Journal of Agricultural Science; Vol. 7, No. 10; 2015
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Protective Role of Some Feed Additives against Dizocelpine Induced Oxidative Stress in Testes of Rabbit Bucks

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Received: June 21, 2015 Accepted: July 28, 2015 Online Published: September 15, 2015

doi:10.5539/jas.v7n10p239 URL: http://dx.doi.org/10.5539/jas.v7n10p239

Abstract

As optimization of farm animals reproductive performance is a main objective, the present study was, undertaken to investigate the possible protective effect of vitamin C, vitamin E and olive pomace against dizocilpine (MK-801) induced oxidative stress and its resultant alterations on antioxidant status, spermiogram, hormonal, enzyme markers and histomorphology of testes of rabbit bucks during first and second month of the experiment. For this purpose thirty six male New Zealand White rabbits bucks were equally and randomly divided into six groups (6 in each) namely control injected with 1.0 ml sterile saline; second group was injected by dizocilpine at a dose 0.1 mg/kg. intraperitoneally (i.p.) for 5 consecutive days, the third group was subjected to dizocilpine and supplemented with "vitamin C" (1 g/L of drinking water) 1 g contain 100 I.U. vit C, while the forth group was subjected to dizocilpine and supplemented with "vitamin E" (50 ml/100 L of drinking water,1ml contain 20 I.U. vit E,) and those of the fifth group were supplemented with "vitamin C" plus "vitamin E". All five groups were fed the same basal diet, while the sixth group was subjected to dizocilpine and fed diet which contains olive cake meal (10%). Results in first month revealed (1) significant decrease in spermiogram, antioxidative parameters, testicular estradiol, testosterone, enzyme markers and clear pathological changes in testes of dizocilpine group; (2) Significant improvement in the measured parameters of groups subjected to dizocilpine and supplemented with "vitamin E", "vitamin C" and olive pomace; (3) significant increase of all measured parameters in the "vitamin C" plus "vitamin E" supplemented group. On the other hand, results at second month showed no difference between all groups in these parameters. Conclusively, it was obvious that the supplementation with vitamin C or/and vitamin E and olive pomace to male rabbits exposed to oxidative stress was associated with improved spermiogram, anti-oxidative parameters, hormonal and testicular enzymatic activities.

Keywords: oxidative stress, olive pomace, rabbit bucks, vitamin C, vitamin E

1. Introduction

The optimization of reproductive performance is one of the main facts that assure high productivity on rabbit farms, and this requires that the management practices take into account the physiology and behavior of the animal since environmental and sanitary aspects interfere with fertility and can impair it (Friggens, 2003).

It is appreciated that rabbit male spermatozoa plays important role in maintainin1g reproductive performance of doe (Bencheikh, 1993).

The term "oxidative stress" refers to an imbalance between the excessive production or accumulation of reactive oxygen species (ROS) and an impaired antioxidant mechanism (Sikka, 2001; Agarwal et al., 2003). Meanwhile, excessive levels of metabolites (reactive oxygen species; ROS) can cause damage of spermatozoa or lipid peroxidation .Also, exposed animal to chemical substance such as injection with dizocilpine (MK-801) induced oxidative stress in rat testis (Ozyurt et al., 2007), blocking glutamatergic-NMDA-receptor complex in the brain. Confirm the expression of functional glutamate transporters in the rat testis (Takarada et al., 2004), which might

play a role in the spermatozoal functions and integrity of spermatogenesis.

Dietary antioxidants factors have special importance in maintenance of growth in a high levels, reproduction and immune-competence in animal production by reducing the bad effects of free radicals and toxic metabolites on animals (Peter, 2007). The antioxidants are believed to play a very important role in the animal body defense system against ROS (Vivek & Surendra, 2006).

Natural antioxidants are constituents of many fruits and vegetables and they have attracted a great deal of public and scientific attention (El Diwani et al., 2009). Boskou, (2006) reported that the dietary antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), phenolic compounds (olive by-product) and carotenoids are well known and there are a surplus of publications related to their role in health. However, the amounts of the protective antioxidant principles present under the normal conditions are sufficient only to cope with the physiological rate of free-radical generation (Ashok & Sushil, 2005).

Increased oxidative stress thus needs supplementation with external sources of antioxidants, as vit. E can have a positive role in increasing semen quality via protecting cell membrane of testes and mitochondria from oxidation (Yue et al., 2010). Also, vitamin C improves the epididymal sperm concentration, serum testosterone and semen quality (Sónmez et al., 2005).

Olive pomace contains small amount of olive oil and consists mainly of water, olive skin, olive flesh, pulp fragments. While, chemical composition of olive pomace is characterized by a high content of crude fiber and sugars (mainly polysaccharides) and moderate values of crude protein, fatty acids (oleic acid), polyalcohols, polyphenols and other pigments (Brunetti et al., 2005). Olive pomace (olive by-product) contain many compounds with antioxidant activity, mostly phenolic compounds which classified into a lipophilic group (tocopherols mainly), a hydrophilic group (phenolic acid and flavonoids) and oleuropein (a non-toxic secoiridoid, which are hindered phenolic chain breaking antioxidants, and the chain breaking antioxidants are highly reactive with free radicals and form stable compounds that do not share in the oxidation chain reaction (Amro, Aburjai, & Al-Khalil, 2002; El Diwani et al., 2009).

2. Materials and Methods

2.1 Animals and Experimental Design

The study was conducted in the experimental rabbitry of Physiology Department, Faculty of Veterinary Medicine Cairo University during the period from January to May 2014 in accordance with the Chinese guidelines for animal welfare and approved by the animal welfare committee of Animal Science College, Zhejiang University.

Thirty six male New Zealand White rabbits of about 5-months age and average body weight of 2500g were randomly selected, used for the experimental work one month later. During this month, the rabbits were gradually adapted to natural mating. The experiment lasted for 2 month (length of spermatogenic cycle in rabbit), bucks were housed individually in commercial cages (55×60×34 cm), equipped with automatic drinkers and j-feeders. Clean and fresh water was available all time.

The whole rabbitry was well ventilated through both natural windows and electric fans and illuminated to 14:10 light dark cycle through natural and fluorescent lighting. The rabbitry average ambient temperature and relative humidity ranged from 20 to 30 ºC and 70-80%, respectively during summer resting period. Basal and experimental diets were formulated to cover the nutrient requirements of rabbits as recommended in NRC (1977). Diets were subjected to chemical analysis according to AOAC (1997), and offered for all animals ad libitum Table 1.

The bucks were equally and randomly divided into six groups (6 in each) namely control which injected with 1.0 ml sterile saline intraperitoneal (i.p.), second group injected by dizocilpine (MK-801 purchased from Sigma-Aldrich, Egypt), at dose 0.1 mg/kg. The dose of MK-801 was dissolved in 1.0 ml sterile saline and was injected daily intraperitoneal (i.p.) for 5 consecutive days, third group was injected by dizocilpine and supplemented with “vitamin C” (1 g/L of drinking water), while the forth group was injected by dizocilpine and supplemented with “vitamin E” in drinking water (50 ml/100 L)(the intenational units of vitamin E contained in 50ml be mentioned) and those of the fifth group was supplemented with “vitamin C” plus “vitamin E”. All five groups were fed the basal diets, while the sixth group was injected by dizocilpine and supplemented with the experimental diet that contained olive pomace (10%).

2.2 Samples Collection

Semen was collected using artificial vagina (Moce et al., 2000). Semen collection was done by using a teaser female and artificial vagina (containing water at 50 °C) that was locally fabricated as described by Herbert and

Adejumo (1995). The ejaculate volumes were recorded (using a graduated collection tube) after removal of the gel mass; Semen pH was determined using pH paper 1-14; Mass motility (MM) was determined by placing a drop of semen on a clean, dry, warm slide and examinining microscopically using thermostatically controlled hot stage adjusted at 38-40 °C. Mass activity of spermatozoa was scored (0-5) according to the intensity of the moving whirls (Moule, 1965) as follows: 0 = no current, 1 = few slow current, 2 = many moderate waves, 3 = many sweeping waves, 4 = numerous vigorous waves, 5 = numerous rapid and vigorous waves. Individual motility was assessed in semen sample diluted with 2.9% sodium citrate dehydrate solution, spread almost evenly under a glass cover slide and examined microscopically using adjusted hot stage at 38-40 °C. Individual sperm motility percent was determined on a subjective scale of 0-100% to the nearest 5% after viewing several microscopic fields. Sperm-cell concentration per ml was measured by counting the number of spermatozoa present on both sides of an improved Neubauer haemocytometer slide (GmbH & Co., Brands twiete 4, 2000 Hamburg 11, Germany). Total sperm output was calculated by multiplying semen ejaculate volume and semen concentration. Assessment of live, dead, and abnormal spermatozoa was performed using an eosin–nigrosin blue staining mixture (Rodríguez-De Lara et al., 2008).

2.3 Samples Analysis

Two bucks from each group one and two months later from beginning of experiment were slaughtered for obtaining the testes. The testes of each buck were dissected and weighed; one testis was kept in liquid nitrogen for testicular enzyme markers, antioxidant parameters and hormonal assay, while the other testis was fixed in 10% formol saline for histomorphology.

The antioxidant parameters and activities of selected testicular enzyme markers (alkaline phosphatase and lactate dehydrogenase) besides sertoli cell index (gamma glutamyl transferase) were estimated in testes using testicular homogenate which was prepared according to the method adopted by Hoden and Sherins (1973); Wherein testicular tissue was homogenized in 0.015 M disodium hydrogen phosphate (Na_2HPO_4), 0.15 M sodium chloride (Nacl), pH 7.8 at 4 °C. All assays were performed within 48 hours after homogenization.

2.3.1 Oxidative and Antioxidant Status

Superoxide dismutase activity (Jewett & Rocklin, 1993), glutathione peroxidase activity (Paglia & Valentine, 1967), glutathione-S-transferase (Habig et al., 1974), total antioxidant capacity (Koracevic et al., 2001) and lipid peroxidation expressed in Malondialdehyde (Yoshioka et al., 1979) were performed using kits purchased from Biodiagnostic Company, Dokki, Egypt.

2.3.2 Hormonal Assay

Radioimmunoassay was used for quantitative determination of testosterone and17beta-estradiol hormones in testes homogenate.

Determination of testicular testosterone was performed according to the method of Jaffe and Behrman (1974) using kits purchased from "TESTO-RIA-CT" Belgium.

Determination of testicular 17beta- estradiol was performed according to the method of Xing et al. (1983) using kits purchased from "IMMUNOTECH".

2.3.3 The Testicular Enzymes

Alkaline phosphatase (ALP) (Teitz, 1970), gamma glutamyl transferase (GGT) (Szasz, 1969) and lactate dehydrogenase (LDH) (Allain, 1973) using kit purchased from Spectrum Diagnostics.

2.4 Histomorphological Studies

For qualitative analysis of testicular histology, the testes samples were fixed for 2 days at 10% formal-saline and dehydrated by passing successfully in different mixtures of ethyl alcohol-water, cleaned with xylene and embedded in paraffin. Sections of tissue (5-6 μm thickness) were prepared by using microtome and stained with haematoxylin and eosin and in neutral deparafinated xylene (DPX) medium for microscopic observations.

2.5 Statistical Analysis

Data are presented as means ±S.E. and analyzed by one way ANOVA using Costate computer program Costat version 6.400 (copyright© 1998-2008 CoHort software) according to the method of Snedecor and Cochran (1980). Groups were compared using the calculated least significant difference test (LSD) at the at P value ≤ 0.05.

Table 1. Composition percentage and analysis nutrients profile of the basal and experimental diets

Ingredients (%)	Basal diet	Exp. diet
Berseem hay	30.0	29.1
Olive (pomace)	-	10.0
Barley grain	21.0	19.0
Yellow corn	5.0	3.0
Wheat bran	21.1	20.0
Soybean meal	17.5	13.5
Molasses	3.0	3.0
$CaCl_2$	1.5	1.5
NaCl	0.4	0.4
Vit. & Min. Premix*	0.3	0.3
DL-Methionine	0.2	0.2
Chemical analysis (%)**		
Moisture	9.4	9.5
Crude protein	17.5	16.5
Crude fiber	14.0	23.3
Ether extract	2.70	6.1
Total Ash	7.10	7.5
Nitrogen free extract	49.30	47.6
Calculated digestible energy (kcal/kg)	2600	2700

Note. *The Rabbit's vitamin and mineral premix/kg contained the following IU/g for vitamins or minerals: A-4,000,000, D3-5000,000, E-16,7 g, K-0.67 g, B1-0.67 g, B2-2 g, B6-0.67 g, B12-0.004 g, B5-16.7 g, Pantothenic acid-6.67 g, Biotein-0.07 g, Folic acid-1.67 g, Choline chloride-400 g, Zn-23.3 g, Mn-10 g, Fe-25 g, Cu-1.67 g, I-0.25 g, Se-0.033 g, and Mg-133.4 g (Rabbit premix).

** Official methods of analysis of AOAC, international (1997).

3. Results

3.1 Effect of Vitamin C, Vitamin E and Olive Pomace Supplementation on Semen Characteristics of Bucks Subjected to Dizocilpine Oxidative Stress (Means ± SE)

The results summarized in Table 2.

One month after induction of oxidative stress, group subjected to dizocilpine showed significant decrease of semen volume, mass motility, individual motility, sperm concentration and live dead ratio. At the same time there was a significant increase in abnormal sperm count of bucks subjected to dizocelpine as compared to the corresponding counts in control. On the other hand, groups subjected to dizocilpine and supplemented with vitamin C, vitamin E and olive pomace showed a significant improvement in these parameters in comparison to the dizocilpine group group supplemented with vitamin C+E showed no significant difference in semen characteristics as compared with the control values. The group subjected to dizocilpine revealed significant decrease of semen volume, mass motility, individual motility, sperm concentration and live dead ratio than those of control.

Results in second month (Table 3) revealed that, group subjected to dizocilpine and groups of dizocilpine and supplemented with vitamin C, or vitamin E and olive pomace showed no differences in the semen characteristics as compared to control values. While, group supplemented with vitamin C+E showed significant variation in all the sperm concentration as compared with control value.

Table 2. Semen characteristics of bucks one month post induction of oxidative stress

Items	Basal level	Experimental groups						LSD
		C	D	DC	DE	DO	C+E	
Semen Volume(ml)	0.70±0.17	0.83±0.07^{ab}	0.50±0.03^{b}	0.60±0.03^{ab}	0.57±0.07^{ab}	0.70±0.03^{ab}	0.90±0.03^{a}	0.26
Semen PH	7.33±0.29	7.34±0.19^{a}	7.36±0.19^{a}	7.30±0.10^{a}	7.47±0.19^{a}	7.39±0.19^{a}	7.43±0.19^{a}	0.82
Mass motility	3.50±0.15	4.17±0.20^{a}	3.17±0.19^{c}	3.35±0.22^{bc}	3.33±0.19^{bc}	3.67±0.11^{b}	4.33±0.10^{a}	0.47
Individual motility%	72.67±0.84	83.00±5.20^{a}	78.67±4.07^{b}	81.00±3.20^{ab}	79.67±3.35^{ab}	79.67±2.01^{ab}	87.33±4.69^{a}	4.01
Sp.conc × 10^{6} (ml)	305.00±11.67	311.33±11.17^{a}	301.67±10.69^{b}	306.33±11.02^{ab}	305.67±12.35^{ab}	306.33±10.51^{ab}	311.67±11.84^{a}	4.91
Live&dead ratio%	75.00±4.67	87.67±2.51^{a}	79.00±3.33^{b}	84.00±2.88^{ab}	81.67±2.51^{ab}	81.33±2.07^{ab}	89.00±3.20^{a}	4.36
Abnormal sperm%	9.33±0.91	10.03±0.67^{ab}	14.33±0.69^{a}	10.33±0.51^{ab}	10.67±0.51^{ab}	11.00±0.58^{ab}	9.02±0.33^{b}	2.99

Note. Different subscripts within a row indicate a significant treatment effect ($P \leq 0.05$) (n: 6 rabbits/group).

C (Control), D (Dizocilpine), DC (Dizocilpine+Vit. C), DE (Dizocilpine+Vit. E), DO (Dizocilpine+Olive Pomace), CE (Vit. C+Vit. E), LSD (Least Significant Difference).

Table 3. Semen characteristics of bucks two month post induction of oxidative stress

Items	Experimental groups						LSD
	C	D	DC	DE	DO	C+E	
Semen Volume	0.90±0.03	0.83±0.07	0.87±0.02	0.80±0.07	0.90±0.03	1.00±0.03	0.26
Semen PH	7.36±0.18	7.34±0.19	7.35±0.17	7.60± 0.29	7.50±0.17	7.33±0.21	0.83
Mass motility	4.00±0.17	3.67±0.18	3.83±0.11	3.67±0.12	3.83±0.10	4.17±0.13	0.59
Individual motility%	86.33±1.07	85.33±2.84^{a}	85.33±1.51	85.67±1.17	85.00±2.67	88.33±3.69	4.55
Sperm.conc × 10^{6} ml	311.07±11.51^{b}	309.89±10.38^{b}	310.67±12.51^{b}	309.93±10.51^{b}	310.33±11.58^{b}	313.93±10.56^{a}	2.71
Live&dead ratio	88.33±2.69	86.67±2.51	86.33±3.84	87.33±2.11	86.67±1.17	91.33±3.07	4.30
Abnormal sperm count	9.67±0.51	10.67±0.51	10.33±0.84	11.33±0.84	10.00±0.67	8.33±0.51	3.35

Note. Different subscripts within a row indicate a significant treatment effect ($P \leq 0.05$) (n: 6 rabbits/group).

C (Control), D (Dizocilpine), DC (Dizocilpine+Vit. C), DE (Dizocilpine+Vit. E), DO (Dizocilpine+Olive Pomace), CE (Vit. C+Vit. E), LSD (Least Significant Difference).

3.2 Impact of Vitamin C, Vitamin E and Olive Pomace Supplementation on Testicular Antioxidant Parameters of Bucks Subjected to Dizocilpine Oxidative Stress (Means ± SE)

Data presented in Table 4 illustrate the average of some antioxidant parameters in testes through the experiment. Values at the first month revealed a significant decrease ($p<0.05$) in superoxide dismutase, glutathione-S-transferase, glutathione peroxide and total antioxidant parameters of rabbits subjected to dizocilpine as compared with control values. While; groups subjected to dizocilpine and supplemented with vitamin C or vitamin E and olive pomace showed a significant improvement in all these parameters when compared with the dizocilpine group. At the same time the results recorded that there was significant increase of all antioxidant parameters in the group supplemented with vitamin C+E as compared with both the control value or dizocilpine group. On the other hand, one month later values recorded no difference between all groups except the group supplemented with vitamin C+E which indicated a significant increase in average activity of enzymes superoxide dismutase, glutathione peroxide, glutathione-S-transferase and total antioxidant capacity as compared with that of the other groups.

Table 4. Impact of vitamin C, vitamin E and olive pomace supplementation on testicular antioxidative parameters of bucks subjected to dizocilpine oxidative stress (means ± SE)

	Parameters	Experimental groups						
		C	D	DC	DE	DO	C+E	LSD
1st Month	SOD (U/g tissue)	613.50±15.17^{b}	421.50±13.17^{d}	566.00±16.67^{c}	555.00±14.85^{c}	567.00±13.6 ^{c}	661.00±16.33^{a}	13.81
	GST (U/g tissue)	11.69±1.16^{a}	5.84±1.13^{c}	10.10±1.09^{b}	10.16±1.05^{b}	10.38± 1.15^{b}	11.95±1.12^{a}	0.56
	GPX (U/g tissue)	96.80±14.16^{b}	56.97±13.46^{f}	77.13±14.13^{e}	80.39±15.27^{d}	85.10±14.41^{c}	112.66±16.44^{a}	1.82
	TAC (µm/L)	2.68±0.19^{b}	0.60±0.12^{e}	1.91±0.20^{c}	1.58±0.11^{d}	1.77±0.17^{cd}	2.95±0.35^{a}	0.22
2 nd Month	SOD (U/g tissue)	645.00±17.67^{b}	639.00±16.67^{b}	640.50±18.67^{b}	642.50±17.17^{b}	644.59±16.83^{b}	679.50±15.50^{a}	4.70
	GST (U/g tissue)	12.25±1.13^{b}	12.14±1.12^{b}	12.27±1.15^{b}	12.29±1.07^{b}	12.34±1.42^{b}	12.95±1.09^{a}	0.14
	GPX (U/g tissue)	108.3±14.14^{b}	106.72±15.08^{b}	107.79±15.31^{b}	109.29±15.14^{b}	110.13±15.39^{b}	117.32±19.11^{a}	3.28
	TAC (µm/L)	2.86±0.14^{b}	2.79±0.12^{b}	2.85±0.19^{b}	2.87±0.15^{b}	2.89±0.35^{b}	3.15±0.11^{a}	0.23

Note. Different subscripts within a row indicate a significant treatment effect ($P \leq 0.05$) (n: 6 rabbits/group).

C (Control), D (Dizocilpine), DC (Dizocilpine+Vit. C), DE (Dizocilpine+Vit. E), DO (Dizocilpine+Olive Pomace), CE (Vit. C+Vit. E), LSD (Least Significant Difference), SOD (superoxide Dismutase), GST (Glutathione-s-transferase), GPX (Glutathione peroxidase), TAC (Total antioxidant capacity).

3.3 Effect of Vitamin C, Vitamin E and Olive Pomace Supplementation on Testicular Lipid Peroxide (Malondialdehyde 'nmol/g.tissue') of Bucks Subjected to Dizocilpine Oxidative Stress (Means ± SE)

At the first month, values of testicular Lipid peroxide (Malondialdehyde) revealed significant increase in group subjected to dizocilpine alone or those supplemented with vitamin C or vitamin E or olive pomace after subjection to dizocelpine as compared with control value (Table 5). The groups supplemented with vitamin C or vitamin E or olive pomace after subjection to dizocelpine showed significantly lower values for MDA as compared to dizocilpine. Meanwhile, group supplemented with vitamin C+E showed significant decrease of malondialdehyde as compared to control value. Furthermore, values at the second month revealed no difference in malondialdehyde between the groups.

Table 5. Effect of vitamin C, vitamin E and olive pomace supplementation on testicular lipid peroxide (Malondialdehyde 'nmol/g.tissue') of bucks subjected to dizocilpine oxidative stress (means ± SE)

Groups	Intervals	
	1st Month	2nd Month
C	15.12 ± 1.04 ^{d}	15.14± 1.16 ^{a}
D	30.00 ± 2.08 ^{a}	15.58 ± 1.11 ^{a}
DC	18.16 ± 1.71 ^{b}	15.48 ± 1.08 ^{a}
DE	17.82 ± 1.09 ^{b}	15.36 ± 1.14 ^{a}
DO	16.75 ± 1.08 ^{c}	15.44 ± 1.16 ^{a}
C+E	14.45 ± 1.07 ^{e}	14.55 ± 1.19 ^{a}
LSD	0.52	0.12

Note. Different subscripts within a row indicate a significant treatment effect ($P \leq 0.05$) (n: 6 rabbits/group). C (Control), D (Dizocilpine), DC (Dizocilpine+Vit. C), DE (Dizocilpine+Vit. E), DO (Dizocilpine+Olive Pomace), CE (Vit. C+Vit. E), LSD (Least Significant Difference).

3.4 Effect of Vitamin C, Vitamin E and Olive Pomace Supplementation on Testicular Hormones Concentration of Bucks Subjected to Dizocilpine Oxidative Stress (Means ± SE)

From Table 6 results of testicular hormones at the first month revealed significant decrease at $P < 0.05$ in rabbits subjected to dizocilpine as compared with control values. Moreover, groups subjected to dizocilpine and supplemented with vitamin C or vitamin E and olive pomace showed significant improvement of these parameters

when compared to those of dizocilpine group. Furthermore, values indicated significant increase of testosterone and estradiol of rabbits supplemented with vitamin C+E as compared with control value and dizocilpine group. On the other hand, values in second month indicate no significant difference between the groups except with the group supplemented with vitamin C+E indicating a significant increase in average testicular hormones concentration in vitamin C+E group as compared with other groups.

Table 6. Effect of vitamin C, vitamin E and olive pomace supplementation on testicular hormones concentration of bucks subjected to dizocilpine oxidative stress (means ± SE)

	Parameters	Experimental groups						
		C	D	DC	DE	DO	C+E	LSD
1st Month	Testosterone (ng/mg tissue)	4.41±0.17^{b}	1.75±0.10^{d}	3.90± 0.11^{c}	3.81±0.12^{c}	3.76±0.30^{c}	4.81±0.02^{a}	0.24
	Estradiol-17β (pg/mg tissue)	3.03±0.11^{a}	1.73±0.10^{e}	2.06± 0.12^{d}	2.18±0.11^{c}	2.25±0.21^{b}	3.08±0.31^{a}	0.06
2nd Month	Testosterone (ng/mg tissue)	4.85±0.37^{b}	4.80±0.22^{b}	4.86±0.11^{b}	4.82±0.19^{b}	4.87±0.29^{b}	5.11±0.14^{a}	0.13
	Estradiol-17β (pg/mg tissue)	3.25±0.13^{b}	3.22±0.12^{b}	3.24±0.11^{b}	3.30±0.13^{b}	3.27±0.14^{b}	3.82±0.02^{a}	0.06

Note. Different subscripts within a row indicate a significant treatment effect ($P \leq 0.05$) (n: 6 rabbits/group). C (Control), D (Dizocilpine), DC (Dizocilpine+Vit. C), DE (Dizocilpine+Vit. E), DO (Dizocilpine+Olive Pomace), CE (Vit. C+Vit. E), LSD (Least Significant Difference).

3.5 Effect of Vitamin C, Vitamin E and Olive Pomace Supplementation on Testicular Enzyme Markers of Bucks Subjected to Dizocilpine Oxidative Stress (Means ± SE)

The results in (Table 7) illustrate that, the values at first month reveal significant decrease (at $P < 0.05$) in average testicular enzyme markers (alkaline phosphatase, gamma glutamyl transferase and lactate dehydrogenase) of rabbits subjected to dizocilpine comparing with their control values. Moreover, groups subjected to dizocilpine and supplemented with vitamin C or vitamin E or olive pomace showed significant improvement in these parameters comparing with the dizocilpine group. While, at the second month the results reveal no difference between the values of testicular enzymes of C, D, DC, DE and DO groups, the group supplemented with vitamin C+E (CE) showed a significant increase in average testicular enzyme markers (ALK, GGT and LDH) over other groups.

Table 7. Effect of vitamin C, vitamin E and olive pomace supplementation on testicular enzyme markers of bucks subjected to dizocilpine oxidative stress (means ± SE)

	Parameters	Experimental groups						
		C	D	DC	DE	DO	CE	LSD
1st Month	ALK (IU/mg tissue)	1199.00±17.67^{a}	671.33±21.17^{c}	1118.00±25.00^{b}	1120±19.67^{b}	1119.33±18.19^{b}	1197.00±18.33a	8.74
	GGT (IU/mg tissue)	84.31±4.85^{a}	44.01±3.16^{b}	80.30±4.65^{a}	79.00±3.45^{a}	79.28±3.32^{a}	84.80±4.05^{a}	4.30
	LDH (IU/mg tissue)	378.50±11.17^{b}	238.00±11.33^{e}	305.00±12.33^{d}	316.50±11.50^{c}	324.40±13.43^{c}	391.00±14.33^{a}	9.24
2nd Month	ALK (IU/mg tissue)	1192.43±19.33^{b}	1190.00±22.33^{b}	1191.06±23.00^{b}	1189.35±19.67b	1192.66±21.84^{b}	1201.33±21.51^{a}	7.06
	GGT (IU/mg tissue)	91.00±3.37^{b}	86.94±2.73^{b}	88.87±3.41^{b}	87.68±3.43^{b}	90.32±3.66^{b}	95.21±3.33^{a}	2.74
	LDH (IU/mg tissue)	386.50±8.17^{b}	383.45±11.33^{b}	385.55±9.33^{b}	384.99±10.17^{b}	386.88±11.67^{b}	395.50±10.50^{a}	7.35

Note. Different subscripts within a row indicate a significant treatment effect ($P \leq 0.05$) (n: 6 rabbits/group).

C (Control), D (Dizcilpine), DC (Dizocilpine+Vit. C), DE (Dizocilpine+Vit. E), DO (Dizocilpine+Olive Pomace), CE (Vit. C+Vit. E), LSD (Least Significant Difference). ALK (Alkaline Phosphatase), GGT (Gamma Glutamyle Transferae), LDH (Lactate Dehydrogenase).

3.6 Effect of Vitamin C, Vitamin E and Olive Pomace Supplementation on Histological Structure of the Testis of Rabbit Does Mated with Rabbit Bucks Subjected to Dizocilpine Oxidative Stress

Histopathological examination of the testes tissue showed atrophy of the tubular structures, degeneration, disorganization of the tubular epithelium and degenerated germinal cells in lumina of the tubules and the spermatozoa were totally absent in the MK-801 group post one month from injection (Figure 1). Moreover, the administration of vitamin C, vitamin E and olive pomace one month later showed interstitial edema with

congestion of testicular blood vessel and marked reorganization of cellular elements (Figure 2). Meanwhile, group supplemented with vitamin C+E (CE) appeared histologically normal (Figure 3). Furthermore, two months later, testes from all groups revealed no pathological alteration and appeared histologically normal (Figure 4).

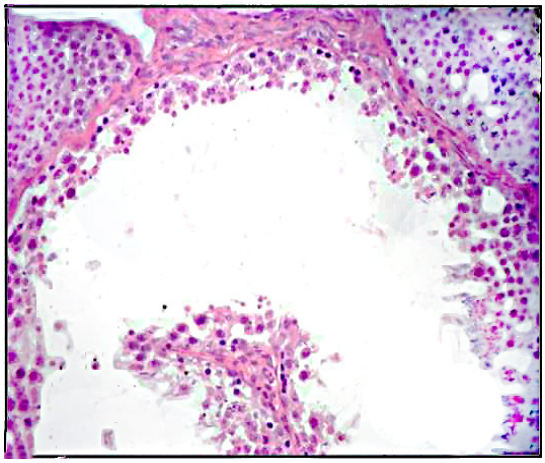

Figure 1. Transverse sections of testis of male New Zealand white bucks of dizocilpine group post one month stained by (H&E) showing disruption in the spermatogenic cell layers H&E stain-X40

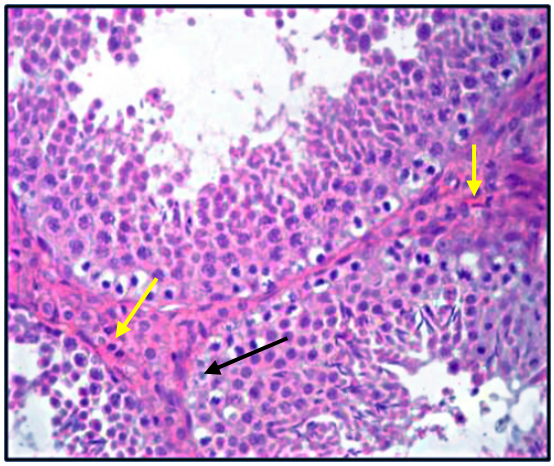

Figure 2. Photomicrograph of seminiferous tubules of testis of male New Zealand white bucks in group subjected to dizocilpine and supplemented with vitamin C or vitamin E or olive pomace for one month simulate each other showing faint pink esenophilic material in interstitial tissue (yellow arrows) with karyorrhexis of spermatogonial cells nuclei (black arrow). H&E stain-X40

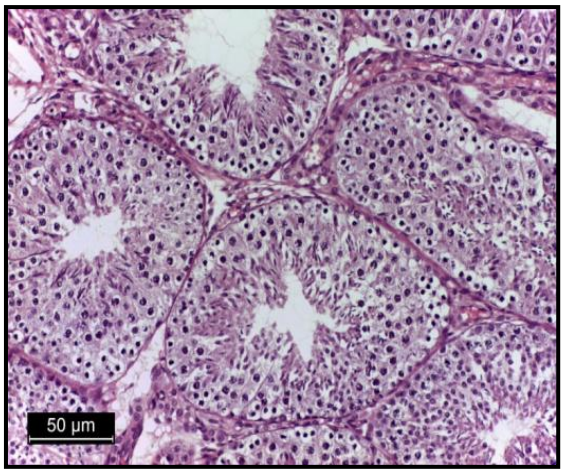

Figure 3. Photomicrograph of seminiferous tubules of testis of male New Zealand white bucks in group supplemented with vitamin C and E for one month showing normal structures of germinal epithelium and interstitial tissue without histopathological changes, H&E stain-X20

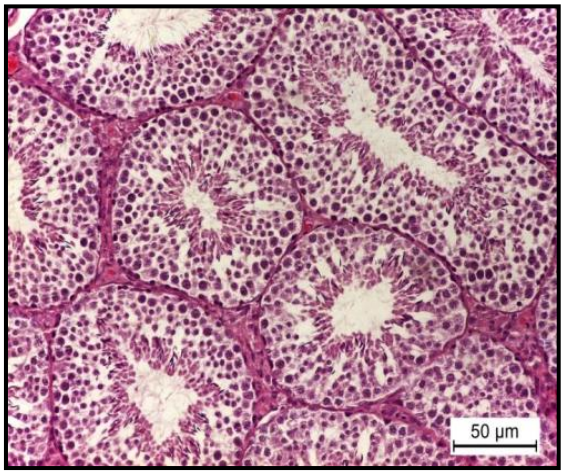

Figure 4. Photomicrograph of seminiferous tubules of testis of male New Zealand white bucks in dizocilpine and Vit.E supplemented group after two month showing no histopathological changes with normally appearing microarchitecture, H&E stain-X20

4. Discussion

Oxidative stress represents an imbalance between the excessive production or accumulation of reactive oxygen species (ROS) and an impaired antioxidant mechanism (Sikka, 2001). Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA (Agarwal et al., 2003). Further, some reactive oxidative species act as cellular messengers in redox signaling which can cause disruptions in normal mechanisms of cellular signaling (Sikka, 2001). The causes of the oxidative stress include environmental factors, chronic inflammation of prostate, auto-immune response to seminal antigens, exposure to toxin or chemical substance (Bozhedomov et al., 2009). Ozyurt et al. (2007) reported that exposing an animal to chemical substance such as injection with dizocilpine (MK-801) induced oxidative stress in rat testis. Excessive ROS can be generated by immotile, abnormal and dead spermatozoa (Silva, 2006). In attempts to reveal this dysfunction, an evaluation of sperm samples was done in the experimental groups to detect counts, live % and abnormalities. Regarding the sperm cell concentration which is considered the most sensitive test for spermatogenesis and it is highly correlated with fertility (El-Kashoury, 2009). In the present study dizocilipine group was found to induce a decrease in sperm concentration and live %; and increase the abnormalities. The reported results coincided with Takarada et al. (2004)

who reported that induction of dizocilipine affected glutamate receptors in testes. It appears that glutamate pathway might play a role in the semen characteristics and integrity of spermatogenesis in the rat testis. Although the precise mechanism of oxidative stress as a result of MK-801 toxicity has not been clarified, the histologic and biochemical analysis results support that an oxidative stress injury occurred in testicular tissues and play a role in decreasing the spermatozoal functions (Parlaktas et al., 2008). The present findings go hand by hand with that of Bailey et al. (2000) and Silva (2006) who reported that a high level of ROS in human seminal plasma is related to poor sperm morphology, poor motility and a low sperm concentration as well as abnormal and dead spermatozoa.

The present investigation recorded a reduction in testicular oxidative stress- antioxidant status of dizocilipine group malondialdehyde was measured, as lipid peroxidation product, and the antioxidant enzymes SOD, GST, GPX and TAC in the testis. The current results are in agreement with those of Hamden et al. (2008) who found that oxidative stress make a fall in testicular antioxidant capacity which appeared by a decrease in both enzymatic antioxidants (superoxide dismutase, catalase and glutathione peroxidase activities) and nonenzymatic antioxidants (copper, magnesium and iron levels). Free radical scavenging enzymes such as catalase, superoxide dismustase are the first line cellular defense enzymes against oxidative injury. The equilibrium between these enzymes is an important process for the effecttive removal of oxygen stress in intracellular organelles (Yeh et al., 2007). This state of oxidative stress was reflected not only by lower sperm counts but also by a decrease in the viability of sperms (Ghosh et al., 2002). The present findings concerning the oxidative status agreed with those of Aybek et al. (2008) who reported similar results concerning testicular SOD and malondialdehyde (MDA). The mammalian sperm plasma membrane, which is rich in polyunsaturated fatty acids, can be easily damaged by the reaction between ROS, such as OH·, and the polyunsaturated fatty acids (Alvarez & Storey, 1995). This mechanism is widely known as the lipid peroxidation reaction (Agarwal et al., 2003) and measured as malondialdehyde level, which is the end-point reaction product of lipid peroxidation (Sikka et al., 1995; Baumber et al., 2000).

The present study revealed that the levels of testosterone and 17beta-estradiol in testes tisues of dizocelpine group when compared with control group. This decrease may be the result of both adecreased total number of Leydig cells and rhythm of androgen biosynthesis by the remaining functional cells these results confirm the previous recorded results of Hurtado de Catalfo et al. (1998), Ballester et al. (2004) and Hamden et al. (2008). Oxidative stress may result in membrane lipid peroxidation and, ultimately, testicular damage and loss of testicular functions. Therefore, estimate of these enzymes (alkaline phosphatase, gammag lutamyl transferase and lactate dehydrogenase) have been recommended as markers for semen quality since they indicate sperm damage (Pesch et al., 2006). The results are agreement with those of Sawiress et al. (2011) who showed that rats under oxidative stress had reduced alkaline phosphatase, gamma glutamyl transferase and lactate dehydrogenase activities. Within the testis, the ALP activity was of significant importance for the process of spermatogenesis, moreover, the decrease in its activity was correlated with the state of germ cell loss (Kumar et al., 2003). The present investigation showed that rabbits subjected to dizocilipine oxidative stress resulted in decrease in ALP activities which correlated with reduction in testicular weights, and sperm counts together with the pathological findings. Testicular LDH is localized in mitochondria of primary spermatocytes and subsequent stages, where it is associated with the maturation of germinal epithelial layer of seminiferous tubules (Sinha et al., 1997). Also LDH was correlated with motility and living sperm where it ensures metabolism of spermatozoa (Pesch et al., 2006). Thus lower activity of LDH pointed to a lower sperm quality together with alteration in spermatogenesis documented by histopathology. Lower counts and live % may not only be attributed to alteration in hormonal profile but also to the injury induced by oxidative stress (Gumieniczek et al., 2008). GGT plays an important role in the protection of spermatozoa from oxidative stress and provides an indicator of a primary testicular and epididymal origin of this enzyme (Hinton et al., 1998). This result appeared to be true since γ GT (Sertoli cell enzyme marker) was altered in the present investigation. Gamma-glutamyl transferase (GGT) measurements have been proposed as a useful diagnostic test in recognition of Sertoli cell dysfunction (Sherins & Hodgen, 1976). Since progressive loss of viability in Sertoli cells was associated with oxidative stress (Gondos et al., 1998), one would speculate a decrease in activities of Sertoli cells which was evidenced in the present study by the decrease in GGT activities. As, Sertoli cells play a crucial role in maintaining high intratubular levels of testosterone (through androgen binding protein) and estrogen production (through aromatase activity), the present study investigated the intratesticular levels of both testosterone and estradiol. Intratesticular estradiol levels were decreased in the dizocilpine group, and this coincided with a decrease in GGT activities.

Antioxidants are believed to play a very important role in the body defense system against ROS (Boxin et al., 2002). Record et al. (2001) who found Antioxidants may be classified into enzymatic antioxidants and non-enzymatic antioxidants (vitamin E, vitamin C and phenolic compoundas olive pomace). In our study, groups

subjected to dizocilpine and supplemented with vitamin C or vitamin E or olive pomace and group supplemented with vitamin CE showed significant improvement in semen characteristics, increase of antioxidant parameters and decrease of lipid peroxide, higher testosterone, estradiol and testicular enzyme markers. The reported results agreed with those of Franchini et al. (2001), Yousef et al. (2003) and Sónmez et al. (2005) who reported that Supplementation with Vit-E increased the viability and concentration of spermatozoa in semen; an effect possibly linked to the antioxidant properties of this vitamin. .Vit-E is important in maintaining the physiological integrity of testis, epididymis and accessory glands (Sönmez et al., 2005; Cerolini et al., 2006), which is critical in spermatogenesis and sperm maturation thus improving sperm quality and quantity. Vit-E has been used in the rabbit, ram and boar to improve semen quality (Tsantarlotou et al., 2002; Sundaraian et al., 2006). Moreover, Luo et al. (2004) found that Vit-E supplementation may improve spermatogenesis through its regulatory action on gonadotropin secretion from the anterior pituitary gland and E2 may be increased due to increased steroidogenesis and inter conversion process between testosterone and estradiol through aromatization.

Vit-C neutralizes ROS and prevents sperm agglutination (Aitken, 2004). The supplementation of ascorbic acid in combination with Vit-E to the buck, in drinking water reduced the production of free radicals in the sperms and improved semen quality, with the greater improvement seeming to be from Vit-E (Yousef et al., 2003). It is a donor of electrons for redox systems, prevents lipid peroxidation, recycles vitamin E and protects against DNA damage induced by hydrogen peroxide radical (Jedlinska-Krakowska et al., 2006). The present results were in agreement with those of El-Tohamy et al. (2012) who showed that supplementation of male rabbits under oxidative stress with ascorbic acid resulted in increase of antioxidant enzymes and decrease of malondialdehyde.

Olive pomace contains many compounds with antioxidant activity, mostly vitamin E and phenolic compounds which are present naturally in vegetables, fruits and grains. They exhibit wide range of physiological properties and possess the ability to reduce oxidative stress and improve testicular function (Middleton et al., 2000). Moreover, Piomboni et al. (2008) reported that natural anti-oxidants such as olive products improve Sperm quality. The present findings go hand by hand with that of Al-Azzawie and Alhamdani (2006) who reported that olive product improved antioxidant enzymes of rabbits under oxidative stress.

5. Conclusion

In conclusion, supplementation of male rabbits with vitamin C, E and olive pomace was associated with improvement of sperm count and all testicular functions (antioxidant parameters, hormones and enzymes) and reduction of oxidative stress. Economically, olive pomace supplementation could be considered as a beneficial tool for maintaining healthy fertility parameters with reduced production costs by 12.75% putting in consideration that 1 kg Yellow Corn costs 1.80 LE/1kg, soya bean costs 3.50 LE/kg and olive pomace costs 0.50 LE/kg.

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