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Original Article 1

Zinc Nanoparticles ameliorated heat stress impacts, improved semen characteristics, and preserved normal antioxidant status and hepatic-renal functions in goat-bucks

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Background
Zinc nanoparticles have several medical and biomedical applications in livestock because of their antioxidant and antimicrobial properties. **Aims:** To improve the reproductive capacity of native male goats during months of heat stress. **Material and methods:** Goat bucks used in the current work (n=8) were divided into control (0.0 mg zinc nanoparticles) and zinc nanoparticles (50.0mg/daily ZnONPs) groups. For 49 days, semen and blood samples were collected during the cold months (Day -42) and at biweekly intervals from the start of the supplementation (heat stress)until 60 days. Sperm mass and individual motilities, live sperm percent, sperm cell concentration, and plasma membrane integrity percent (hypo-osmotic swelling test, HOST) were determined for each animal in the control and treated groups. Malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase, total proteins, albumin, globulin, creatinine, aspartate transaminase (AST), and alanine transaminase (ALT) were measured in the blood samples. **Results:** ZnONPs synthesized by the green method using thyme aqueous extract were amorphous and had a diameter of <20nm. Compared to the control, the sperm mass motility of the ZnONPs group increased on Days 15 ($P<0.001$), 45 ($P<0.05$), and 60 ($P<0.001$). The progressive sperm motility of the ZnONPs group was higher than controls on Days -42-30 ($P<0.05$) and 45-60 ($P<0.001$). Live sperm %and sperm cell concentrations of ZnONPs group increased ($P<0.001$) throughout the experiment compared to controls. The number of HOSTs of ZnONPs increased on Days 15-30 ($P<0.05$) and days 45-60 ($P<0.001$). Malondialdehyde of supplemented bucks were lower than controls on days -42 ($P<0.01$), 15-30 ($P<0.01$), and 60 ($P<0.01$). The total antioxidant capacity of intreated bucks was higher than that of the controls on Day 30 ($P<0.01$). Higher SOD activity of treated bucks was observed on Days -42, 15 ($P<0.01$), and 60. The catalase activity of treated bucks was lower than that of controls on days 30 ($P<0.05$), 45, and 60 ($P<0.0001$). Total proteins and globulins of treated bucks showed lower concentrations than controls but higher albumin except on day 15 for total proteins and day 60 for albumin. Lower creatinine levels were observed in treated bucks on days 45 and 60. Slightly higher AST and ALT ($P>0.05$) were observed in treated bucks on Day 15. **Conclusions:** The recommended daily allowance of ZnONPs improved semen characteristics, reduced oxidative stress, improved total antioxidant status, and preserved normal hepatic and renal functions.

Keywords: Heat stress, semen parameters, oxidants-antioxidants, hepatic-renal functions, goats

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Introduction

Zinc is an important trace element in animal bodies. It is involved in most body enzymes such as phosphatases, carbonic anhydrase, and alcohol dehydrogenase, among those controlling reproduction[1]. It is a cofactor in many biological processes for maintaining good health, immunity, and production, promoting growth, and improving reproduction. Zinc bioavailability is very low therefore, it is recommended in apparently healthy individuals to avoid its deficiency [2]. Organic and inorganic zinc supplementations are important for normal spermatogenesis and oxidative antioxidant status [3]. In rams, supplemented zinc improved normal testicular growth, functions, and semen production[4] and kept optimal sperm characteristics [5], and reproductive hormones due to the inclusion of zinc finger proteins in the

nuclear receptors for steroids [6]. Dietary zinc supplementation in either organic form [7], zinc sulfate in Beetal bucks [8], zinc sulfate in rams [9], or both [3] improved antioxidant status, testicular blood flow, androgens, and semen traits. Supplementing rams with zinc nanoparticles improved semen parameters and the antioxidant status of heat-stressed rams [10].

Oral zinc is non-toxic however, zinc toxicity has been observed in animals supplemented after administrating higher doses of zinc [11]. High doses of zinc nanoparticles caused toxicity in rats by interfering with copper and iron utilization and adversely affecting HDL cholesterol concentrations [12]. Heat stress has become a global challenge that needs global collaboration to minimize the impacts of global warming and its adverse effects on male goats' fertility [13]. In bucks, summer heat stress reduces testicular dimensions, semen parameters, and androgens [13]. Male livestock animals can cope with heat stress through efficient testicular thermoregulatory mechanisms to preserve healthy spermatogenesis[14].

Several antioxidants were supplemented to reverse the impact of heat stress on germ cell damage and apoptosis such as melatonin [15], vitamin C [16], and resveratrol [17]. The effect of supplementing zinc nanoparticles in reversing the impact of heat stress semen parameters, and antioxidant status has not been reported. Therefore, this study aims to explore the anti-heat stress effect of orally supplemented zinc nanoparticles on semen parameters, oxidants-antioxidant status, and blood biochemical of bucks.

Materials and methods

Ethical approval

The study protocol was approved by the Animal Care and Ethical Use Committee of the Faculty of Veterinary Medicine at Cairo University (Approval ID.Vet-CU-18042024921).

Animals housing and management

Eight about one-year-old native male goats (N=8) weighing 25 to 45kg were housed in semi-open yards under daylight and temperature at EL-Azhar's Faculty of Agriculture research farm (Cairo, Al Azhar University). Bucks were divided into a control (n=3) and a supplemented group (ZnONPs, n=5) and both groups underwent comprehensive clinical, andrological, and ultrasonographic examinations to select healthy animals before being included in the current research. The animals were routinely vaccinated and dewormed, and they (according to NRC recommendations), were fed (1-2% BWT) concentrates (51% yellow corn, 24% wheat bran, 16% cottonseed meal, 6% soybean meal, 1.7% limestone powder, 0.8% salt, 0.5% mineral salts, and yeasts). Wheat straw and green clover (Trifolium alexandrinum) were available ad libitum. Also, fresh water and mineral licks were available ad libitum.

Bucks' heat stress assessment

The temperature-humidity index (THI) was determined using the equation of Mader et al. [18] which was recommended to be suitable for subtropical Indian goats. During the experimental period (May and June 2024), goats were subjected to climatic high-stress THI > 80 [19].

Synthesis of zinc nanoparticles (ZnONPs)

Zinc was prepared using the green synthesis method by the aqueous extract of Thymus oregano leaves with zinc acetate salt[20].

Experimental design

All bucks were supplemented with the basal diet. The nano-zinc group (ZnONPs) supplemented their daily requirement of zinc [21]with a 5.0 mLoral solution containing 50 mg ZnONPs (10mg/ml) for seven weeks[10].

Semen collection and evaluation

An artificial vagina (length 11 cm, diameter 6 cm, Minitube, Germany) with hot water adjusted at 42°C and lubricated with gel was used in the early morning for semen collection. Immediately after collection, semen was transferred to the laboratory and placed in a warm water bath at 37°C. The sperm concentration, motility, viability, abnormalities, plasma membrane, and acrosomal integrity were evaluated for collected semen samples from each animal in all treated and control groups at the studied period from Day -42 before heat stress to Day 60 after supplementation withdrawal during heat stress[9, 20].

Blood sampling

After each semen collection, blood samples (5ml) were collected from the jugular vein into both plain tubes and sera were harvested and stored at -20°C for analysis.

Hormone assaying, blood biochemical, oxidants, and antioxidant measurements

Malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase, total proteins, albumin, globulin, creatinine, aspartate transaminase (AST), and alanine transaminase, (ALT) were calorimetrically assessed using commercialkits (Bio-diagnostic, Dokki, Egypt).

Statistical analysis

Data are presented as Mean ± SEM (Standard error of the Mean). A simple one-way analysis of variance (ANOVA) was carried out to study the effect of time intervals within control and treated groups. The independent sample T-test was used to

compare the treatments at each time interval during Days -42, 15, 30, 45, and 60. Repeated measure Univariate Generalized model (2 treatment × 5 time-intervals) was performed using the SPSS program, version 26. Duncan's Multiple Range test was used to compare between significant means at P<0.05.

Results

The prepared zinc by the aqueous extract of thyme plant appears as aggregates by the scanning microscope (Fig. 1A), were spherical of size < 20 nm by transmission electron microscope (TEM, Fig. 1B), and amorphous by TEM (Fig. 1C) with X-Ray diffraction (XRD, Fig. 1D), negatively charged (-11.4) with small size by zeta potential (Fig 1E, F).

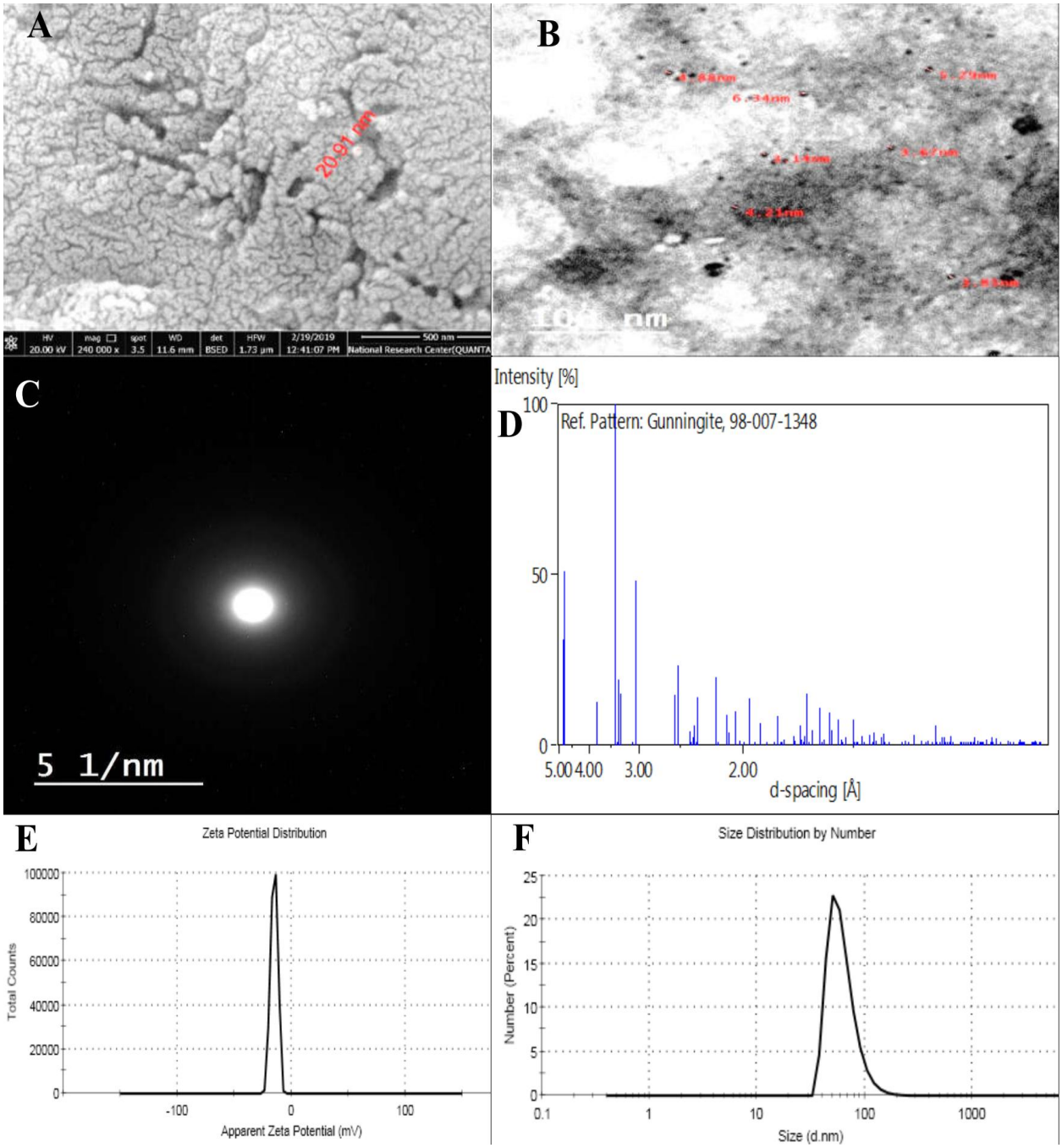

Fig. 1 The characterization of zinc nanoparticles with scanning electron microscope (SEM, A), Transmission electron microscope (TEM, B), The amorphous zinc oxide nanoparticles (C), X-ray diffraction (XRD, D), and Zeta potential size distribution electric charge (E) and Size determination (F)

The sperm mass motility declined (P<0.05) in ZnONPs on Day 30 and increased on Days 45 and 60 (Fig. 2A). Control bucks showed increased sperm mass motility from Day 30 to Day 60.

Compared to the control group, mass motility increased in ZnONPs throughout the experiment except on Day 30. The sperm individual motility %

ascended linearly (P<0.001) in both control and ZnONPs treated groups from the beginning to the end of the experiment with a significant increase (P<0.05) in ZnONPs treated groups throughout all days of the experiment (Fig. 2B).

The live sperm % increased (P<0.001) from Day 42 to Day 60 in the control and treated groups. Higher (P<0.001) values of live sperm % were observed in ZnONPs compared to the control animals (Fig. 2C) during all days of the experiment. Sperm cell concentrations of the treated bucks were higher than the control group (P<0.001) from Day 42 to Day 60 with no significant changes within the control groups during the experiment and significantly low (P<0.001) values in the treated animals on Days -42 and 30 (Fig. 2D). The plasma membrane integrity (PMI) using sperm hypo-osmotic swelling test (HOST) showed linear (P<0.001) increase in both treated and control groups with significantly increased (P<0.05) percentages in the ZnONPS treated group from Day 15 to Day 60 (Fig. 2E).

The levels of malondialdehyde (MDA, Fig. 2F) increased in the control group from Day -42 to Day 60, except for Day 45 which showed a significant decrease. In the control bucks, higher MDA (P<0.001) were observed on Days -42 to 30 compared to a low level on Day 60 and the lowest level on Day 45. ZnONPs group showed higher (P<0.001) MDA only on Day 15.

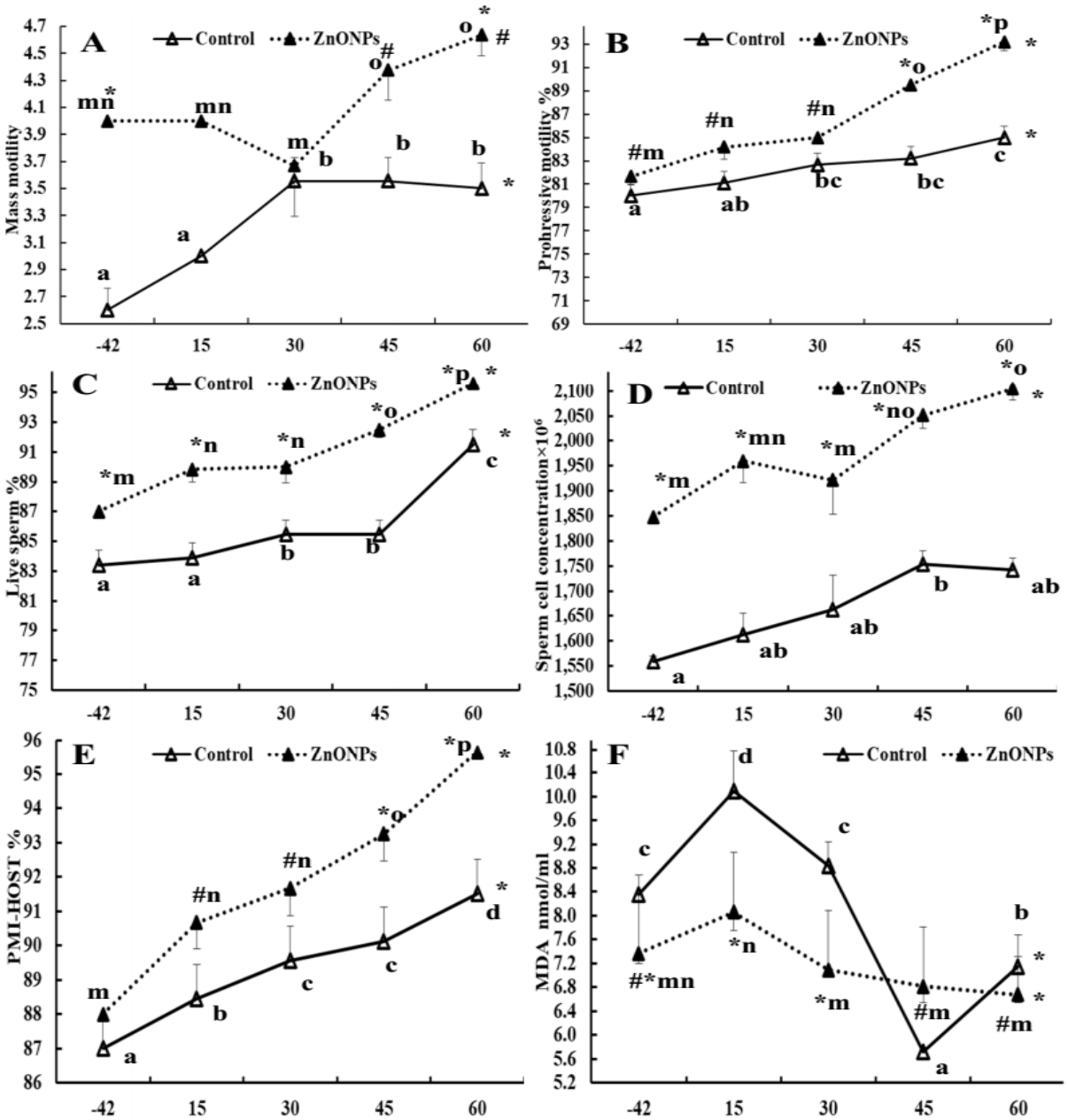

Fig. 2 Sperm mass motility score 1-5 (A), progressive motility % (B), live sperm %(C), sperm cell concentration $\times 10^6$ (D), sperm plasma membrane integrity by the hypo-osmotic swelling test (HOST, E), malondialdehyde (MDA nmol/ml, E), # means significant at P<0.05, * means significant at P<0.001, #* means significant at P<0.01,
Superscript letters (a, b, c, d) indicate significant differences within control, and (m, n, o, p) indicate significance within zinc oxide nanoparticles (ZnONPs) treated groups.

It can be observed from Figure (3A) that the total antioxidant capacity (TAC) was influenced by time intervals in both control and ZnONPs treated groups, with the lowest (P<0.0001) TAC occurring on Day 30 in control bucks and Day 45 in treated ones (Fig. 1A). In comparison to control bucks, TAC increased (P<0.01) in treated bucks on Day 30 but decreased (P<0.0001) on Day 45 and sustained low levels on Days -42 (P>0.05), 5, and 60 (P<0.01).

Time intervals had an impact on superoxide dismutase (SOD in control (P<0.0001) and treated (P<0.01) bucks. SOD declined in treated bucks (P<0.01) from before the start of the supplementation (Day -42) to reach the lowest concentration on Day 60 (Fig. 3B) but in control bucks, it reached the highest activity on Day 3 and the lowest one on Day 60. Higher SOD activity in treated bucks could be noticed on Day -42, Day 15 (P<0.01), and Day 60 than in controls (Fig. 3B). Time of the experiment influenced catalase activity in either control (P<0.0001) or treated (P<0.01) bucks (Fig. 3C). Lower catalase activity can be observed in treated bucks from Day 30 (P<0.05) to Day 60 (P<0.001) than control. The maximum highest catalase activity in control bucks was seen on Day 60 (P<0.001), but those of treated bucks were observed on all periods except Day 45.

The total protein levels increased (P<.0001) in control bucks from Day 30 to reach a maximum value on Day 60 (Fig. 3D). However, higher total proteins (P<0.05) were recorded in treated bucks on all days of the experiment, except on Day -42. Control bucks obtained higher total proteins on days 30, 45, and 60 compared to days -42 and 15. Compared to treated bucks, higher total protein levels were recorded in control bucks on Day -42, Days 30 (P>0.05), 45 (P<0.01), and 60 (P<0.0001). Albumin levels were influenced (P<0.001) by time intervals in both control and treated bucks (Fig. 3E). Both the control and treated bucks had higher albumin levels on Days 45 and 60. Treated bucks had higher albumin levels than control bucks on Days 15 (P<0.05) and Day 45 (P>0.05). On Day 60, the treated bucks showed a lower albumin level. Control and treated bucks showed a significant impact of experimental time intervals on globulin levels (Fig. 3F). Control bucks had the highest globulin levels on Day 30 but, the treated bucks had high globulin levels on Day 15 and Day 30. Compared to the control bucks, the supplemented group showed low globulin on Days -42 (P<0.0001), 15 (P>0.05), 30 (P<0.05), and 45 (P<.0001 and 60 (Fig. 3F). Total antioxidant capacity (TAC in mM/L, A), Superoxide dismutase (SOD in U/mL, B), catalase U/L (C), total proteins g/dL (D), albumin g/dL (E), globulin (g/dL, E), # means significant at P >0.05, * means significant at P<0.05, ** means significant at P<0.01, *** means significant at P<.0001, superscript letters (a, b, c, d) indicate significant difference within control and (m, n, o) indicate significant within zinc oxide nanoparticles (ZnONPs) treated groups.

Creatinine levels in both control and treated bucks were influenced (P<0.001) by the time of the experiment (Fig. 4A). Differences in creatinine levels in control and treated bucks were evident on Day -42 (P<0.001) and Day 60 (P<0.01) which increased in treated bucks on Day -42 and decreased on Day 60. Creatinine of treated bucks reached the highest value (P<0.0001) on Day 15 compared with other time intervals. Creatinine levels in the control bucks reached higher values on Days 15 and 60 (Fig. 4A). ALT of control bucks was impacted (P<0.0001) by the time intervals with a non-significant (P<0.0001) increase on Day 30 and a significant increase on Day -42 in comparison to the treated animals (Fig. 4B). Compared to the controls, a significant (P<.01) increase in the levels of ALT in treated bucks was ~~is~~ observed on Day 45that tended to be high (P>0.05) on Day 15. AST levels in the control and ~~or~~ treated groups were affected by time (P<0.0001). Higher AST levels were observed in control bucks on Day 30 and treated bucks on Days -42 and 30. AST tended (P>0.05) to increase in control bucks on Day 30 compared to the treated bucks (Fig. 4C) and tended (P>0.05) to be lower than that of the controls on Day 15 (Fig. 4C).

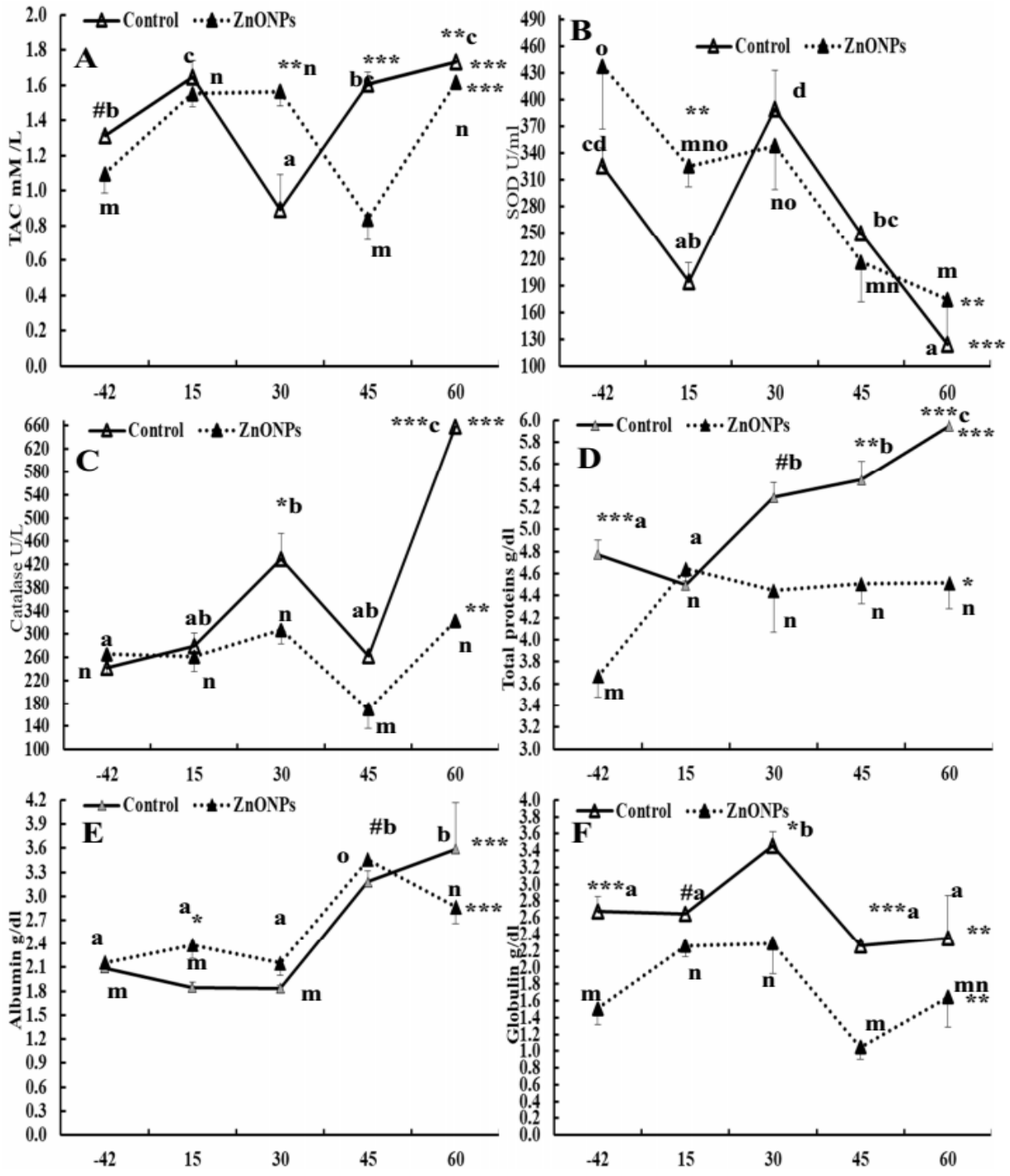

Fig. 3 Total antioxidant capacity (TAC in mM/L, A), Superoxide dismutase (SOD in U/mL, B), catalase U/L (C), total proteins g/dL (D), albumin g/dL (E), globulin (g/dL, E), # means significant at P >0.05, * means significant at P<0.05, ** means significant at P<0.01, *** means significant at P<.0001, superscript letters (a, b, c, d) indicate significant difference within control and (m, n, o) indicate significant within zinc oxide nanoparticles (ZnONPs) treated groups.

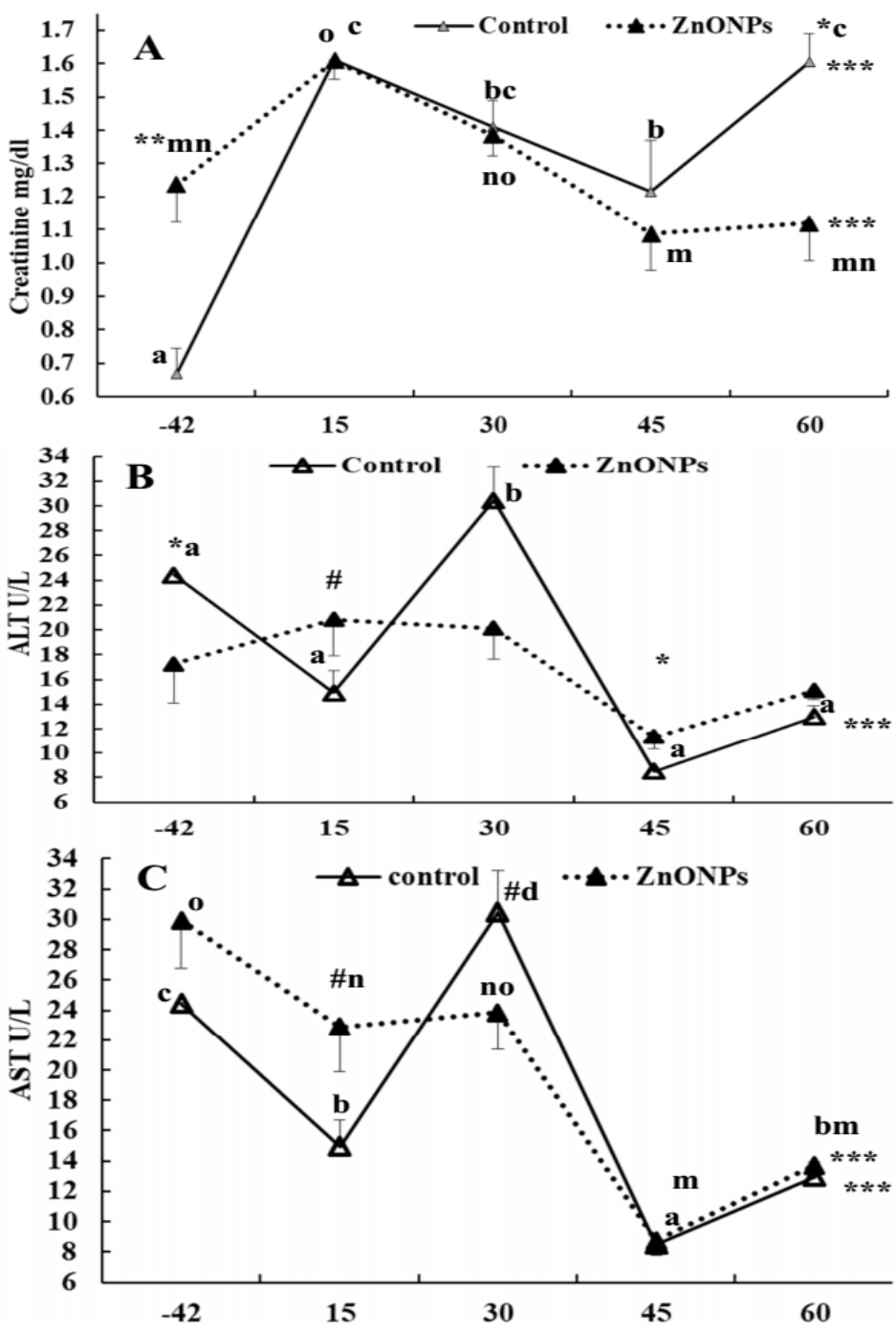

Fig. 4 Creatinine mg/dL (A), Alanine transaminase (ALT in U/L, B), Aspartate transaminase (AST in U/L, C), # means significant at P>0.05; * means significant at P<0.01, ** means significant at P<0.001, *** means significant at P<.0001, superscript letters (a, b, c, d) indicate significant difference within control and (m, n, o) indicate significant within zinc oxide nanoparticles (ZnONPs) treated groups.

Discussion

The size of the–zinc nanoparticles prepared in this study using thyme aqueous plant extract with zinc acetate was lower than that obtained in our previous study using zinc sulfate [9]. Higher ZnONPs (40-70 nm) prepared by the green method using *Mangifera indica* seeds were cylindrical with antioxidant activities [23]. Those prepared by *Hyssops officinalis* leaves and flowers showed antiangiogenic and anti-inflammatory properties and had spherical shapes of 20-40 nm [24]. In agreement with our results, ZnONPs prepared by *Berberis aristata* had nearly similar diameters but were 5-25 nm but of needle shape with antibacterial and antioxidant activities [25]. The low negative zeta potential of the synthesized ZNONPs confirmed the aggregation of the particle obtained by scanning microscope [26]. Similarly, a low negative zeta potential of -19.3 was obtained when

ZnONPs were prepared using zinc acetate and *Pelargonium odoratissimum* aqueous leaf extract [27]. The improvement in sperm progressive motility %, live sperm %, sperm cell concentration, and plasma membrane integrity determined by the hypo-osmotic swelling test (HOST) in ZnONPs supplemented bucks were also observed in rams supplemented orally with 40 and 80 mg ZnONPs for 49 days during the non-breeding season [10]. Moreover, Beetal bucks supplemented daily with 50 and 100 mg ZnONPs improved sperm cell concentrations, progressive sperm motility percentage, and live sperm percentage but a higher dose of 15 mg /animal reduced the sperm cell concentrations due to increasing the semen volume but maintained high sperm motility similar to 100 mg/animal [8].

In agreement with the results of improvement in sperm mass motility, sperm progressive motility, live sperm percentage, sperm plasma membrane integrity, and concentration obtained after supplementing native bucks with 5 mg ZnONPs for 49 days, Beetal bucks supplemented with 50, 100, and 200 mg zinc sulfate for 90 days showed non-significant improvement in the sperm cell concentration and live sperm percentage by supplementing bucks with 50 and 100 mg ZnSO4 [8]. They also recorded increased semen volume by supplementing 100 and 200 mg of zinc sulfate and improved sperm progressive motility percentage by supplementing three doses [8]. Supplementing 50 and 100 mg of dietary zinc improved the reproductive performance of Teressa goats [28]. In addition, dietary zinc sulfate was better than organic zinc when both were supplemented for 85 days to improve all semen parameters in prepubertal rams [3]. In contrast to our results, semen parameters did not improve in rams supplemented with zinc sulfate and folic acid [9]. Although zinc supplementation did not improve all semen parameters, zinc nanoparticles supplemented to rams at 40 and 80 mg per animal daily for 49 days improved ~~the~~ sperm progressive motility percentage, sperm viability, plasma membrane integrity, volume, and concentration with increasing doses of zinc [10]. Supplementation with ZnONPs improved semen parameters in rams [10] and bucks in this study, but also the supplementation of cryopreserved semen with zinc nanoparticles improved the post-thaw semen traits in ~~the~~ rams [20] and goats [29].

The decrease in malondialdehyde levels (MDA) as an indicator for lipid peroxidation in animals supplemented with 50 mg ZnONPs of this study throughout the heat stress time confirms the protective effects and antioxidants prepared by using aqueous *P. odoratissimum* leaves extract *in vitro* [27]. A similar decrease in MDA was recorded after three months of supplementing prepubertal rams with zinc sulfate rather than organic zinc [3] and in thioacetamide-intoxicated rats [30]. The increase in superoxide dismutase enzyme (SOD) activity in bucks of this study on Days -42, 15, and 60 agree with the increase of SOD activity in Beetal bucks supplemented with dietary metal and organic zinc [8], prepubertal rams supplemented with zinc sulfate for three months [3], and rams supplemented with 10 and 50 mg ZnONPs for 49 days [10]. In the current study, the increased total antioxidant capacity (TAC) in heat-stressed bucks 30 days after the daily supplementation with 50 mg ZnONPs is similar to the increase of TAC in rams supplemented with 80 mg ZnONPS which counteracted the effect of half of this dose that did not increase it [10] and increased in prepubertal rams supplemented with zinc sulfate [3]. The decreased catalase activity in the bucks of this study lies in agreement with low catalase activity in thioacetamide-intoxicated rats supplemented with ZnONPs at three different doses three times weekly for two months [30].

Regardless of the increase in total proteins from the start of ZnONPs supplementation until the end of the study, their levels were lower than those of the controls, except on Day 15. Similarly, total protein declined in growing Boer-cross goats supplemented with 200 mg of zinc sulfate heptahydrate [31]. In this study, an increase in albumin in ZnONPs-supplemented bucks during the 49 days of supplementation was also observed in growing prepubertal Boer-cross goats supplemented with 100 and 200 mg zinc sulfate heptahydrate for three months [31].

In agreement with the non-significant alterations in creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in the bucks of this study during the supplementation period, creatinine, ALT, AST did not change in Boer-cross goats supplemented with 100 and 200 mg zinc sulfate heptahydrate for three months [31] or Beetal bucks supplemented with organic and inorganic zinc [8] and reduced creatinine, AST, and ALT in thioacetamide-intoxicated rats [30].

Conclusions

Under heat stress conditions, zinc nanoparticles in the daily requirement dose improved semen parameters and decreased lipid peroxidation, and antioxidant enzyme activities while preserving normal hepatic and renal functions.

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