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ORIGINAL ARTICLE

Energy drinks leading to infertility by causing cytotoxic injury of the ovaries - A randomized controlled animal trial

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ABSTRACT

Aim: To observe energy drink induced ovarian cytotoxicity and possible protective effects of co-administration of omega 3 fatty acids.

Methods: The experimental study comprised of 36 adult female albino rats was conducted at anatomy department, PGMI, Lahore. Animals were divided into 3 groups. Group A was control. Groups B received energy drink. Group C received omega 3 along with energy drinks once daily orally for 30 consecutive days. Rats were sacrificed under deep anesthesia on 30th day. Ovaries were extracted and sections were stained with H&E stains. Results were analyzed using SPSS. A *p*-value < 0.05 was considered statistically significant.

Results: Animals administered with energy drink showed marked pyknosis of graafian follicular cells and oocyte nuclei when compared with control and energy dink plus omega 3 treated animals. Vacuolization of the follicular cells was also more pronounced in energy drinks treated group (p <0.001). Ovarian stroma of energy drink treated group showed marked vascular congestion and tissue infiltration as compared to the omega 3 plus energy drink treated groups..

Conclusion: Oxidative stress induced by the energy drinks is the basic cause behind the toxic change seen in ovarian histo-architecture. Omega-3 significantly decreased these detrimental effects of energy drink due to its antioxidant and anti-inflammatory action. This study can be a pioneer in guiding the population about the harmful effects of energy drinks on ovarian morphology, leading to cytotoxicity of oocyte and ultimately leading to infertility.

Keywords: Energy drinks; Omega-3; Cytotoxicity; Oxidative stress; Carbonated Beverages; Infertility; Caffeine

INTRODUCTION

The new millennium has ushered in a wave of synthetic, caffeinated beverages known as energy drinks intended to energize and specifically targeting the youth market[1]. The increasing use of energy drinks has emphasized on their popularity and controversy. Researches have been conducted, comparing their advertised benefits to the possibly critical health risks[2]. The lack of monitoring oversight and policies has led to the violent marketing of these beverages toward youngsters, students and players for performance-enhancing and restorative effects[3].

The manufacturers of energy drinks attribute their effects of enhanced alertness, cognition and energy to the unique combination of the ingredients including caffeine, taurine, glucuronolactone, guarana extract, ginseng, amino acids, vitamins, acidity regulators and carbohydrates.[4] Total caffeine content of ED is increased by the extracts like guarana, kola nut, yerba mate, and cocoa that combines and interacts with caffeine to promote a "synergistic effect" that enhances caffeine effects and increases its half-life[5]. Taurine also enhances the effects of caffeine in energy drinks[6].

Previous research works indicate that the major body organs are highly vulnerable from ED intoxication. ED cause distinct pathological injuries to the renal tubules and glomerular necrosis. The glomeruli were converted into shrunken, abnormally shaped structures[7]. Pancreatic islets of Langerhans of rats administered ED displayed significant necrotic cytotoxic changes, including vacuolization and nuclear karyolysis.[6] Intake of high levels of sugar in the ED is the reason of numerous damaging health effects. This includes higher insulin resistance and development of metabolic disorders including obesity and diabetes[8].

Previous studies indicated that after 4 weeks administration of energy drinks Liver function enzymes were elevated in the rat's serum. Light and electron microscopic results discovered clear loss of normal liver architecture along with vascular congestion and leukocytic infiltration among the hepatocytes.[9] Digestive organs especially stomach has been reported to be potential target of oxidative stress caused by ED. Caffeine intake leads to gastrointestinal irritation as evident by increased incidents of stomach upset and diarrhea[10] (On et al 2010). ED treated rats showed marked desquamation of gastric mucosa, destruction of gastric epithelium and damage to the physiological barrier protecting gastric mucosa from luminal contents[6].

The effects of ED on the reproductive system had not been documented in details. Maternal consumption of high dose caffeine during gestation significantly decreases fertility and reproductive competence of the rat's offspring.[11] A study conducted on ovariole number and body size of *d.melanogaster* demonstrated that animals fed on energy drink media has reduces body weight and reduced number of ovarioles as compared to the animal fed on natural media.[12] Frequent miscarriage and low birth weight have been associated with a caffeine intake of 300 mg/day.[13] Cultured human sertoli cells exposed to caffeine exhibited a decrease in the antioxidant capacity of the sertoli cells in a dose dependent manner. The pro-oxidant potential of caffeine causes a synchronized rise of protein oxidative injury[14].

Omega 3 fatty acids are a subgroup of polyunsaturated fatty acids (PUFA). In our study we used fish oil omega 3 and looked for possible beneficial effects of EPA and DHA. Recommendations have been made for omega 3 supplement for pregnant females, females hoping to conceive, nursing mothers, infants, and vegetarians/vegan[15].

PUFA exert several positive effects in human reproduction system. Oocytes contain high levels of poly unsaturated fatty acids. They are used as an energy source for oocyte maturation and during the time of embryonic development before implantation The omega 3 content of oocyte affects oocyte maturation, its cryopreservation and subsequent developmental ability.[16] Omega 3 fatty acids help in increasing gestational age and improving fetal birth weight when given prenatally to pregnant mothers. Omega 3 are well proved to be a good energy source to the oocytes and embryos in early developmental stages. They support the development of the oocyte by enhancing and supporting the fatty

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acid composition of oocyte lipids, and by modifying the prostaglandins and other metabolites concentrations in the follicular fluid surrounding the oocyte[17].

There is significant evidence that dietary omega 3 supplementation positively influences the biosynthetic pathways that are involved in synthesis of prostaglandins and steroidogenesis. This helps in up regulation of reproductive function. PUFA content of the cell membranes of sperm and oocyte is vital for fertilization. EPA and DHA also give sperm plasma membrane the fluidity that helps to contribute in the membrane fusion events during fertilization[18].

Previous work[6] showed that Omega-3 succeeded in protecting the stomach mucosa and the pancreas from the deteriorating effects of ED induced pathological changes. Study was conducted for 4 weeks and the results significantly proved that omega 3 holds therapeutic beneficial effects on energy drink induced pancreatic and gastric dysfunction. It is evident from the literature review that ED has toxic effects on various organs. Omega 3 has been proven to protect against the ED induced organ toxicity. Keeping this in mind the current study was designed to explore the possible cytotoxic effects of caffeinated beverage on ovaries and possible protective role of omega 3 against ED induced ovarian damage.

The objective of the study was to evaluate the morphological effects of fish oil omega 3 fatty acids (EPA/DHA) on energy drink induced ovarian cytotoxicity in adult female albino rats.

MATERIALS AND METHODS

An experimental randomized Control Trial was conducted at Experimental Research Laboratory, Anatomy department, Postgraduate Medical Institute (PGMI) Lahore. The study protocol was approved by the Ethical Committee of PGMI, Lahore and Advanced Studies and Research Board of University of Health Sciences, Lahore. In order to detect a difference of 70% - 90% in the histological changes, a sample size of 36 female albino rats was needed at a significance level of 5% with a Power of the study of 90%.

Procedure: A total of thirty six adult female albino rats of Wister strain with approximate weight of 130 – 180 grams and gaining sexual maturity at 55-60 days were obtained. The rats were weighed before start of experiment and examined properly for any gross morbidity before the beginning of the experimentation. Rats were divided into three equal groups by using random number generator.

Group A: (n=12) Control group: Rats were given distilled water orally at the time of dose administration to rats of experimental groups for 30 consecutive days.

Group B: (n=12) ED treated group: Rats were given ED 10 ml /kg body weight (equivalent to 7.5 ml/rat) by gavage method once a day for 30 consecutive days.

Group C: (n=12) ED and OMEGA 3 treated group : Rats were given ED10 ml /kg body weight (equivalent 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 once a day for 30 consecutive days.

Therapeutic agents used in this study are energy drink (red bull) and omega 3: Energy drink and omega 3 were administered orally through gavage method. The dose of therapeutic agents (energy drink and omega 3) and their duration of administration were selected according to the protocol of previously conducted studies.[6]

Dissection and tissue sampling: At the end of experimental period the rats were weighed. 24 hours after administration of last dose all rats were sacrificed under deep anesthesia. Ovaries (right and left) were identified and dissected out. All animals were euthanized by decapitation and their bodies were disposed-off by burying in Postgraduate Medical Institute, Lahore. Ovaries were washed and after fixation tissue processing was done. Slides were made for histological examination of the ovaries. The slides were stained with eosin and hematoxyline stains. Qualitative parameters including oocyte nuclear pyknosis, granulosa cell nucleus pyknosis, theca folliculi vacuolization and stromal vascular congestion were measured by selecting 3 fields of vision per slide rotating slide clock wise.

Photographic magnification (photographs of slides): (ACCU-SCOPE 3000-LED Microscope) with 10X and 40X magnifications with digital head camera mounted microscope was used for taking the photographs of slides.

Statistical analysis: The observations were recorded in MS Word® and Excel(R) data sheet. Data was entered and analyzed using SPSS 21.0 (Statistical Package for Social Sciences). Normality of data was checked with Shapiro Wilk Test. Frequency and percentages were calculated for qualitative variables. Chi-square test was applied to observe the association of categorical variables with the groups. A *p*-value ≤ 0.05 was considered as statistically significant.

RESULTS

The animals were weighed at the start and end of experiment and examined carefully throughout the experiment. Rats remained healthy throughout the experimental period. There was no morbidity or mortality among the groups.

Granulosa cell nucleus pyknosis of Graafian follicle: Granulosa cell of control group A appeared polyhedral in shape with central rounded nucleus. Whereas in group B all rats had abnormal granulosa cell nuclei which appeared pyknotic and karyorrhectic and nuclear debris were scattered throughout the degenerating zona granulosa. Statistically significant association between Granulosa cell nuclei of different groups was observed by using Chi-square test. In group B, all rats had abnormal Granulosa cell nuclei whereas in group C (25%) rats had abnormal Granulosa cell nucleus.

Oocyte nucleus pyknosis of Graafian follicle: Oocytes of rats of control group (A) showed large eccentric nucleus with finely dispersed chromatin and one or more large nucleoli (Fig.). Chi square test showed that there was an association between Oocyte nucleus and groups. There was no abnormal Oocyte nucleus seen in group A. In group B, all rats had abnormal oocyte nucleus. Nuclei appeared pyknotic, karyorrhectic and karyolysed. In group C, 3 (25.0%) rats had abnormal Oocyte nucleus. Rest of the animal showed normal cellular architecture (Table 1, Fig.1,2).

Parameter		Group A		Group B		Group C		p value
		n	%	N	%	N	%	
Granulosa cell nucleus	Normal	12	%	0	100%	09	25%	<0.001*
	Pyknotic	0		12		03		
Oocyte nucleus	Normal	12	0%	0	100%	09	25%	<0.001*
	Pyknotic	0		12		03		
Theca folliculi	Normal	12	0%	0	100%	10	16.6%	<0.001*
	Vacuolized	0		12		2		
Stromal vascular congestion	Present	11	8.33%	0	100%	11	8.33%	<0.001*
	Absent	01		12		01		

Chi square test *p value ≤ 0.05 is considered statistically significant

Figure 1: Photomicrograph of ovarian section of mature graafian follicle in group A showing normal oocyte(black arrow) with normal eccentric nucleus surrounded by many layers of polyhedral granulosa cells (yellow arrow). Degenerated graafian follicle in group B shows shrunken oocyte (black arrow) surrounded by degenerating layers of granulosa cells (yellow arrow). Granulosa cell have darkly stained pyknotic nuclei. Theca interna cells show vacuolization (Blue arrow). Group (C) showing a mature regenerating graafian follicle with oocyte (black arrow) surrounded by many layers of granulosa cells (yellow arrow). H&E. X10

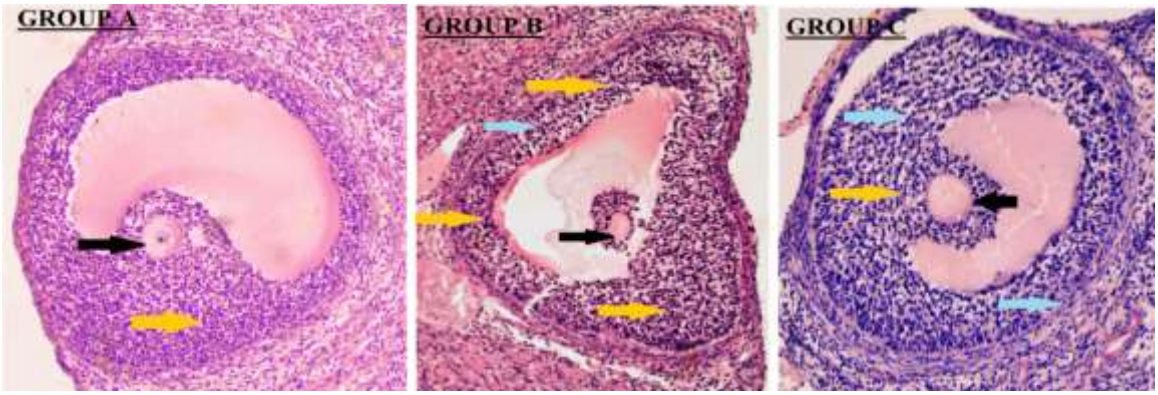
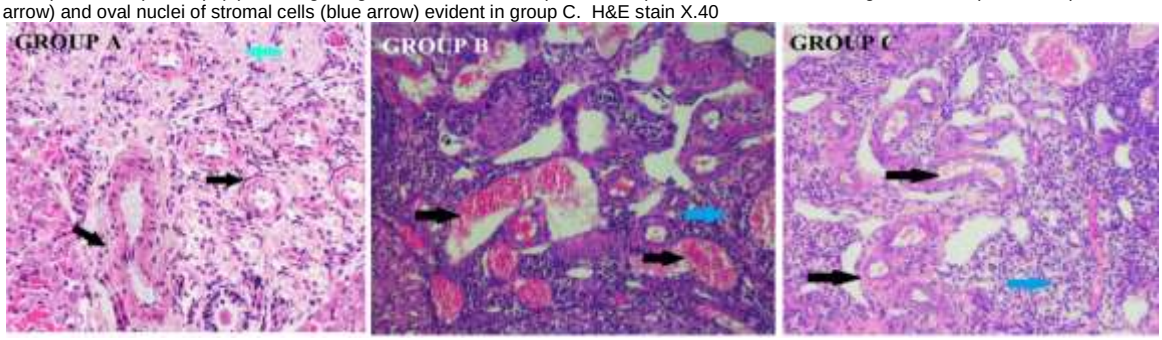


Figure 2: Photomicrograph of stroma of the ovary. Group (A) showing normal blood vessels (black arrow) lined by intact endothelium and oval nuclei of stromal cells (blue arrow). Group (B) showing congested blood vessels (black arrow). Stromal cells show elongated nuclei (blue arrow). Normal blood vessels (black arrow) and oval nuclei of stromal cells (blue arrow) evident in group C. H&E stain X.40



Theca folliculi vacuolization of Graafian follicle: Theca folliculi of control group (A) showed two distinct layers, one having polygonal shaped, steroid producing cells with collagen fibers and capillaries called theca interna. Theca externa, the other layer contained collagen bundles with smooth muscle cells. There was no theca folliculi vacuolization seen in group A. In group B, all rats had theca folliculi vacuolization. In group C, 2 (16.6%) rats had theca folliculi vacuolization. Chi square test showed that there was an association between theca folliculi vacuolization and groups (Table 1).

Stromal blood vessel congestion: Chi square test showed that there was an association between stromal blood vessel congestion and groups. There was no blood vessel congestion or stromal hemorrhage seen in group A. In group B, all rats had blood vessel congestion, disrupted endothelial lining and stromal hemorrhage. In group c only 1 (8.3%) rat shows vascular congestion (Fig.1,2

DISCUSSION

Drinking energy drink (ED) is a becoming a popular practice amongst youth for variable situations like compensation of deficient sleep and increase psychoactive performance while studying.[19] The toxic effects of ED are mainly due to its caffeine content and most importantly due to the combined enhanced effects of its various components with the caffiene[20].

Albino rats used in this study were the most standardized of all laboratory animals. They are specifically known for their greater sensitivity to most of drugs. They are easy to handle being small in size. They can withstand long periods of experimentation because of lack of their vomiting centers[21].

In this study, ovarian sections of rats administered ED for 30 days showed significant histological changes in Graafian follicles with degenerated oocytes & granulosa cells along with desquamation of some of the granulosa cells within follicular cavity. The granulosa cell and oocyte of the mature follicle in ED treated animals showed nuclear disruption. Nuclei appeared pyknotic, karyorrhectic and karyolysed. The nuclear debris were scattered throughout the degenerating zona granulosa. This supported the results of Mubarak who reported that submandibular gland secretory cells of rats treated with red bull for 8 weeks demonstrated hyperchromatic nuclei with atypism and pleomorphism[22]. He attributed these premalignant changes to the toxic action of caffeine and preservatives (sodium benzoate) contents of red bull. Similar finding were found in rat kidneys with pyknotic nuclei and loose of chromatin materials in cells of proximal and distal convulated tubules after 4 weeks administration of Power Horse.[7] These changes were attributed to ED related depletion of ATP, thus leading to cellular death.

Omega 3 is known specifically for reducing the number and intensity of caspase3 immune expression, thus indicating apoptotic cellular rescue. This indicates its protective effect in Group C (ED plus Omega 3). These findings are in accordance with previously published work which stated that Omega 3 FA inhibit apoptotic gene expression and DNA fragmentation.[23] Thus, they are favorable for averting apoptosis of gastric mucosa and pancreatic acinar cells caused by oxidative stress.[6]

Vacuolization within theca folliculi and ovarian stromal cells might be the signs of ovarian toxicity and cell degeneration in ED treated group (B). This was in agreement with previous studies where rats showed loss of normal liver architecture along with

marked vacuolization of hepatocytes after administration of PH[8,24]. Liver tissue showed marked inflammation along with lipid droplet accumulation in cells. These vacuoles could be liable for collecting harmful substances and inhibiting them from obstructing the biological activities of these cells.[24] The vacuolization of ovarian cells was not remarkable in ED + Omega 3 treated group (C) revealing the possible protective effect of omega 3 in our study A study done by Suresh and Das indicated that Omega3 PUFA may decrease inflammatory vulnerability and diminish the inflammatory reaction in the pancreatic islet cells by reducing cytokine production[26].

Congestion of blood vessels with stagnant blood cells & disrupted endothelium causing hemorrhage within ovarian stroma in ED treated group B may be due to the prevention of prostaglandin synthesis which could have regulated blood flow. These findings are in consensus with previous who reported extravasated red blood cells, vessel congestion and dilatation after treatment with energy drinks[8, 27]. This stromal vascular congestion might be credited to the microcirculatory disturbances that established due to the interaction of caffeine and taurine contents of the energy drinks[22].

These abovementioned adverse stromal findings were not observed in ED plus Omega 3 treated group. This commensurate with previous study done on the protective effects of omega 3 on ED induced gastric and pancreatic toxicity. They proposed anti-inflammatory activity of Omega3 causing reduction in the proinflammatory mediators, thus maintaining the vascular integrity[6].

In the present study, ovarian sections of rats treated with ED for 30 days showed significant atrophic changes, including many atretic follicles with degenerated oocytes and granulosa cells. There was increase number of atretic corpora lutea. Stroma of the ovary appeared vacuolated with focal hemorrhage and congested blood vessels.

Induction of oxidative stress in the tissue is a possible mechanism of ED harmful effect, and the anti-inflammatory and antioxidant activity of Omega3 could be a possible protective mechanism.

Moreover the present study will produce awareness of the hazards of the energy drinks. The food regulating agencies should take the responsibility and potentially regulate the availability of these beverages. Their sale and market availability should be monitored. Especially their easy access to the students must be discouraged.

CONCLUSION

The above discussed results clearly indicate that energy drink administration leads to atrophic and cystic ovarian changes. These cytotoxic cellular changes can be a leading cause of infertility in younger population excessively consuming these beverages. Oxidative stress induced by the combined ingredients of the ED is highly cytotoxic. Co administration of omega 3 fatty acids helps in preservation and maintenance of ovarian tissue architecture by acting as anti-oxidant agent and thus, counter balancing ED induced cellular cytotoxicity.

Further research must be carried out on such animal model with simultaneous assessment of serum caffeine levels, hormonal and immuno biochemical changes.

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