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ORIGINAL ARTICLE

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Effects of Dietary Supplementation with either Zinc Oxide or Nano-Zinc Oxide during Post Maturation on some Physiological and Biochemical Parameters in Mature V-line Male Rabbits

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Abstract
The current work aimed to investigate the effects of dietary supplementation with either Zinc Oxide (ZnO) or nano-Zinc Oxide (n-ZnO), during post maturation, on some biochemical parameters in mature V-line male rabbits. A total of 30 V-line male rabbits (5 months old) were divided into three equal groups; control group received basal diet, ZnO group received basal diet supplemented with 60mg ZnO/kg and n-ZnO group received basal diet supplemented with 60 mg nano-ZnO/kg for 3 months. At 6th month old, body weight (BW), body weight gain (BWG), food consumption (FC), food conversion ratio (FCR), triiodothyronine (T3), thyroxine (T4), testosterone, and semen characters as well as serum and seminal plasma levels of Zinc, superoxide dismutase (SOD) and catalase (CAT) were recorded monthly. At the end of 8th month old, androgen receptor (AR) gene expression and microscopical picture of testicular tissue were recorded. Results revealed that ZnO group exhibited significant ($P \leq 0.05$) improvement in all studied parameters. Moreover, n-ZnO group showed significant improvement over ZnO group in all studied parameters at the 6th month of age, and in Zinc level throughout the experimental period. However, at the 7th and 8th months of age n-ZnO group, exhibited significant ($P \leq 0.05$) adverse effects on all the studied parameters. It could be concluded that ZnO or nano-ZnO improves production and reproduction in mature V-line male rabbits. However, the effect of nano-ZnO was time dependent, since, it induced drastic effects after the 6th month of age.

Keywords
Androgen Receptor Gene, Antioxidants, Testosterone, Nano-Zinc Oxide, Zinc Oxide

1. Introduction
Zinc is counted as a vital trace element that plays an essential role in the antioxidant activity inside animal tissues beside its roles in the metabolic and other physiological activities **(Hassanin et al., 2020)**. It has a wide variety of biological roles including catalytic, structural and/or regulatory functions **(King and Keen, 1999).** Zinc is the most common catalytic ion in cell cytoplasm which takes part in cellular signals and divisions **(Farooq et al., 2020).** It is vital for ionization and polarization of some enzymes as carbonic anhydrase, carboxy peptidase A, alkaline phosphatase and phospholipase C. which, participate in protein and carbohydrate metabolism **(Andreini and Bertini, 2012).** Additionally, **Chrastinova et al., (2015)** illustrated that cellular genetic material (DNA and/or RNA) needs Zinc element either in their structures or in their whole activities including transcription and genes expressions. As a regulator, Zinc element controls expression profiles of RNA polymerases, alcohol dehydrogenase, carbonic anhydrase, glutamic dehydrogenase, carboxypeptidase, lactic dehydrogenase and alkaline phosphatase **(King and Keen, 1999; McDonald et al., 2002).** Regarding to hormonal actions, Zinc is a basic constituent in wide verities of hormones as thymulin, testosterone, prolactin, somatomedin and other growth factors **(Maret, 2017).** Likewise, it was mentioned that dietary Zinc oxide enhanced growth performance parameters such as live body weight, feed consumption, weight gain and feed conversion ratio **(Hassan et al., 2017; Kamel et al., 2020).**

Substantial development has been achieved in the employment of nanotechnology science in many fields particularly mineral nutrition **(Hassan et al., 2017).** Nanomaterials are characterized by small size, high bioavailability, wide surface area per unit mass which make them more reactive inside the cell **(Onyeaka et al., 2022).** Nano ZnO (n-ZnO) has special physical and chemical properties and it can penetrate some of the body's barriers so that it can achieve its beneficial effect more easily **(Jiang et al., 2018).** In this context, supplementation of rabbit ration with nano Zinc causes improvement in feed efficiency, growth and reproduction **(Kamel et al., 2020).** Furthermore, **Elkatcha et al., (2017)** found that nano-Zinc supplementation at different levels including; 30, 45 and 60

ppm/kg improves final body weight, B.W.G. and food conversion rate in poultry. Other study recorded that, dietary supplementation with 30mg/kg n-ZnO can enhance growth performance, lipid profile, immunity and antioxidant status of growing rabbits under heat stress conditions **(Kamel et al., 2020).** Moreover, it has been mentioned that n-ZnO improve antioxidant status as, it reduces Malondialdehyde (MDA) and increases superoxide dismutase (SOD), catalase (CAT) activity and total antioxidant capacity (TAC) **(Abdel-Monem et al., 2021).**

Based on the aforementioned studies, the present study was designed to declare the effect of ZnO and n-ZnO as feed additives on a complex set of growth and reproductive performance parameters in mature V-line male rabbits during pre and post maturation. The growth performance trials involve body weight (B.W.), body weight gain (B.W.G.), food consumption (FC) and food conversion ratio (FCR). Besides, serum and seminal levels of Zinc, SOD and CAT were investigated. In addition, serum levels of triiodothyronine (T_3) and thyroxine (T_4) were also recorded as an indicator for metabolic activities. Also the study extended to investigate reproductive performance trials include determination of testosterone level in serum, examination of AR (AR) gene expression, evaluation of semen characters and microscopical findings of testicular tissue.

2. Materials and Methods

All animal experimental procedures were conducted at the Animal Experimental Unit of Physiology department, Faculty of Veterinary Medicine, Beni-Suef University. The animal experiments were displayed according to the guide lines of Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Beni-Suef University **(Ethical Approval Number 021-182).**

2.1. Animals

A total of 30 V-line clinically healthy male rabbits (2 months old) with an average body weight of 1.5–2Kg, were used in this study. Animals were randomly divided into 3 comparable groups (10/each) and received the following diets for about 6 months, as follows:

1- Control group was fed on basal diet,
2- ZnO groups were fed on basal diet supplemented with 60 mg ZnO/kg
3- n-ZnO groups were fed on basal diet supplemented with 60 mg n-ZnO/kg.

At the age of 5 months which is considered as the age of sexual maturity according to **(Shehab, 2022).** Rabbits were housed in galvanized wired cages and received the following diets for 6 months throughout pre and post-maturation period. All rabbits were kept under the same controlled environmental, managerial and hygienic conditions. In this respect; animals were reared under controlled hygienic environmental conditions. Balanced pelleted diet and fresh water were offered to animal ad libitum. The basal pelleted diet contained corn gluten, soybean meal, yellow corn, minerals and vitamins premix, and molasses. The calculated chemical components of the diet were 17% crude protein, 12.6% crude fiber, and 2500kcal digestible energy/kg diet according to **NRC (1991).**

2.2. Chemicals

ZnO was obtained from **Alpha Chemika Company, INDIA.** Then, it was converted into n-ZnO in Nanomaterial research laboratory, Faculty of Postgraduate Studies for Advanced Science, Beni-Suef University. The obtained n-ZnO was previously characterized as described by **Farghali et al., (2007)** and **Abdelmohsen et al., (2017).**

2.3. Experimental Design

All animals (30 rabbits) were randomly distributed into 3 equal groups (15 rabbits/group) and received the following diets for six months (pre- and post-maturity period). The first group received a basal diet, while the second and third groups received a basal diet supplemented with 60mg ZnO/kg and 60mg n-ZnO/kg respectively. After the end of the first experiment that implemented during pre-mature stage at the 3rd, 4th and 5th months of age, 5 rabbits/group were sacrificed for determination of AR gene expression and microscopical findings of the testis in growing V-line male rabbits **(El-Anwar et al., 2023).** Moreover, only 30 male rabbit (10 rabbits/group) were used in the current experiment throughout the post maturation period at the 6th, 7th and 8th months of age.

2.4. Blood Samples

At the end of the 6th, 7th and 8th months of age, blood samples were separately collected via the ear vein from each rabbit in all groups. Blood samples were left to clot and followed by centrifugation at 3000rpm to obtain the sera that kept at -20˚C for further biochemical analysis **(Al-Dobaib et al., 2007).**

2.5. Semen Samples Collection and Evaluation

Semen samples were collected twice weekly from each buck in all groups using an artificial vagina and a female teaser rabbit during the 6th, 7th and 8th months of age (mean value for 4 semen specimen were obtained monthly/each buck) **(Attia and Kamel, 2011).** The temperature of the inner rubber sleeve of the artificial vagina was adjusted at 41-43ºC. Each buck was previously trained for semen collection technique, in a preliminary period during the 5th month of age **(Bezrra et al., 2019).** fresh semen samples were examined for pH value, semen volume (ml), individual motility (%), sperm concentration (X 10^6/ml), total sperm count/ejaculate as well as live sperm and dead sperm (%), primary, secondary and total sperm abnormalities (%). The average of each parameter was calculated monthly for each group **(Hafez and Hafez, 2002).** In addition, an individual seminal plasma specimen from the last semen sample was collected monthly (6th, 7th and 8th months old) from each buck after centrifugation at 3000rpm and kept at -20˚C for biochemical analysis **(Younan et al., 2017).**

2.6. Measurements

2.6.1. Growth Performance

Growth performance trials; BW, BWG, FC and FCR were recorded monthly during the period of maturity starting from the 6th month till the end of the experiment (8th month old) according to **McDonald et al., (1995).**

2.6.2. Biochemical Parameters

The following biochemical parameters were measured as follows;

1- Zinc concentration in rabbit sera and seminal plasma were estimated, according to **Johnsen and R.Eliasson (1987)** using Zinc kit (µg/dl), spectrum-diagnostics Co., EGYPT.
2-Superoxide dismutase (SOD) in sera and seminal plasma were estimated, according to **Nishikimi et al., (1972)** using SOD kit (U/mL), bio-diagnostic Co., EGYPT.
3- Catalase (CAT) in sera and seminal plasma were estimated, according to **Aebi (1984)** using CAT kit (U/L), bio-diagnostic Co., EGYPT.
4- Triiodothyronine (T3) and Thyroxine (T4) were measured in sera, according to manufacture structure using T3 kit (ng/ml), Abbkine Co., China and T4 kit (ng/ml), My Biosource Co., USA) respectively.
6- Testosterone was measured in sera, according to **Karabulut and Gulay, (2020)** by using testosterone kit (ng/mL), cusabio Co., USA.

2.6.3. Microscopical Examination of Testicular Tissue

At the end of the 8th month age, five representative rabbits from each group were euthanized randomly to obtain left testis fixed in 10% buffered formalin (BF) for at least one day. Then, specimens were histological prepared according to **Bancroft and Layton (2013)**. It worth to mention that, right testes were used for AR gene expression, as follows:

2.6.4. Determination of AR Gene Expression

Total RNA was obtained from rabbit testis by one-step reverse transcription-quantitative PCR (RT-qPCR) protocol using GoTaq® 1-Step RT-qPCR System [(a,b)] , promega, USA according to manufacturer's instructions. The primer sequences that used for amplification of Androgen receptor (AR) and ß-actin (as a reference gene) were F: 5′-TCC ACC TCC TCC AAG GAC AGT-3′, R: 5′-CCA ACG CCT CCA CAC CCA A-3′ and F: 5′-TCC TTC CTG GGC ATG GAG TC-3′, R: 5′-GGA TGT CCA CGT CGC ACT TC-3′ 5′-GGA TGT CCA CGT CGC ACT TC-3′, respectively **(El-dawy et al., 2016)**. Analysis of RT-qPCR result was determined as fold change according to **Yuan, et al., (2006)**.

2.7. Statistical Analysis

All data for the three groups during the period of maturity starting from the 6th month till the end of the experiment (8th month old) were expressed as mean ± standard error of mean (SEM) by using SPSS for windows, version 25, USA was used for data analyses depending upon one-way analysis of variance (ANOVA) to detect significance among tested groups at P≤ 0.05. Also, Duncan's test **(Duncan, 1955)** was used to confirm significant difference among tested groups.

3. Results

3.1. Effect of ZnO and n-ZnO Dietary Supplementation on Growth Performance Trials in Mature V-line Male Rabbits

The obtained data **(Table, 1)** showed that, ZnO supplementation induced significant (P≤0.05) improvement in all the growth performance trials including; B.W., B.W.G. and FCR throughout the current maturation experiment, in comparison with that of the control group. However, the obtained results revealed that the effects of n-ZnO is time dependent since, prolonged dietary supplementation of n-ZnO was recorded to induce significant (P≤0.05) drastic effects **(Table, 1).** In this respect, significant reduction in the BW was noted in n-ZnO dietary supplemented group in comparison with ZnO group on 7th and 8th months of age. In addition, n-ZnO supplementation group induced significant hazard effects in BWG and FCR throughout maturation period in comparison with ZnO group, as well as on the 7th and 8th months of age in comparison with that of control group. Meanwhile, the results of FC, throughout the maturation period, were nearly similar in all groups.

Table 1. Effect of either ZnO or n-ZnO dietary supplementation on Growth performance trials in V-line male rabbits (Mean±SE).

Parameter	Age (Months)	Control (1st Group)	ZnO (2nd Group)	n-ZnO (3rd Group)
BW (g)	6	4017.80±13.00 ^{A}	4309.00± 30.32 ^{B}	4454.80± 19.79 ^{C}
	7	4684.20± 23.72 ^{A}	5058.30± 46.03 ^{B}	4881.20± 30.94 ^{C}
	8	5257.80 ± 21.54 ^{A}	5750.20± 41.53 ^{B}	5222.90± 35.37 ^{A}
BWG (g)	6	608.70±4.89 ^{A}	685.90 ±19.97 ^{B}	585.00 ±19.99 ^{A}
	7	666.60± 20.52 ^{A}	749.30± 27.40 ^{B}	426.4 ± 15.25 ^{C}
	8	573.80± 26.73 ^{A}	691.90± 20.24 ^{B}	341.70 ± 13.52 ^{C}
FC (g)	6	2404.49±32.64 ^{A}	2355.07 ±25.91 ^{A}	2353.87±25.93 ^{A}
	7	2549.26±29.79 ^{A}	2515.16±26.97 ^{A}	2521.06±22.31 ^{A}
	8	2883.41±47.29 ^{A}	2809.72±13.76 ^{A}	2894.32±27.49 ^{A}
FCR	6	3.95± 0.05 ^{A}	3.46 ± 0.12 ^{B}	4.06 ± 0.14 ^{A}
	7	3.85 ± 0.09 ^{A}	3.39 ± 0.12 ^{B}	5.98 ± 0.22 ^{C}
	8	5.13 ± 0.26 ^{A}	4.09 ± 0.12 ^{B}	8.57 ± 0.29 ^{C}

-In the same row, values having different capital letters differ significantly (P≤0.05).

3.2. Effect of ZnO and n-ZnO Dietary Supplementation on Biochemical Parameters in Serum and Seminal Plasma of Mature V-line Male Rabbits

The current study showed that ZnO dietary supplementation induced significant (P≤0.05) increase in the levels of Zn, SOD and CAT in serum **(Table 2)** and seminal plasma **(Table, 3),** as comparable with the control group, throughout the maturation period. However, n-ZnO supplementation significantly (P≤0.05) increased levels of SOD and CAT in both serum **(Table, 2)** and seminal plasma **(Table, 3)** at the 6th month of age only of the maturation period. It was also noted that, n-ZnO dietary supplemented group exhibited significant (P ≤ 0.05) increase in the serum **(Table, 2)** and seminal plasma **(Table, 3)** levels of Zn all over the maturation period. The current results cleared that the positive effects of n-ZnO on the above mentioned parameters seemed to be higher than that of ZnO. On the contrary, significant (P≤0.05) hazard effects were observed in the levels of SOD and CAT in both serum **(Table, 2)** and seminal plasma **(Table, 3)** of n-ZnO dietary supplemented group at the 7th and 8th months of age.

Regarding the serum levels of thyroid hormones, it was found that ZnO didn't induce any significant change in T_3 and T_4 during maturation period as compared to control group. Likewise, n-ZnO dietary supplementation didn't exhibit any significant change at the 6th month of age, while, at the 7th and 8th months of age, significant (P≤0.05) hazard effect were obtained on both hormones, as compared to both control as well as ZnO groups **(Table, 2).**

Concerning AR gene expression at 8th month of age, it appeared that ZnO induced significant (P≤0.05) increase in AR gene expression while n-ZnO induced significant (P≤0.05) adverse effects, as compared to control group **(Table, 2).**

The current results **(Table, 2)** showed a significant (P≤0.05) improvement in serum testosterone levels in the ZnO supplemented group throughout maturation period as compared to control group. Moreover, n-ZnO group showed significant (P≤0.05) an improvement in serum testosterone levels only at the 6th month of age in comparison with control as well as ZnO treated group. However, at the 7th and 8th months of age, significant (P≤0.05) reduction in serum levels of testosterone was observed.

Table 2. Effect of either ZnO or n-ZnO dietary supplementation on serum biochemical parameters and AR gene expression in mature V-line male rabbits (Mean±SE).

Parameter	Age (Months)	Control (1st Group)	ZnO (2nd Group)	n-ZnO (3rd Group)
Zinc µg/dl	6	91.64 ± 1.45 A	103.27 ±1.34 B	111.00 ±1.67C
	7	89.43 ±2.46 A	104.61 ± 1.96 B	112.60±1.81C
	8	92.62±2.29 A	102.29 ± 1.07 B	108.92± 1.65C
CAT U/L	6	259.60± 4.08 A	361.60± 2.98 B	396.40 ± 5.36C
	7	292.80±8. 53 A	387.20±7.16 B	247.20± 5.38C
	8	306.80±6.02 A	394.80± 6.41 B	250.80± 2.52C
SOD U/mL	6	1.40 ± 0.04 A	1.82±·.02 B	2.01 ± 0.05C
	7	1.24 ± 0.02 A	1.83 ± 0.03 B	1.09 ± 0.02C
	8	1.28± 0.04 A	1.92 ±·.02 B	1.13 ± 0.02C
T3 ng/ml	6	1.09 ± 0.02 A	1.13 ± 0.04 A	1.20 ± 0.07A
	7	1.17 ± 0.09 A	1.22± 0.02 A	0.89 ± 0.05B
	8	1.03 ± 0.05 A	1.07 ± 0.02A	0.86 ± 0.04B
T_4 ng/ml	6	5.77 ± 0.08A	5.81 ± 0.3A	5.90 ± 0.09A
	7	6.13± 0.23A	5.87 ± 0.31A	4.21 ± 0.13B
	8	5.83 ± 0.22A	6.17 ± 0.15A	4.51 ± 0.16B
Testosterone ng/mL	6	2.91 ± 0.06 A	3.96 ± 0.05 B	4.37 ± 0.13C
	7	4.08 ± 0.11 A	4.75 ± 0.10 B	3.51 ± 0.04C
	8	4.28 ± 0.09 A	4.92 ± 0.08 B	3.88 ± 0.07C
AR gene expression	8	1.02 ± 0.03 A	2.03 ± 0.03 B	0.69 ± 0.01C

In the same raw, values having different capital letters differ significantly (P≤0.05).

Table 3. Effect of either ZnO or n-ZnO dietary supplementation on seminal plasma biochemical parameters in mature V-line male rabbits (Mean±SE).

Parameter	Age (Months)	Control (1st Group)	ZnO (2nd Group)	n-ZnO (3rd Group)
CAT U/L	6th	130.00 ± 3.70 A	159.2 ± 3.22B	196.40 ±4.68 C
	7th	172.20 ± 2.50 A	201.00 ± 3.55B	119.20 ±1.70 C
	8th	136.00 ± 3.55 A	173.40± 3.11 B	112.60± 1.87 C
SOD U/mLs	6th	1.31 ± 0.03 A	1.67 ± 0.03 B	1.98 ± 0.06 C
	7th	2.11 ± 0.09 A	2.73 ± 0.03 B	1.73 ± 0.04 C
	8th	1.92 ± 0.07A	2.13± 0.07 B	1.75 ± 0.03 C
Zinc µg/dl	6th	124.23 ± 2.30 A	141.47 ± 2.74 B	162.95± 4.06 C
	7th	119.71 ± 1.37 A	144.20 ± 3.51 B	176.04±3.44 C
	8th	122.65 ± 2.24 A	149.95 ± 3.70 B	169.34 ± 4.14 C

In the same raw, values having different capital letters differ significantly at (P≤0.05).

3.3. Effect of ZnO and n-ZnO Dietary Supplementation on Semen Characters of Mature V-line Male Rabbits

Table (4) showed that, ZnO dietary supplementation induced significant (P≤0.05) improvement in semen characters as indicated by an increase in the values semen volume (ml), individual motility (%), sperm concentration (X 10^6/ml), sperm output and live sperm (%) as well as decreased dead sperm (%), primary (%), secondary (%) and total (%) sperm abnormality, as compared to control group throughout maturation period. Moreover, n-ZnO group showed significant (P≤0.05) improvement in semen characters only at 6th month of age in comparison with control as well as ZnO treated group. Meanwhile, significant drastic effects were noted at the 7th and 8th months of age.

Table 4: Effect of either ZnO or n-ZnO dietary supplementation on semen characters of mature V-line male rabbit (Mean ± SE):

Parameter	Age (Months)	Control (1st Group)	ZnO (2nd Group)	n-ZnO (3rd Group)
Volume	6	0.53 ± 0.01A	0.63 ± 0.02B	0.71 ± 0.02 C
	7	0.60 ± 0.01 A	0.67 ± 0.01 B	0.49 ± 0.01 C
	8	0.67± 0.01 A	0.72 ± 0.01B	0.48 ± 0.01 C
Individual M.%	6	75.70 ± 0.29 A	77.85 ± 0.33 B	80.05 ± 0.51C
	7	76.50 ± 0.63 A	79.15 ± 0.41 B	69.05 ± 0.80 C
	8	78.10 ± 0.38A	81.10 ± 0.45 B	67.75 ± 0.75 C
Sperm conc.×10^6/ ml	6	213.95 ± 2.87A	223.55 ± 1.52B	233.90 ± 1.76C
	7	226.80 ± 1.17 A	237.75 ± 1.95 B	212.25 ± 1.75C
	8	225.25 ± 1.91 A	239.40 ± 1.69 B	209.45 ± 1.57C
Sperm output ×10^6/ejaculate	6	113.33 ± 1.77 A	141.34 ±3.89 B	165.08 ± 2.74 C
	7	135.94 ± 3.20 A	158.32 ± 3.39 B	102.51± 2.72 C
	8	150.43 ± 2.91A	172.36 ± 3.45 B	103.11 ± 2.34 C
Live sperm%	6	79.75 ± 0.38 C	84.20 ± 0.46 B	86.70 ± 0.48 C
	7	81.15 ± 0.34 A	86.35 ± 0.44 B	73.70 ± 0.79 C
	8	80.90 ± 0.33 A	87.00 ± 0.53 B	69.45 ± 0.57 C
Dead sperm%	6	18.95 ±0.22 A	15.00 ± 0.37 B	12.65 ± ·,28 C
	7	17.70 ± 0.24 A	13.60 ± 0.29 B	26.05 ± 0.62 C
	8	17.55 ± 0.24 A	14.40 ± 0.39 B	31.15 ± 0.49 C
1ry sperm Abnormality%	6	2.50 ± 0.09 A	2.10 ± 0.10 B	1.75 ± 0.10 C
	7	2.45 ± 0.08 A	1.79 ± 0.09 B	4.65 ± 0. 18 C
	8	2.40 ± 0.08A	1.9٥ ± 0.09 B	4.9٥ ± 0.19 C
2ry sperm Abnormality%	6	16.55± 0.23 A	15.15 ± 0.18 B	13.05 ± 0.16 C
	7	14.10 ± 0.29A	11.50 ± 0.27 B	18.20± 0.38 C
	8	14.05 ± 0.27A	12.30± 0.26 B	18.88 ± 0.40 C
Total sperm Abnormality%	6	19.05 ± 0.24 A	17.20 ± 0.22 B	14.78 ± 0.18 C
	7	16.55 ± 0.31A	13.28 ± 0.30 B	22.85 ± 0.36 C
	8	16.50 ± 0.28A	14.25 ± 0.31 B	23.15± 0.45 C

In the same row, values having different capital letters differ significantly (P≤0.05).

3.4. Effect of Dietary ZnO and n-ZnO Supplementation on Microscopical Picture of Testicular Tissue at the 8th Month of Age of V-line Male Rabbits

At the 8th month age, seminiferous tubules (ST) of control group showed normal spermatogenic activity and many of them were packed with spermatozoa **(Fig. 1).** Furthermore, ST of ZnO group show spermatogenic activity and they were highly packed with spermatozoa in comparable with control group **(Fig. 2).** In addition, ST of n-ZnO group showed necrobiotic changes of spermatogenic cells especially spermatid and spermatozoa and there were an increased number of multinucleated giant cells in the lumen of ST. The density of spermatid and spermatozoa was lower in ST of n-ZnO group in comparison to the control group **(Fig. 3).**

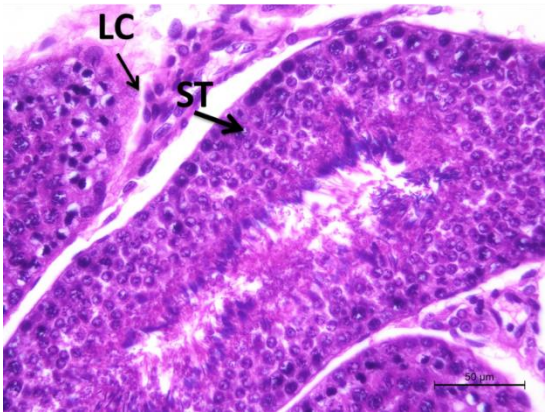

Fig.1. ST of control group revealed a marked spermatogenic activity and many of them were packed with spermatozoa (H and E, X400).

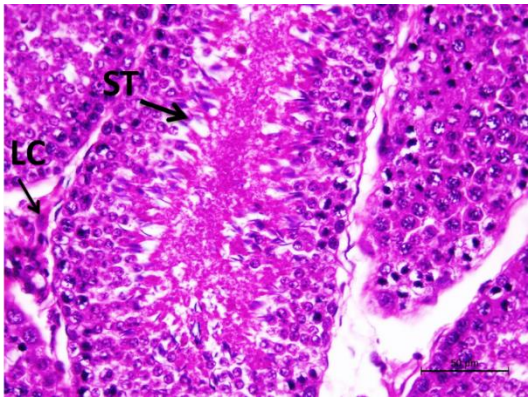

Fig.2. ST of Zinc oxide group show spermatogenic activity and many of them were highly packed with spermatozoa in comparable to control group (H and E, X400).

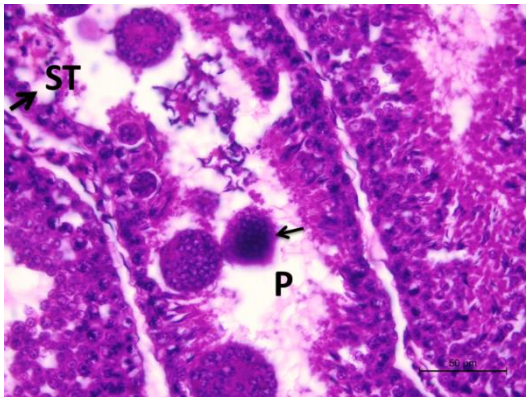

Fig. 3. ST of n-ZnO group show necrobiotic changes of spermatogenic cells especially spermatid and spermatozoa and the number of multinucleated giant cells per ST were increased as well as the density of spermatid and spermatozoa was lower in comparison to the control group (H and E, X400).

4. Discussion

The obtained data **(Table, 1)** showed that ZnO supplementation induced significant ($P \leq 0.05$) improvement in all the growth performance trials including; B.W., B.W.G. and FCR, while the results of FC not significantly changed, throughout maturation period (6th, 7th and 8th months of age), in comparison with that of the control group. Similarly, it was observed that Zinc supplementation enhance growth performance including BW and BWG at dose of 40µg/kg dry matter in cross breed New Zealand white and California white adult does at 6 months old for 60 days **(Alikwe et al., 2011).** Also, several previous studies recorded the beneficial effects of Zinc supplementation in ration of growing rabbits on growth performance trials at different doses. In this context, **Kamel et al., (2020)** documented that Zinc supplementation at a dose of 50mg/kg dry matter in ration of growing Egyptian Baladi rabbits (5 weeks old) for 8 weeks was essential to support normal growth performance and prevent deficiency conditions during a steady state. In the same line, it was reported that 100mg Zinc/Kg ration improved both BW and FCR as compared with control group in growing New Zealand White male at for 8 weeks **(Al-Sagheer et al., 2020).** It was reported that Zn is essential for many physiological functions inside animal body and vital for growth as well as it is essential for the action of more than 200 metallo enzymes **(Tako, 2019).** In this context, the positive effects of Zinc may be due to its role in polymeric organization of DNA and RNA, which are reliable for the growth and development of skeleton and synthesis of body proteins **(Chrastinova et al., 2015).** In contrast, other reports did not record any significant effect on growth of NZW rabbits when fed diet supplemented with ZnO at a dose 50, 100, 200, and 400 mg ZnO/kg ration for 8 weeks **(Selim et al., 2012)** and Zinc bacitracin at a dose of 83mg/V-line rabbit per day for 81 days **(Attia et al., 2015).** These results were attributed to probabilities that Zinc absorption inside the intestinal lumen may prevented by some antagonists such as excess amount of Ca, Fe, unabsorbed fat and dietary phytate that present in plant based diets such as wheat bran,

soybean meal and cottonseed meal **(Meshreky et al., 2015; Ognik et al., 2016)**.

Concerning the effect of n-ZnO dietary supplementation on growth performance trials throughout the maturation period (6th, 7th and 8th months of age); the present results **(Table, 1)** showed that n-ZnO group exhibited significant (P≤0.05) increase in B.W. at the 6th and 7th month of age, in comparison with that of control, as well as the 6th month only as comparable with ZnO group. Furthermore, results of FC throughout the maturation period were nearly similar among different groups. These findings support the results of our recent study, which revealed that n-ZnO supplementation in growing V-line male rabbits induced significant improvement in all of the previously mentioned growth performance trials **(El-Anwar et al., 2023).** Similar findings were obtained when n-ZnO was supplemented in premature New Zealand White rabbits at a dose of 30 mg n-ZnO/kg ration **(Hassan et al., 2017)** and 60mg n-ZnO/kg ration **(Tag-El Din, 2019)** as well as in Egyptian Baladi rabbit at a dose of 30 mg n-ZnO/kg ration **(Kamel et al., 2020)**. It is Noteworthy to mention that n-ZnO significantly improved the production of testosterone **(Abdel-Wareth et al., 2022)** and growth hormones **(Hassan et al. 2017)** which were reported to be vital for many physiological processes relating to growth **(Sinha et al., 2014)**. Moreover, the positive effect of n-ZnO over that of ZnO may be attributed to its physicochemical characteristics such as great specific surface area, high surface activity and strong adsorbing ability **(Jiang et al., 2018))**.

However, the obtained results revealed that effect of n-ZnO is time dependent since, it induced significant (P≤0.05) reduction in the BW was recorded in n-ZnO group in comparison with ZnO group at the 7th and 8th months of age. Beside, significant (P≤0.05) drastic effect in BWG and FCR in the n-ZnO group throughout maturation period in comparison with ZnO group, as well as at the 7th and 8th months of age in comparison with that of control group. These drastic effects may be due to the effect of n-ZnO supplementation is time dependent, since it induced hazards effect after a long term of supplementation (6 months). Similarly, it was found that long term exposure of mice to 50 and 500 mg/kg n-ZnO diets showed minimal toxicity and retard their body weight due to its bioaccumulation inside the body organs **(Wang et al., 2016)**. Furthermore, **Hong et al., (2014)** concluded that n-ZnO administration at high repeated doses (400mg/Kg B.W.) caused retardation in growth performances of rat dams. The authors attributed this effect to the dose dependent effect of n-ZnO that induced repeated irritation to internal organs and cytotoxicity that retard the maternal body weight. In addition, it was certified that intra-peritoneal treatment of male rabbit with n-ZnO at a dose of 600 mg/kg B.W. induced increase in oxidative stress and ROS (reactive oxygen species) that disrupt cell metabolism, antioxidant enzymes function, inhibit cell response to antioxidant as well as resulted in DNA damage and cell degeneration **(Taha and Ismail, 2023)**. Besides, n-ZnO physicochemical characteristics, such as being sufficiently small to penetrate the cell membranes and their large surface area enhance their cytotoxicity and bioaccumulation of n-ZnO inside the body **(Deng et al., 2009 and Seok et al. 2013)**.

The current results showed that ZnO dietary supplementation induced significant (P≤0.05) increase in serum levels of Zn, SOD and CAT in comparable with control group, throughout this experiment **(Table, 2)** and in our previous growing experiment at the 3rd, 4th and 5th months of age **(El-Anwar et al., 2023)**. Similarly, it was found that ZnO supplementation significantly (P≤0.05) increase Zinc level, as the supplemented Zinc acts to compensate the unavailable Zinc inside the animal body **(Kuckova et al., 2021)**. It was also, mentioned that Zinc enters in the constitution of more than 240 enzymes and it is considered as a vital component for SOD, which was included in the cellular scavenging of free radicals and ROS **(Prasad, 2008)**. Furthermore, **Milinković-tur, et al., (2009)** and **Kuckova et al., (2021)** stated that supplementation of animal diet with Zinc leads to an increase in the activity of antioxidant enzymes such as CAT and GSH-Px and SOD. Besides, Zinc was considered as an antioxidant since; it diminishes the O-2 formation via inhibition of NADPH oxidase (an electron donor) that facilitates O_2 formation **(Prasad et al., 2004; Jarosz et al., 2017)**.

It was also noted that, n-ZnO group induced significant (P≤0.05) increase in the serum levels of Zn throughout the experimental period **(Table, 2)** periods. However, it's effect on SOD and CAT in serum appeared to be time dependent, as these parameters increased significantly (P≤0.05) in serum during the previous growing experiment **(El-Anwar et al., 2023)** as well as at the 6th month of age only of the maturation period **(Table, 2).** In addition, the positive effects of n-ZnO on the above mentioned parameters seemed to be higher than that of ZnO. These findings were in the same line with previous studies that recorded the positive correlations between n-ZnO dietary supplementation and serum levels of Zinc **(Hassan et al., 2017)** as well as SOD and CAT **(Hafez et al., 2020; Kamel et al., 2020).** It was suggested that, the positive results of n-ZnO on the above mentioned parameters appeared to be partly supported by higher absorption of n-ZnO as a result of its physicochemical characteristics, such as being sufficiently small to penetrate the cell membranes and their large surface area **(Hassan et al., 2017; Hafez et al., 2020)**.

On the contrary, significant (P≤0.05) decrease was observed in serum levels of SOD and CAT at the 7th and 8th months of age in n-ZnO dietary supplemented group. It was observed that studies concerning the effects of long term n-ZnO dietary supplementation on serum antioxidants activities in rabbits appeared to be scanty. However, the current results come in the same line with **Adeniyi et al., (2023)** who found that intra-peritoneal repeated doses of n-ZnOat a dose of 80µg/kg for 4 weeks in rats led to increase ROS and decrease levels of SOD and CAT. Likewise, it was found that long term exposure to nano-Zinc oxide through supplementation in diets showed minimal toxicity, increase ROS and decrease levels of antioxidant enzymes **(Wang et al., 2016)**. In this context, it was mentioned that ROS and their metabolites induce bad effects on DNA, proteins, lipids and anti-oxidative enzymes; including SOD and CAT **(Pujalte et al., 2011)**. Furthermore, the previously mentioned physicochemical characteristics of n-ZnO enhance their bioaccumulation throughout the period of supplementation and cytotoxicity in a dose **(Taha and Ismail, 2023)** and time **(Wang et al., 2016)** dependent manner.

On the other hand, the effects of ZnO as well as n-ZnO dietary supplementation on the levels of Zn, SOD and CAT in seminal plasma during maturation period of V-line rabbit, data in **Table (3)** showed that the changes in seminal plasma levels of the above mentioned parameters come in the same line with their levels in the serum **(Table, 2).** In this context, it was found that dietary Zn as well as n-ZnO supplementation enhances antioxidant enzymes activities in seminal plasma of rabbit buck **(El-Gindy et al., 2023)**. Also, dietary Zn supplementation increased Zn and antioxidant enzymes activities levels in seminal plasma of buck **(Amen and Al-Daraji, 2011),** boriler male chicken **(Rahman et al., 2014)** and stallion **(Liu et al., 2020)**.In opposition, the low values of SOD and CAT in seminal plasma **(Table, 3)** were exhibited in the n-ZnO dietary supplemented group at the 7th and 8th months of age, comes in concomitant with their levels in serum **(Table, 2).** These findings might be support the positive correlation between biochemical component that found in serum and those in seminal plasma, so seminal plasma is considered as a reflecting mirror for the levels of serum constituents **(Talluri et al., 2017).**

Concerning the effect of ZnO and n-ZnO dietary supplementation on thyroid hormones, results of the current work **(Table, 2)** revealed that, ZnO supplementation didn't induce any significant changes in serum levels of T3 and T_4 during maturation period as compared to control group. Likewise, n-ZnO supplemented group didn't show any significant changes at 6th month age. In this context, Zn **(Teixeira et al., 2020)** as well as nano-Zinc **(Hameed et al., 2023)** were found to play an important role in maintaining normal levels of TRH, TSH, T_3 and T_4 and enhance metabolism as, Zinc has stimulating effect on some endocrine glands such as pituitary gland and hypothalamus. It was also, postulated that Zn deficiency is concomitant with increased expression of hepatic T4-5'-monodeiodinase enzyme activity which inhibit thyroid hormones **(El-sisy et al., 2008)**. However, at 7th and 8th months of age, n-ZnO induced significant ($P \leq 0.05$) hazards effect on serum levels of both hormones, as compared to both control as well as ZnO groups. In this regard, few studies in rats showed that intra-peritoneal injection with n-Zinc at a dose of 30 and 60mg/Kg for 7, 14, and 28 days provoked significant decrease in serum levels of T_3 and T4 **(Luabi et al., 2019)**. In light of this point the author added that, the high nano-Zinc intake may induce toxicity in a time and dose dependent manner via reducing the regulatory effect of pituitary gland on thyroid gland and decreasing production due to the prolonged supplementation period. In addition, high n-ZnO can increase oxidative stress and ROS that disrupt cell metabolism and antioxidant enzymes function **(Yin et al., 2010)**.

The current results showed that, ZnO group exhibited significant ($P \leq 0.05$) improvement in serum testosterone levels throughout the previous growing period **(El-Anwar et al., 2023)** as well as in the current maturation **(Table, 2)** experiments, in comparable with control group. Similarly, **El-Masry et al., (1994)** recorded that, Zinc sulphate supplementation in the diet of NZW mature male rabbits (12-15 months old) at a dose of 35mg/kg diet for 3 months significantly improved serum testosterone level. Moreover, it was observed thatoral administration of Zinc gluconate in male mature rats at a dose of 5mg/kg. BW for 90 days **(Darago et al., 2020)** or intra-peritoneal injection of Zinc (5mg/kg BW) twice weekly for 8 successive weeks led to an increase in serum testosterone level **(Goma et al., 2021)**. In this context, Zinc plays a vital role in production of gonadotrophin releasing hormone and consequently, secretion of testosterone from the Leydig cells **(Saaranen et al., 1987)**. Additionally, Zinc was reported to lower the blood concentrations of cholesterol and directed it for the production of testosterone **(Reza et al., 2014)**. Additionally, it was also noted that Zinc stimulates the transformation of testosterone into its biologically active form (5α -dihydrotestosterone) **(Ali et al., 2007)**.

On the other side, n-ZnO dietary supplemented group exhibited significant ($P \leq 0.05$) increase in the serum levels of testosterone throughout the previous growing period **(El-Anwar et al., 2023)** as well as at 6th month of age only of the maturation period **(Table, 2)** as compared to ZnO as well as control groups. Similarly, previous studies documented that n-ZnO dietary supplementation induced significant increase in serum testosterone levels over that of its bulk form (ZnO), suggesting, higher absorption and bioavailability of n-ZnO **(Hafez et al., 2019; Kamel et al., 2020; Goma et al., 2021).** Meanwhile, at the 7th and 8th months of age, n-ZnO dietary supplementation induced significant ($P \leq 0.05$) decrease in serum levels of testosterone **(Table, 2).** These results might be a reflection to the prolonged exposure to n-ZnO throughout the 3 months of the growing period till the 6th month of maturation period. In this respect, it was suggested that n-ZnO prolonged exposure through diet supplementation induced minimal toxicity, increased ROS, decreased levels of antioxidant enzymes that resulted in DNA damage, degeneration and apoptosis in interstitial cell of Leydig, consequently decreased testosterone production **(Wang et al., 2016; Goma et al., 2021; Taha and Ismail, 2023).**

It was known that, Androgen receptor is a single nuclear receptor that controls androgens actions via acting as a ligand-dependent transcription factor **(Mangelsdorf et al., 1995).** The present work cleared that, ZnO supplementation exhibited significant ($P \leq 0.05$) increase in AR gene expression at the 8th **(Table, 2)** month of age, as compared to control group. It was found that data concerning the influence of ZnO and n-ZnO dietary supplementation on AR gene expression in mature male rabbits found to be scanty. However, it was reported that intra-peritoneal injection of ZnO at low doses in rats led to improve the expression of AR gene and increased serum testosterone level **(Darago et al., 2020 and Goma et al., 2021).** These effects might be attributed to the fact that Zinc is considered as an antioxidant; inhibiting oxidation of lipids, proteins, DNA and consequently prevent inhibition of gene expression **(Kulbacka, et al., 2009).** On the other side, at the 8th month of age, n-ZnO supplementation displayed significant ($P \leq 0.05$) reduction in the AR gene expression when compared to control as well as ZnO groups **(Table, 2),** while it induced an improvement at the end of the growing period (5th month of age) **(El-Anwar et al., 2023).** The results of the current experiment considered as a consequence for the long term of n-ZnO supplementation that induced positive effects at the 5th month of age while, hazards effect occurred at the 8th month of age. In this context, **Gomaet al. (2021)** recorded an inhibition of AR expression and other fertility-related genes in rat supplemented with a high dose of n-ZnO. These effects might attributed to the higher physicochemical characters of n-ZnO and long term exposure whichenhance their bioaccumulation that caused increased ROS, cytotoxicity and DNA damage, consequently inhibiting AR gene expression **(Kamel et al., 2020; Gomaet al., 2021; Taha and Ismail, 2023).**

It was found that, there was a direct relationship between semen characters and the above mentioned parameters including; serum testosterone levels, Zn concentration and antioxidant activities **(Saylam and Cayan, 2020; Abdel-Wareth et al., 2022).** In this context, it was found that oxidative stress decreased serum levels of antioxidant enzymes and increased ROS that resulted in DNA damage, degeneration and apoptosis in interstitial cell of Leydig, consequently decreased testosterone production that resulted in impaired spermatogenesis and other sperm parameters relating to sperm quality **(Majzoub et al., 2017, Saylam and Cayan, 2020; Taha and Ismail, 2023).** The current experiment showed a significant ($P \leq 0.05$) improvement in semen characters throughout the maturation period in the ZnO group as well as at the 6th month of age in n-ZnO group, as compared to control group **(Table, 4).** These findings come in concomitant with the improvement in serum testosterone level **(Table, 2)** as well as Zn and antioxidant enzymes levels in serum **(Table, 2)** and seminal plasma **(Table, 3).** Moreover, several studies in rabbits bucks documented that Zinc supplementation in ration at different doses (35 to 150 mg/kg) was associated with the improved spermatogenesis and semen characters **(Moce et al., 2000; Oliveira et al., 2004; Cheah and Yang, 2011).** Additionally, optimal Zinc concentration in seminal plasma was reported to enhance semen characters and antioxidant enzymes activity of the sperm **(Roy et al., 2013).** Furthermore, it was found that Zn supplementation might improve testicular activity and steroidogenesis via its stimulating effect on the hypothalamus and the pituitary in a dose dependent manner **(Arangasamy et al., 2018).** Also, the positive effect of Zinc might be attributed to the involvement of Zinc in many biochemical processes and physiological functions such as function of structural proteins, enzymes and hormones which are vital for growth and development **(Bao et al., 2009).** As well, it increases the synthesis of DNA and RNA through stimulating the activity of DNA and

RNA polymerases, so improves spermatogenesis and semen characters **(Moce et al., 2000; Oliveira et al., 2004; Cheah and Yang, 2011).**

Moreover, n-ZnO group exhibited significant (P≤0.05) improvement over that of ZnO group in the semen characters **(Table, 4)** at the 6th month of age as well as in the previous growing experiment (3rd, 4th, 5th months of age) **(El-Anwar et al., 2023).** Likewise, **Abdel-Wareth et al., (2020)** found that supplementation of male rabbit diet with 100mg n-ZnO/kg diet improves semen characteristics and testosterone concentration. The positive biological action of n-ZnO might be attributed to its physicochemical properties that exert its superior efficacy as compared with ZnO **(Hafez et al., 2020).** Conversely, n-ZnO supplementation induced significant (P≤0.05) hazard effects in examined semen characters at the 7th and 8th months of age, as compared to both control and ZnO groups. These results support the time dependent effect of n-ZnO supplementation that induce bioaccumulation and cytotoxic effect. Also, **Halo et al., (2021)** documented that n-ZnO supplementation in rabbit ration induced bad effects on the semen picture especially sperm motility and viability parameters when supplemented at high doses. Similarly, **Goma et al., (2021)** observed that rat supplemented with a high dose of n-ZnO diversely affects semen characteristics; sperm motility, viability and epididymal sperm count as well it increased sperm abnormalities and dead sperm %. These harmful effects might be due to n-ZnO bioaccumulation, cytotoxicity, increased ROS, and decreased antioxidant and testosterone serum levels **(Wang et al., 2016; Taha and Ismail, 2023).**

Concerning microscopical picture of the testicular tissue at the end of the maturation period; at the 8th month age, showed that ST of ZnO group showed normal spermatogenic activity and many of them were highly packed with spermatozoa as compared to control group **(Fig. 2).** These result are in the same line with **Goma et al., (2021)** who found that ZnO supplementation enhance mitotic activity of ST. Also, **Oliveira et al., (2004)** documented that supplementation of ZnO at different doses (50-150mg/kg) increases spermatozoa concentration. These findings might be attributed to the involvement of Zinc in many biochemical processes and physiological functions which are necessary for growth and development **(Bao et al., 2009).** Also, Zinc increases the synthesis of DNA and RNA by stimulating the activity of DNA and RNA polymerases, so increases spermatogenesis and improves semen characters **(Moce et al., 2000; Oliveira et al., 2004; Cheah and Yang, 2011).**

However, ST of n-ZnO group showed necrobiotic alterations in the spermatogenic cells especially spermatid and spermatozoa accompanied with a high number of multinucleated giant cells in the lumen of ST **(Fig. 3).** In addition, the density of spermatid and spermatozoa was lower in ST of n-ZnO group as comparable with the control group **(Fig. 1).**Similarly, **Wang et al., (2016)** and **Goma et al., (2021)** found that n-ZnO supplementation had harmful effect on testicular tissue in a dose and time-dependent manner including; shedding germinal epithelial (pachytene stage) in the lumen of ST, shrinkage of ST, vacuolar degeneration of the germinal epithelium and Sertoli cells. These harmful effects might be due to n-ZnO bioaccumulation, cytotoxicity, increased ROS and decreased antioxidant, AR gene expression as well as serum testosterone levels **(Wang et al., 2016; Taha and Ismail, 2023)**.

5. Conclusion

Based on the results of the current work, it can be concluded that ZnO supplementation at 60mg/kg ration could improve rabbit growth performance and reproduction while n-ZnO could induce hazards effects when increase duration of supplementation at the same dose.

6. Authors Contributions

All authors participated equally to the design of the research, methodology, statistical analysis of results, and writing of the manuscript.

7. Conflict of Interest

The authors stated that there is no conflict of interest.

8. References

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