (62.14 ± 4.33 to 63.21 ± 1.32), pampiniform coloration % was increased in the supplemented G1 group at Day 30 (78.31 ± 5.26) and at Day 60 (80.47 ± 7.33).

3.2 | Testosterone levels

As depicted in Figure 3, means of plasma testosterone levels in G1 were significantly increased at D 30 and D 60 in comparison with G0 and G2. Moreover, plasma testosterone levels showed a significant ($p < .05$) decrease in G2 at D 30.

3.3 | Semen parameters

The effects of supplementation of ration with zinc sulphate and folate on ram semen parameters were summarized in Table 1. Percentages of individual motility, alive, normal sperm, structural membrane integrity and acrosome integrity were significantly higher

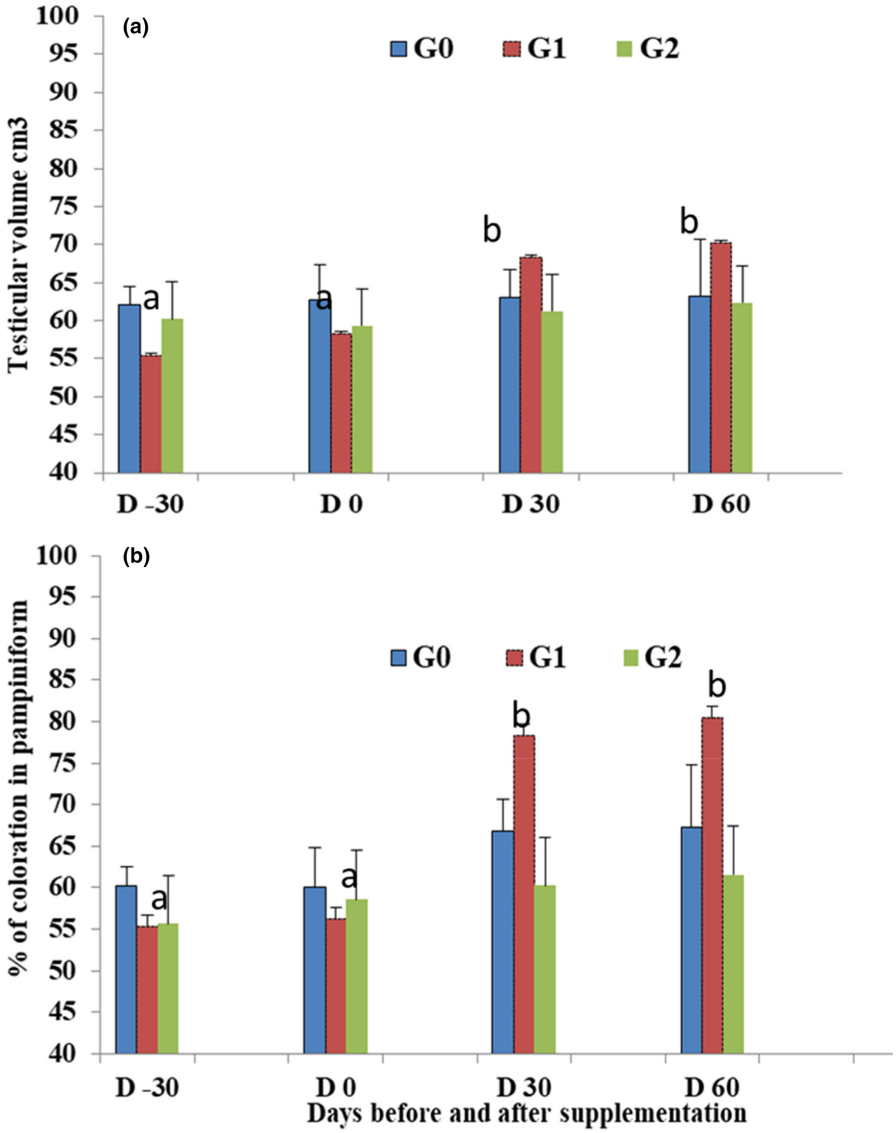

FIGURE 2 Mean ±*SEM* of rams testicular volume (a) using the equation for ellipsoid organ and pampiniform coloration % (b) measured in both control and supplemented rams with zinc sulphate ($ZnSO_4$, 0.4 mg/kg bw), and folic acid (0.02 mg/kg bw) and rams supplemented (with $ZnSO_4$, 0.8 mg/kg bw), and folic acid (0.04 mg/kg bw) during days before and after supplementation

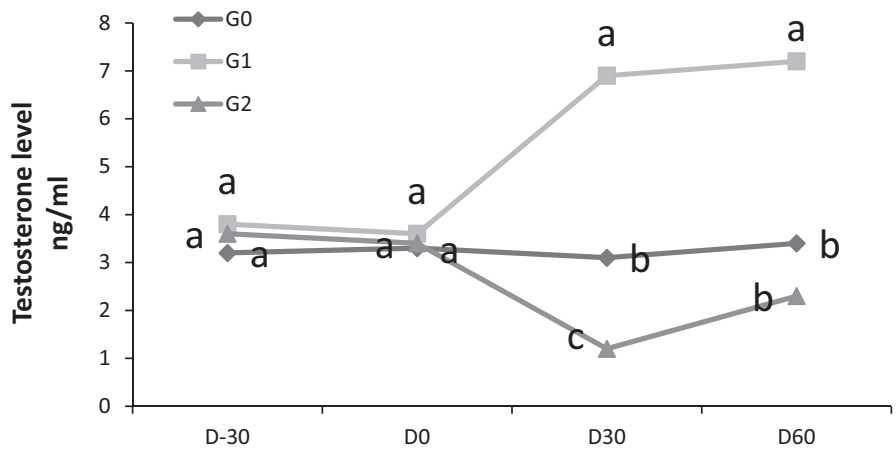

FIGURE 3 Mean ± *SEM* of monthly plasma testosterone concentrations in Ossimi rams after dietary zinc sulphate and folic acid formula supplementation (G0; $n = 5$ control; G1; $n = 5$; G2; $n = 5$) at different time points (Days −30, 0, 30 and 60) in Ossimi rams. Means with different superscripts are significantly different at $p < 0.05$

($p < .05$) at D 30 and D 60 in G1 in comparison with G0 and G2 respectively. Semen parameters had no significant difference between G0 and G2 at D 30 and D 60. However, G2 showed the lowest values compared to other groups (G1 and G0 respectively).

4 | DISCUSSION

Many studies have reported the negative effect of heat stress in the summer season on the testicular haemodynamics in rams (Hedia

TABLE 1 Effect of zinc sulphate and folic acid combination as an oral supplementation on the ram semen parameters

Time	D−30			D0			D30			D60		
Semen parameters	G0	G1	G2	G0	G1	G2	G0	G1	G2	G0	G1	G2
Individual motility %	70.34 ± 1.35	72.22 ± 1.17	70.53 ± 1.52	72.12 ± 1.35	71.43 ± 1.25	73.04 ± 1.32	72.65 ± 1.42	80.43 ± 1.37[a]	70.25 ± 136	73.24 ± 1.04	84.09 ± 1.55[a]	72.43 ± 1.32
Live sperm %	72.14 ± 1.22	74.37 ± 1.32	72.25 ± 1.28	75.52 ± 1.47	73.49 ± 1.22	75.81 ± 1.44	76.34 ± 1.25	82.73 ± 1.54[a]	72.85 ± 1.22	77.64 ± 1.62	87.45 ± 1.23[a]	75.84 ± 1.72
Abnormal sperm %	20.21 ± 1.37	19.77 ± 1.21	18.19 ± 1.45	19.01 ± 1.21	18.20 ± 1.81	18.16 ± 1.23	18.88 ± 1.13	15.01 ± 1.57[a]	23.34 ± 1.08	19.35 ± 1.47	12.43 ± 1.45[a]	21.42 ± 1.65
Viability %	68.53 ± 1.42	69.13 ± 1.72	68.43 ± 1.27	70.24 ± 1.45	71.04 ± 1.35	71.78 ± 1.35	71.83 ± 1.57	74.23 ± 1.15[a]	69.04 ± 1.13	71.34 ± 1.52	79.24 ± 1.18[a]	69.45 ± 1.37
Acrosome integrity %	74.51 ± 1.32	73.15 ± 1.72	72.04 ± 1.35	75.02 ± 1.12	74.89 ± 1.28	73.34 ± 1.21	76.22 ± 1.38	81.34 ± 1.13[a]	70.21 ± 1.52	74.17 ± 1.05	86.44 ± 1.25[a]	72.43 ± 1.12

Note: $N = 60$ (15 ejaculates; 4 replicates).

[a]Values in each parameter are different at least at $p < .05$ among the three groups. Semen quality parameters (Individual motility %, Live sperm %, Viability % (HOST), Abnormal sperm % and Acrosome integrity %) after dietary zinc sulphate and folic acid formula supplementation (G1; $n = 5$; G2; $n = 5$) versus control (basal diet only; G0; $n = 5$) at different time points (Days −30, 0, 30 and 60) in Ossimi rams.

et al., 2020) and goat bucks (Samir et al., 2018) and on the semen quality in rams (De et al., 2017) and bulls (Brito et al., 2003). Oxidative stress is one of the most imperative causes accounted for the deteriorative effect of heat stress on the male reproductive performance (Hüttemann et al., 2011; Ishii et al., 2005). The current study investigated, for the first time, to determine the ameliorative effect of a potent antioxidant formulation (zinc sulphate and folic acid; ZnF) on the testicular blood flow parameters of rams housed under environmental heat stress in the summer season. The findings of the current study are in line with the hypothesis that a dietary zinc sulphate and folic acid combination affects the testicular blood perfusion especially in the hot humid conditions. The provision of such information is critical for improving the animal productivity and as a tool for assisting the future resolution of various difficulties related to sheep fertility.

In this current investigation, the PI of the proximal supratesticular branch is almost affected by supplementation than the distal branch, and this could be related to the different blood flow pattern with different velocity in different locations (Souza et al., 2015), as ZnF (G1) caused significant reductions in Doppler indices of testicular and penile arteries (RI and PI) which have negative relationships with tissue vascular perfusion downstream (Ginther, 2007, Abdelnaby et al., 2021). Reduced RI and PI readings imply lower blood flow resistance, resulting in increased testicular perfusion and a constant supply of oxygen and nutrients to the testis (Dickey, 1997; Ginther, 2007). The ways through which dietary ZnF improves the Doppler indices were not explained in the current study; nonetheless, two possible explanations may discuss this improvement. Firstly, dietary ZnF may affect the testicular haemodynamics through elevating serum gonadotropins (FSH and LH; Hafiez et al.,1989) and testosterone in rams (Ghorbani et al., 2018) that explain the possible effect of ZnF on the hypothalamic–pituitary–gonadal axis through elevating the GnRH pulsatile secretions that has indirect effect on the testicular function (El-Shalofy & Hedia, 2021). Secondly, zinc, as a potent antioxidant, has the ability to scavenge the free radicals and inhibition of the ROS production via NADPH oxidase inhibition (Prasad et al., 2004). Also, zinc is a cofactor of the superoxide dismutase enzyme (SOD) that catalyses the break of O_2^- to H_2O_2 (Prasad et al., 2001; Wang et al., 2018). Furthermore, zinc encourages the production of metallothionein, cysteine-rich compound, and is a profound OH scavenger. Moreover, zinc activates the antioxidant proteins and enzymes such as SOD, glutathione (GSH) and catalase (Bao et al., 2013; Wang et al., 2018). Additionally, nitric oxide (NO), synthesized in the vascular endothelial cells, is speedily disabled by ROS especially superoxide anion radical to form peroxynitrite (Kissner et al., 1997). Exogenous administration of antioxidants, such as zinc and folic acid, can efficiently hunt ROS from the blood vessels before its rejoining with NO ensuing higher blood NO bioavailability (Thakor et al., 2010). Authors reflect the improvement in the testicular haemodynamics following ZnF dietary supplementation may be accredited in part to elevated levels of NO that is well known as a strong vasodilator (Lissbrant et al., 1997). NO is produced from L-arginine by nitric oxide synthase enzyme

(NOs) in different peripheral tissues, including the testicular blood vessels and seminiferous tubules (Rodeberg et al., 1995). Some studies conveyed critical roles of NO in the vasculature tone regulation and testicular haemodynamics (Lissbrant et al., 1997; Sharma et al., 1998). Nonetheless, further studies are needed for the precise explanation of the exact mechanism through which ZnF affects the in vivo blood vessel tone regulation. In the current study, doubled dosed ZnF (G2) did not alter the testicular blood perfusion (RI and PI) or the testicular volume when compared to control group. These non-significant results could be attributed to the pro-oxidant effect of the higher doses of Zn on the SOD and GPx (Rahman et al., 2014) that led to decrease in its antioxidant effect with subsequent low NO bioavailability via oxidative inhibition of NOs enzyme (Thakor et al., 2010).

The assessment of the ram testicular volume is very critical in order to determine the reproductive development of animal, and our findings revelled an elevation in testicular volume that affected by supplementation with no difference between both testes, which is contrast to some studies in stallion (Kavac et al., 2003) and dogs (Souza et al., 2014). Similar study reported that zinc deficiency has been associated with delay in the testicular development which reflects the role of zinc administration in enhancement of the testicular volume (Emmanuel et al., 2019). Estimation of the plexus coloration percentage is of a great challenge, as this tool could be used for the future fertility prediction. In this study, the coloration percentages were increased after one month of supplementation in the supplemented males. Similarly, it was recently established that males with higher plexus coloration are more fertile than males with low levels of coloration in the pampiniform plexus region (Ribeiro et al., 2020).

In this study, the measurement of the both branches of ram testicular arteries was performed to estimate Doppler indices in order to show the phasic pattern of blood flow wave in both control and supplemented animals, as both testis showed a pattern of blood flow monophasic waveform pattern. Similarly, many studies reported the testicular phasic form in humans and dogs (Gumbsch et al., 2002; Tanriverdi et al., 2006), but another study concluded that there was a biphasic testicular blood flow pattern in stallions (Pozor & McDonnell, 2004). These results could be helpful to estimate the pattern of normal blood flow.

Improvement in the plasma testosterone levels was found in the present study (G1). Mansour et al. (1989) speculated a strong reduction in the 3 beta-hydroxysteroid dehydrogenase activity which is important in testosterone production in rats that may show the strong correlation between the zinc and testosterone synthesis. In addition, mice received zinc deficient diet exhibited lower zinc concentration in leydig cells and down-regulated zinc transporter 7 (expressed in steroidogenic acute regulatory protein in leydig cells) that led to decreased serum testosterone levels (Chu et al., 2016).

Our results suggested that ZnF had a synergistic effect which appeared as an improvement in testicular blood supply and semen traits. Moreover, zinc acts as an important cofactor for dihydrofolate reductase and gamma-glutamyl hydrolase, which are vital enzymes for metabolism of the folate (Favier et al., 1993). Results of the current study revealed that dietary supplementation of ZnF improved semen quality parameters which is in accordance with previous results (Egwurugwu et al., 2013; Raigani et al., 2014). This positive effect might be attributed to the potent anti-oxidative properties of ZnF against ROS during oxidative stress. ZnF could ameliorate the oxidative state through the following pathways: firstly, zinc has a potent scavenging action on ROS (Prasad, 2008); secondly, zinc preserves the normal function of the antioxidant enzyme especially super oxide dismutase (Agarwal & Sekhon, 2010); thirdly, zinc prevents the lipid, protein and DNA oxidation through occupying the binding sites for iron and copper and also protects proteins from oxidation via binding to its sulphydryl groups (Raigani et al., 2014). In addition being a potent antioxidant, folate plays a vital role in DNA and protein synthesis, is essential for normal spermatogenesis and prevents the chromatin damage (Ebisch et al., 2006; Wong et al., 2002).

In the current study, doubling the dose of dietary ZnF had no significant effect on the studied semen traits. Our findings are in agreement with previous studies (Carpino et al., 1998; Danscher et al., 1978; Egwurugwu et al., 2013). The lack of improvement in semen quality after ZnF dietary inclusion might be attributed to the effect of zinc ions on calcium ions, and the increase in Zn ions resulted in more occupation of the specific binding sites for CA ions which are necessary for motility, viability and activation of spermatozoa (Busselberg, 1995; Chyb et al., 2000; Maisse et al., 1995).

5 | CONCLUSION

Dietary supplementation of heat-stressed rams with zinc sulphate (0.4 mg/kg bw) and folic acid (0.02 mg/kg bw) combination improved testicular haemodynamics (by RI and PI decrement) and volume, testosterone levels and semen quality. However, further studies are needed to clarify the actual pathways that enable ZNF to improve the testicular blood flow dynamics.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Aya M. Fadl involved in semen collection, evaluation and statistical analysis, and writing—review and editing. Hossam R. El Sherbiny involved in writing—review and editing. Elshymaa A. Abdelnaby involved in Doppler scanning, supervision, visualization, data curation and editing.

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created in this study, there is no repository number for our data.

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How to cite this article: Fadl, A. M., Abdelnaby, E. A., & El-Sherbiny, H. R. (2022). Supplemental dietary zinc sulphate and folic acid combination improves testicular volume and haemodynamics, testosterone levels and semen quality in rams under heat stress conditions. *Reproduction in Domestic Animals*, 57, 567–576. https://doi.org/10.1111/rda.14096