Protective Effects of Omega-3 Fatty Acids on Energy Drink Induced Ovarian Cytotoxicity in Adult Female Albino Rats: A Randomized Controlled Trial

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Keywords: Caffeine; Carbonated Beverages; Cytotoxicity, Cytotoxins, Docosahexaenoic Acids, Eicosapentaenoic Acid, Energy drinks, Infertility, Omega-3, Ovarian Diseases

doi: 10.37978/tijfs.v5i1.329

Submitted: October 13, 2020
Accepted: December 15, 2020
Published Online: January 18, 2021

How to cite this: Ilyas, M., Ahmad, M., Jawad, H., Siddique, N., Shahper, L. and Ghouri, M.H. 2021. Protective Effects of Omega-3 Fatty Acids on Energy Drink Induced Ovarian Cytotoxicity in Adult Female Albino Rats: A Randomized Controlled Trial. Int J Front Sci, 5(1).

Significance:

The widespread use of energy drinks and similar caffeine containing beverages has increased exponentially all over the world, mainly due to their marked advertisements. These beverages are emerging as a major health risk due to lack of awareness in the general population about their adverse health risks. It was uncertain if the energy drinks can be responsible for decreased fertility by damaging the gonadal organs. This study provides an estimate of the prevalence of ovarian tissue damage caused by using energy drinks.

ABSTRACT

Background: Energy drinks (EDs) are commonly used to prevent fatigue, enhance physical, and cognitive performance. Its administration induces toxic effects in body. Omega-3 is an antioxidant and anti-inflammatory agent that helps in proper functioning of immune system. Objectives of this study were to evaluate the morphological effects of fish oil omega 3 fatty acids (Eicosapentaenoic acid / Docosahexaenoic acid) on energy drink induced ovarian cytotoxicity in adult female albino rats.

Methods: The study was conducted at animal house, Anatomy department, Postgraduate Medical Institute, Lahore from January to march 2019. ARRIVE guidelines were followed for conduct of animal study. Ethical approval was obtained from PGMI, Lahore and Advanced Studies and Research Board of University of Health Sciences, Lahore. The study comprised 36 adult female albino rats divided into 3 groups i.e., control, energy drink and omega 3 treated. Rats were sacrificed, ovaries extracted, and sections were stained with H&E and PAS. SPSS version 21.0 were used.

Results: Statistically significant difference was present in gross parameters between the control and experimental groups. Energy drink administration caused a decrease in diameter of mature graafian follicle and diameter of the oocyte. Disruption in basement membrane was more pronounced in Energy drink treated group.

Conclusion: Energy drinks were found to cause cytotoxic effects on ovarian and oocyte morphology, ultimately leading to infertility. Omega 3 reduces the extent of damage caused by the intake of energy drinks.

Introduction

Changed pace of life, pervasive haste and the need of being available twenty-four hours a day cause an increasing number of population to access a group of beverages called the caffeinated energy drinks (EDs) (1). The term "energy drink" (ED) refers to beverages believed to reduce fatigue, increase a person's physical performance, enhance personality and improve individual's cognitive performance. EDs are most widely used by college students, athletes, night drivers (2). The uprising of energy drinks has highlighted both their popularity and controversy, comparing their advertised benefits of enhanced alertness, cognition and energy as compared to the possibly critical health risks (3). The first beverage of this type was introduced in the market in Austria in 1987 and ten years later similar beverages were introduced in the United States of America that comprised of high caffeine and taurine concentrations (2). EDs are widely available in markets now under different brand names. Power horse, Red Bull, code red and monster are few names among some of the popularly used energy drinks with an annual worldwide business of several billions (1).

ED's have gained a widespread popularity since their first appearance all around the globe. The manufacturers attribute these enhanced effects to the unique mixture of the ingredients including caffeine, taurine and glucuronolactone (4). Apart from active ingredients energy drinks also contain guarana extract, ginseng, additional amino acids, vitamins including niacin, pantothenic acid, B6, B12, acidity regulators (sodium bicarbonate, magnesium carbonate) and carbohydrates to complete the list of supposedly helpful ingredients (5). ED brands available commercially have high caffeine content leading to insomnia, anxiety, headache and tachycardia (6). ED's contain extra caffeine through extracts like guarana, kola nut, yerba mate, and cocoa. These additives increase the total caffeine content and efficacy of ED (7). The high sugar content is known to contribute to obesity (8). Body weight gain with use of ED is due to the higher rate of catabolism and leads to a higher rate of lipid storage in the adipose tissue (9). Adverse cytotoxic effects of ED have been evaluated extensively. EDs lead to Hydropic changes in epithelium, intertubular hemorrhage and inflammatory changes in renal tubules (10). Pancreatic cells of rats administered

ED displayed significant necrotic changes, vacuolization and nuclear karyolysis along with congested blood vessels and perivascular infiltration. ED treated rats showed marked gastric ulcers, glandular atrophy and vascular congestion and damage to the physiological barrier between gastric lumen and underlying mucosa (11). The effects of ED on the reproductive system have been documented in a lesser number. The results of another animal study clearly revealed that maternal consumption of high dose caffeine during gestation and lactation significantly decreased weight of the ovary and number of primordial follicles. It also reduced fertility and reproductive competence in the Wistar rat's offspring (12).

Omega-3 fatty acids are a group of polyunsaturated fatty acids (PUFA) (13). In our study we used fish oil Omega-3 and looked for possible helpful effects of fish oil omega-3, Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These two Omega-3 fatty acids cause significant biochemical and physiological changes in the human body (14). The Food and Drug Administration (FDA) has recommended that a maximum adults dose of 3 grams per day of combined DHA and EPA can be safely consumed (15). The most extensively available dietary source of EPA and DHA is fish oil extracted from herring, mackerel, salmon, anchovies and sardines (16). Omega-3 PUFAs are believed to decrease the synthesis of different inflammatory cytokines, including tumor necrosis factor alpha, interleukin-1, and interleukin-6 (17). EPA helps in proper functioning of inflammatory systems. Prostaglandins effecting as anti-inflammatory action are made directly from EPA. It lowers risk of unnecessary inflammation and inflammation related diseases (16). Recommendations also have been made for DHA intake for pregnant women, females hoping to conceive, nursing mothers, infants, and vegetarians/vegan (18). The Omega-3 is major constituent of oocyte cell membrane (17).

The mean diameters of the ovarian mature graafian follicle were increased in dairy cows fed on a diet rich in omega-3. It reduced Prostaglandin F2a (PGF2) synthesis which improves embryo survival. Omega-3 enriched diet during late gestation results in longer gestational period in humans (19). After artificial insemination, dairy cows that were fed on a diet higher in Omega-3 showed a higher pregnancy rate and pregnancy losses were significantly reduced (20). Supplementation with omega-3 caused prolonged gestation length followed by an improved birth weight and better rate of neonatal survival. There is also an improvement of milk composition of cows due to PUFA supplementation (21). Endogenous n-3 PUFA's have proved to be a significant energy source to the oocytes and early developing embryos. They improve oocyte development by influencing the fatty acid composition of oocyte lipids, and by modifying the prostaglandins and other metabolites' concentrations in the follicular medium in the surrounding of the oocyte (20).

Atrophic and cystic degeneration of ovaries lead to infertility which is a global health issue. The study was conducted to evaluate the cytotoxic effects of ED and to establish the possible ability of Omega-3 to protect ovarian tissue from ED induced oxidative stress. This explains importance of omega-3's important reproductive role by maintaining ovarian follicular integrity. This study was conducted after the observation regarding increasing use of energy drinks in our community, especially by the young population including students. There have been many researches regarding harmful health effects of these beverages but the author observed that toxic effects of these drinks are still not researched extensively in the field of reproductive health.

Materials and Methods

Study Design & Settings: Open-Label Randomized Control Trial conducted at Experimental Research Laboratory, Anatomy department, Postgraduate Medical Institute (PGMI) Lahore. The study protocol and procedures were approved by the Ethical Committee of PGMI, Lahore and Advanced Studies and Research Board of University of Health Sciences, Lahore. ARRIVE guidelines were followed for conduct of research on lab animals.

Subjects: 36 adult female albino rats of Wister strain weighing 130 – 180 grams and achieving sexual maturity at 55-60 days were obtained from animal house of PGMI. Animals were weighed and examined thoroughly for any gross morbidity. They were individually kept in climate-controlled conditions of temperature 22 ± 0.5˚C, humidity (50%±10%), and 12 hours light / dark cycles and were provided standard rat food and water ad libitum. Rats were kept for a week for acclimatization.

Randomization & Masking: Rats were distributed into three equal groups (A, B & C) using random number generator. The participants and investigators were not blinded to the intervention being administered. Group A was Control group while the other two groups (B & C) were experimental groups. The therapeutic reagents used were energy drink and Omega-3. The doses and their duration of administration were selected according to protocol of previously conducted study (11).

Procedures: The control group (A) was given distilled water orally for 30 days. Energy drink treated group (B) was given ED 10 ml /kg body weight (equivalent to approximately 7.5 ml/rat) once a day for 30 days. Energy

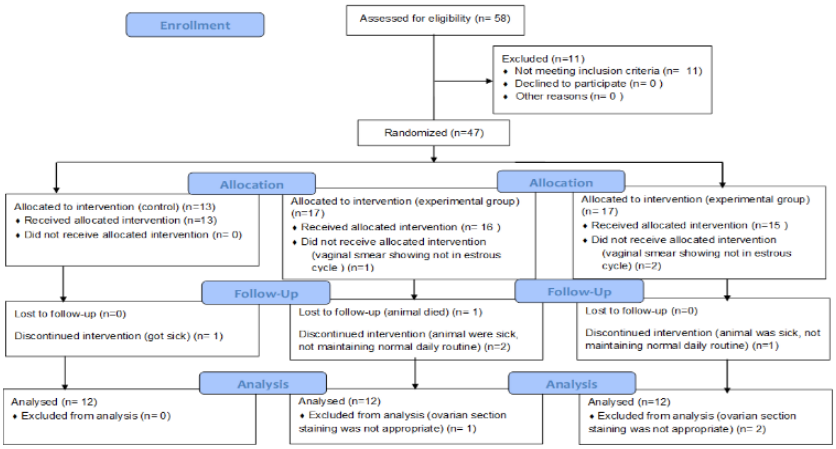

Figure 1 *CONSORT Flow Diagram*

drink and Omega-3 treated group (C) was given ED 10 ml /kg body weight (equivalent to 7.5 ml/rat) daily along with Omega-3 at a dose of 300 mg/Kg body weight once a day for 30 days (Table 1). Drugs were administered orally by gavage method. The exact dose of energy drink and omega 3 was adjusted according to the exact weight of individual animal and was given through gavage needle. Rats were weighed 24 hours after administration of last dose and were sacrificed under deep anaesthesia. Ovaries (right and left) were dissected out. Weight of both ovaries was recorded carefully on electronic weighing scale. Volume of organ was obtained by immersing organ in water in a calibrated test tube. The difference between two readings of the level of water was calculated as volume of ovary. All the animals were euthanized by decapitation. Their bodies were disposed-off by burying in burial ground at PGMI, Lahore.

Outcomes (Histological Analysis)

Ovaries were washed with normal saline and fixed in 10 ml of neutral 10% buffered formalin. Automatic tissue processor was used for tissue processing and then embedded in paraffin. 3 – 4 µm thick serial paraffin sections were obtained from the paraffin blocks and stained with standard hematoxylin and eosin (H&E) and PAS reagent for the histopathological examination. Slides were examined under light microscope (ACCU-SCOPE 3000-LED Microscope) at 10X and 40X magnifications. Diameter of mature Graafian follicle (µm) and oocyte of Graafian follicle (µm) were calculated by using ocular micrometer. For measuring diameters, two measurements were taken. The second measurement was obtained by placing micrometer at a right angle from the midpoint of the first measurement. Average was taken and documented. Follicular diameters were calculated from the outermost wall of the thecal layer, if present. In case of absence of thecal layer, the diameter was measured from the outermost layer of granulosa cells. The diameter of oocyte was measured including the zona pellucida, when present (22). Basement membrane was assessed for intact and disrupted state in PAS stained slides.

Table 1 *Experimental grouping of animals, their doses and duration*

Groups	Number of animals (n)	Intervention and dosage	Duration
A Control Group	12	food and water *ad libitum*	30 consecutive days
B Energy Drinks treated group	12	ED10 ml /kg body weight (equivalent to 7.5 ml/rat) by gavage method	30 consecutive days
C Energy Drinks + OMEGA-3 treated group	12	ED10 ml /kg body weight (equal to 7.5 ml/rat) daily along with omega 3 at a dose of 300 mg/Kg body weight (0.5-0.4 ml/rat) orally by gavage method	30 consecutive days

Statistical Analysis: Data was analyzed using SPSS 21.0. Normality of data was checked with Shapiro Wilk Test. Mean ± SD was calculated for quantitative variables. One-way ANOVA was applied to determine the mean difference among the groups. Post Hoc Tukey Test was applied for multiple comparisons. Chi square was used to observe association among qualitative variables. A p-value ≤ 0.05 was considered as statistically significant.

Sample Size of the study was calculated as 36 using the following formula:

$$n = \frac{Z_{\beta}^{2}[p_1(1-p_1)+p_2(1-p_2)]}{(p_{2-}p_1)^2}$$

Where p1 is the anticipated proportions of regular menstruation in Omega 3 group, p2 is the anticipated proportions of regular menstruation in control group and Z β^2 is the desired power of study.

Results:

Effects of ED on body weight: At the start of the experiment the mean body weight of animals in all groups was not significantly different (p-value = 0.699) whereas there was a statistically significant difference in mean body weights among groups at the end of the experiment ($p < 0.001$). (Figure 2)

For multiple comparisons, post hoc Tukey test showed that final weight gain in group B and C were significantly higher as compared to control group. (Table 2)

The comparison of mean paired ovarian weight and volume in all groups was significantly significant (p< 0.001) (Figure 3).

Table 2 *Pair wise comparison of final body weight among groups*

Multiple Comparison					
S. No.	Groups I	Groups J	Mean Difference (I-J)	Std. Error	p-value
1	A	B	-29.0000	4.0507	.000*
		C	-21.0000	4.0507	.000*
2	B	C	8.0000	4.0507	.134

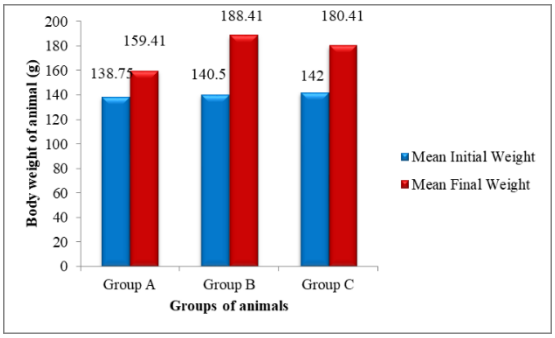

Figure 2 *Comparison of initial and final weight amongst animals of group A and B*

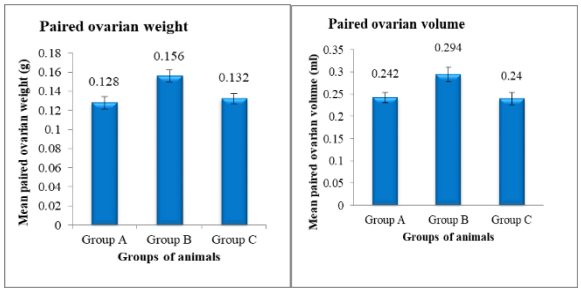

Figure 3 *Bar charts showing comparison of paired ovarian weight and volume (ml) among groups A, B and C. Error bars indicating ± SEM.*

Effect of ED and omega 3 on weight and volume of ovaries: Post hoc Tukey test showed that paired ovarian weight and volume in groups B was significantly higher as compared to control and ED + Omega-3 treated group. (Table 3).

Table 3 *Pair wise comparison of paired ovarian weight (g) among groups*

Multiple Comparison						
Parameter	S. No.	Groups I	Groups J	Mean Difference(I-J)	Std. Error	p-value
Mean paired ovarian weight (g)	1	A	B	-0.02841	.00853	0.006*
			C	.03842	.00853	0.886
	2	B	C	.05217	.00853	0.019*
Mean paired ovarian volume (ml)	1	A	B	-0.0519	.01989	0.035*
			C	0.0025	.01989	0.991
	2	B	C	0.0544	.01989	0.026*

Effect of ED and Omega-3 on diameter of Graafian follicle: The mean diameters (µm) of Graafian follicle in all groups were calculated by micrometry at 10X magnification. The mean diameter of Graafian follicle in all groups were significantly different (p-value = 0.001) (Figure 4)

For multiple comparisons, post hoc Tukey test was used which showed that diameter of Graafian follicle in group B (Figure 6) was significantly lower as compared to

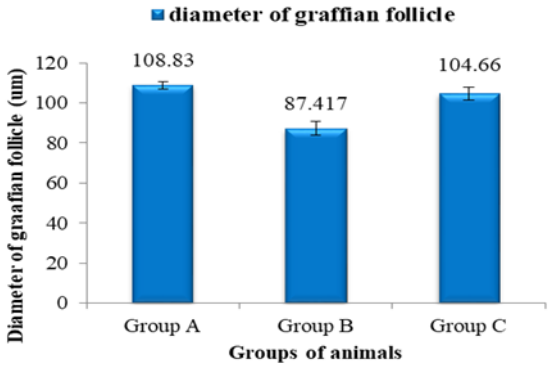

Figure 4 *Bar chart showing comparison of mean diameter of Graafian follicle (um) of ovaries among groups A, B and C. Error bars indicating ± SEM*

remaining all groups. However, no significant difference was found in the diameter of Graafian follicle among groups A (Figure 6) and C (Figure 6) (Table 4).

Effect of ED and Omega 3 on Diameter of Oocyte of Graafian follicle: The mean diameters (µm) of Oocyte of Graafian follicle in all groups were recorded during micrometry at 40 X magnification. Mean diameter of Oocyte of Graafian follicle in all groups were significantly different (p-value < 0·001) (Figure 5).

Table 4 *Pair wise comparison of mean diameter (µm) of Graafian follicle among groups*

Multiple Comparison						
	S. No.	Groups I	Groups J	Mean Difference (I-J)	Std. Error	p-value
Mean Diameter (µm) of Graafian Follicle	1	A	B	21.416	4.173	0.000*
			C	4.166	4.173	0.483
	2	B	C	-17.250	4.173	0.001*

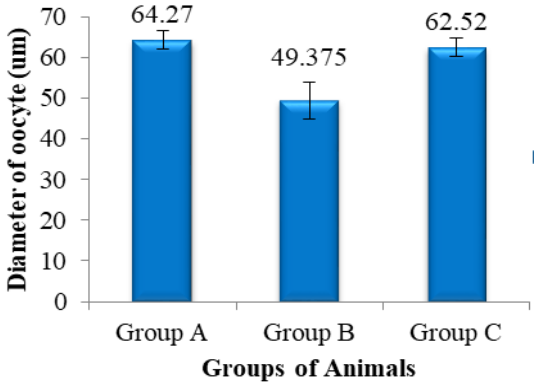

Figure 5 *Bar chart showing comparison of mean diameter of oocyte (um) of ovaries among groups A, B and C. Error bars indicating ± SEM*

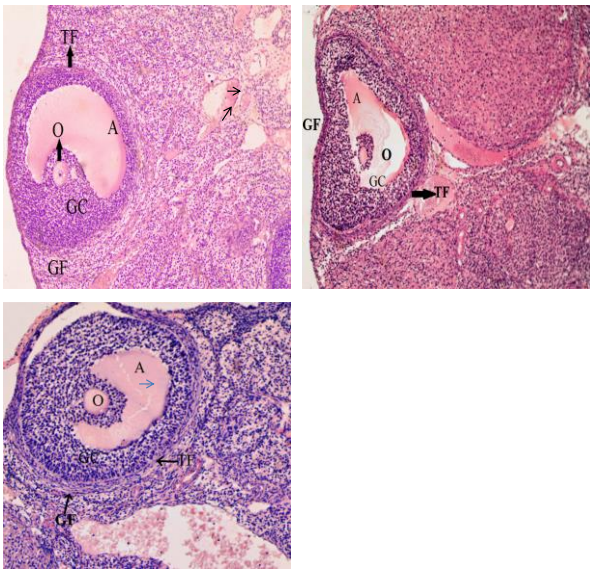

Figure 6 *Photomicrograph of ovarian section of group (A) mature Graafian follicle (GF) showing normal oocyte (O) with normal eccentric nucleus surrounded by many layers of polyhedral granulosa cells (GC). Theca folliculi (TF) containing polyhedral theca interna cells. In comparison group (B) showed degenerated Graafian follicle (GF) with shrunken oocyte (O) surrounded by degenerating layers of granulosa cells (GC). Theca follicular (TF) cells show vacuolization. Group (C) showing a mature regenerating graafian follicle (GF) with oocyte (O), antrum (A) and surrounded by many layers of granulosa cells (GC). H&E. X10*

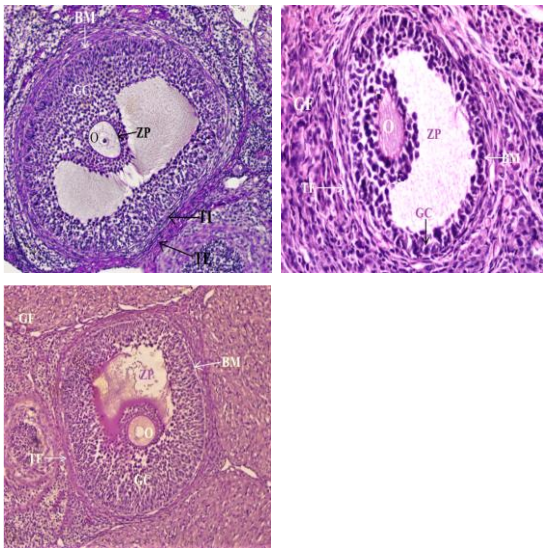

Figure 7: *Photomicrograph of an ovarian section from control group (A) showing Graafian follicle(GF) containing oocyte (O) with intact zona pallucida (ZP) surrounded by layers of granulosa cells (GC) with intact magenta colored basement membrane (BM).Theca folliculi containing polyhedral theca interna cells (TI) and vascular theca externa having fusiform fibroblast nuclei (TE). In comparison group (B) showing Graafian follicle (GF) containing degenerated oocyte (O) and degenerated zona pallucida (ZP). Oocyte is surrounded by degenerated granulosa cells (GC) with discontinuous magenta colored basement membrane (BM). Theca folliculi (TF) containing vacuolated theca interna cells. Group (C) shows Graafian follicle (GF) containing oocyte (O) with intact zona pallucida (ZP). Granulosa cells (GC) have intact magenta colored basement membrane (BM).Theca folliculi (TF) containing polyhedral theca interna cells. PAS stain X.40.*

For multiple comparisons, post hoc Tukey test was used which showed that diameter of Oocyte of Graafian follicle in group B (Figure 6) was significantly lower as compared to remaining all groups. However, no significant difference was found in the diameter of Oocyte of Graafian follicle among groups A (Figure 6) and C (Figure 6) (Table 5).

Basement membrane of Graafian follicle: Graafian follicle of Control group (A) showed magenta coloured intact basement membrane (a distinct boundary between zona granulosa and theca folliculi) with PAS stain. There was no abnormal or disrupted basement membrane seen in group A (Figure 7). All the rats showed normal zona pallucida (surrounding membrane of oocyte). In group B, all rats had disruption of basement membranes of mature graafian folliculi. Zona pallucida was also disrupted in all the animals of group B (Figure 7). In group C, only 3 (25%) rats had disrupted basement membrane of Graafian follicle (Figure 7). Chi square test showed that there was an association between disruption of basement membrane of mature Graafian follicle and groups (Table 6)

Table 5: *Pair wise comparison of Mean Diameter (µm) of Oocyte of Graafian follicle among groups*

Multiple Comparison						
	S. No.	Groups I	Groups J	Mean Difference (I-J)	Std. Error	p-value
Mean Diameter (µm) of Oocyte of Graafian Follicle	1	A	B	14.895	4.452	0.006*
			C	1.750	4.452	0.919
	2	B	C	-13.145	4.452	0.015*

Discussion

The adverse effects of ED are largely ascribed to its caffeine content and the possible adverse reactions due to the combined enhanced effects of its various components causing a pro-oxidant environment (23). Omega-3 was used to assess its protective role as it acts in preventing

Table 6 *Distribution of basement membrane of Graafian follicle among groups*

Rats	Basement membrane		Percentage disruption	P value
	Intact	Disrupted		
Group A	11	01	8.3%	<0.001*
Group B	00	12	100%	
Group C	09	03	25%	

oxidative stress and production of numerous physiological anti-inflammatory mediators (24).

Present study showed a gradual normal increase in body weight of control group. A statistically significant percentage weight gain in ED treated and ED plus omega-3 treated groups was observed. This increase final weight gain is due to high catabolism rate induced by the glucose component of the ED. High components of sugars in the ED leads to increased availability of insulin in body that cause an increased rate of lipid storage in the adipose tissues. Results were similar to previous published work (25, 26). Contradictory reports showed the animals given energy drinks added diet had a decreased body weight gain (27).

Increase in mean paired ovarian weight and volume of rats treated with ED was in agreement with previous data (26) showing a significant increase in brain weight of adult rats treated with ED for 30 days. Increase in the weight and volume of the organ resulted due to the swellings of the ovarian tissue based on the cytotoxic effects of the ED on the cells of the ovary. A cytotoxic edema result in the swelling of the tissue. This caused degeneration of the follicles and formation of cystic structures resulting in increased net organ weight and volume. Rats who were caffeine fed for 4 weeks showed an increase in their testicular weight relative to the body weights (28, 29). In contrast to this research reported that the consumption of a higher caffeine dose caused a significant decrease in ovarian weight (12).

Ovarian weight of ED plus omega-3 treated rats was near to the control group, thus proving that the anti-inflammatory effects of omega 3 reduced the ovarian tissue damage and prevented edema of the ovaries. This is in accordance with published work (30) showing that ovarian weights of dairy cows after treatment with omega-3 remained same as that of the control group.

Diameter of the graafian follicle and oocyte as measured by the ocular micrometer was markedly decreased in ED treated group. It was in consensus with previous study, (12) demonstrating that caffeine ingestion causes a decrease in number of cells and cellular death by interfering with follicular cell division. High dose caffeine consumption caused reduction of the seminiferous tubules' diameter due to caffeine induced inhibition of seminiferous cords (28). Similar observation was made on testis of male rats after 40 days of caffeine intake (29).

Omega-3 maintained the oocyte and follicular diameter by decreasing inflammatory vulnerability and suppressing cytokine production. Studies showed that the use of omega-3 supplements in dairy cows for 15 days duration caused an increased number of ovarian follicles and an increase in their mean diameters, mostly due the anti-inflammatory effects of omega-3 (31).

PAS-stained ovarian sections of rats administered with ED demonstrated a weak to mild PAS-positive reaction. The Graafian follicles demonstrated disrupted (45%) or discontinuous basement membrane (55%). PAS-stained ovarian sections of rats treated with ED plus Omega-3 demonstrated a moderate PAS-positive reaction with intact basement membrane. This confirms role of omega-3 in maintaining basement membrane integrity for survival of ovarian follicle and increasing their number. Omega-3 supplementation positively influences phospholipid fatty acid composition of ovarian follicular and cumulus cells, maintaining the integrity of oocyte membranes, thus improving the oocyte number and quality (32). The cows fed with PUFA n-3 supplemented diet for 15 days had better membrane integrity and oocyte structure than control (31). Contradictory reports showed adverse effects on the morphological appearance and membrane integrity of embryos fertilized from oocytes after exposure to an environment high in polyunsaturated fatty acids (33).

Conclusion

In this study it was observed that the Omega-3 fatty acids have succeeded, to certain extent, in protecting the ovaries from the deteriorating effects of the ED induced histopathological changes. In short, the current study demonstrated that ED induce ovarian cytotoxicity whereas; Omega-3 can significantly attenuate these adverse effects. The possible mechanism of ED harmful effect is the induction of oxidative stress in the tissue, and the anti-oxidant and anti-inflammatory property of Omega-3 could be a possible protective mechanism. From the foregoing results, it is clear that omega-3 fatty acids being anti-oxidant and anti-inflammatory have maintained ovarian tissue architecture, remodeled basement membranes and significantly reduced degeneration of graafian follicles by counter balancing ED induced oxidative stress. Hence it can be recommended as a protective agent against ED induced ovarian damage due to easy, rapid and safe dietary administration, especially with the increasing use of ED among students and athletes.

Conflict of interest: Authors declare no conflict of interest.
Disclosure: None
Human/Animal Rights: No human or animal rights were violated during this study.

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