# EVALUATION OF TOXIC EFFECTS OF CAFFEINATED ENERGY DRINKS ON THYROID GLAND OF ADULT MALE ALBINO RATS: ASSESSMENT OF APOPTOSIS AND REGENERATION

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## ABSTRACT

**Background:** Young adults and adolescents are rarely aware of the negative effects of caffeinated energy beverages. **Aim of the work:** to investigate the hazards of caffeinated energy drinks on the thyroid gland in albino rats by biochemical, histological, and immunohistochemical examinations, and examining the signs of the apoptosis/regeneration rate. **Material and methods:** 24 adult albino rats were classified into 3 groups; Group 1 were kept on basal diet and distilled water, Group 2 and Group 3 were given energy drinks in 2 different doses for 14 days. Rats in Group 2 were given a daily low dose of caffeinated energy drinks daily (10 mg/kg), while the Group 3 of rats were given a daily high dose of caffeinated energy drinks (20 mg/kg). Finally, T3, T4 and TSH were measured, histopathology and immunohistochemical study using ki-67 and caspase-3 were performed. **Results:** the mean free T3 and T4 in (groups 1, 2 and 3) showed statistically significant higher values. There was a significant difference regarding ki-67 in the 3 studied groups; the mean ki-67 score showed the highest value in group 3 followed by the control group and group 2 respectively, while there was a non-significant difference regarding caspase 3 in the 3 studied groups. **Conclusion:** oral consumption of energy drinks was associated with significantly overexpression of Ki-67 in thyroid gland suggesting a proliferative response to thyroid gland injury and significant increase in free t3 and t4 in group 2.

**Keywords:** *Energy Drinks, Thyroid Gland, ki-67, Caspase-3.*

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## INTRODUCTION

Because of their strong caffeine content and marketing, energy drinks (ED) stand apart from other soft beverages and sports drinks, they can help with performance enhancement and fatigue relief ***(Oddy and O'sullivan, 2018)***. Cans of Red Bull® are available in capacities of 250 ml (or 80 mg of caffeine) or 355 ml (or 113.6 mg of caffeine). It has about 32 milligrams of caffeine per 100 milliliters. It contains sugar, salt, taurine, guarana, caffeine, and vitamin B6. The most significant ingredient is caffeine (1, 3, 7-trimethylxanthine) ***(Costa et al., 2018)***.

Caffeinated energy drinks (CEDs) have been linked with a number of unfavorable health effects, including headaches, tremors, palpitations, arrhythmias, anxiety, restlessness, and sleeping problems ***(Gubina-Vakulyck et al., 2020)***.

High amounts of caffeine have been demonstrated to affect a number of hormonal secretions and may alter the levels of pituitary gonadotrophins and thyroid hormones, among other hormones. Caffeine also stimulates the cardiovascular and neurological systems. Caffeine is considered an inhibitor of phosphodiesterase, and can affect the hormonal release or suppression through raising cyclic adenosine monophosphate (cAMP), or by indirect pathway, as it can inmpact neurotransmitters and, in turn, hypothalamic clearance factors ***(Arnaud, 2011; Khudhair and Abdulkareem, 2021)***.

Research on the relationship between thyroid hormones and caffeine is still ongoing ***(Zheng et al., 2023)***. In earlier studies on animals, intraperitoneal administration of coffee in rats resulted in the release of hypothalamic growth inhibitory hormone, which suppressed TSH secretion.

Nevertheless, no research has been done on how long-term coffee use affects the thyroid gland ***(Zhao et al., 2023)***. Additionally, it has been shown that 50 mg/kg of caffeine may lower TSH and GH concentrations. After four hours, the fall in TSH was followed by decreased levels in T3 and T4 ***(Da Silva and Associates, 2017)***.

High doses of caffeine have been shown in certain studies to directly trigger cellular death; however, because of the unfavorable side effects of high doses, clinical verification of this finding has not been achievable ***(He et al., 2003)***.

A well-known marker of nuclear cell proliferation, Ki-67's expression is linked to the compensatory activation of cell proliferation in response to tissue damage ***(Tkachenko et al., 2018)***. The enzymes known as caspases are members of the cysteine protease group. Effector caspases, such as caspase 3, are in responsible for initiating apoptosis. An increase in apoptotic activity is indicated by a rise in tissue caspase 3 concentrations ***(Slawinski et al., 2018)***.

## THE AIM OF THE WORK

Few studies, to our knowledge, have examined the impact of caffeinated energy drinks on the thyroid gland, hence, this work aimed to investigate the toxic effect of caffeinated energy drinks consumption on thyroid gland of male albino rats for 14 days and to evaluate the morphology of the thyroid gland, as well as the features of apoptosis/regeneration. T3, T4 and TSH were measured histopathology and immuno-histochemical study using ki-67 and caspase-3 were performed.

## MATERIAL AND METHODS

The current work is an animal experimental study. It was carried out at the Pathology, Forensic Medicine & Clinical Toxicology departments, and Animal House at Cairo University's Faculty of Medicine. Cairo University's Institutional Animal Care and Use Committee (IACUC) gave its approval for the experiment and issued an ethical approval number: **CUF- III-66-23.**

**Sample size**

Calculating the power and sample size for a non-inferiority intervention study using a 0.05 alpha error, 0.80 power, 95% confidence interval, and 0.1 non-inferiority margin with an enrollment ratio of 3.

A sample size of 24 rats, plus an additional 10% to account for the follow-up period, was determined to analyze the harmful effects of CEDs on the thyroid glands of rats. Each group consists of eight rats.

**Study design**

24 adult male albino rats weighing an average of 150 g each were housed in separate cages. With a mean temperature of 25 ± 2 °C and a 12 h × 12 h light/dark photo cycle, the rats were housed in normal, healthy conditions. Three groups of rats (each with eight rats) were created: Rats in the control group (G1) were fed distilled water and a baseline diet for the duration of the study.

Rats in the low dosage ED ingestion group (G2) were given a daily dose of 10 mg/kg.

The high dose ED ingestion group (G3) of rats were given a daily dose of 20 mg/kg.

This study used the energy drink brand "Red Bull," and over the period of 14 days, energy beverages were given by gavage ***(Akande and Banjoko, 2011)***.

**Collection of samples and Biochemical analysis:**

A sample of blood was obtained from inferior vena cava from each rat at the completion of the experiment, 24 hours after the last dose in order to collect blood sample for T3, T4, and TSH biochemical tests. At last, the thyroid glands were taken from the animals, stored in 10% neutral buffered formalin, and sent for pathological analysis after the animals were sacrificed.

**Histopathological examination:**

Processing of the received tissue was performed by dehydration in graded alcohols of increasing concentrations, followed by clearance in xylene and finally embedding in paraffin. The paraffin-embedded blocks were sectioned at 4 µm and stained by routine hematoxylin and eosin stains for routine examination. ***(Gubina-Vakulyck et al., 2020)***. The degree of congestion, the follicles' colloid content, the proliferation and cytoplasmic vacuolization of the follicular cells, and the existence of any fibrosis or inflammatory response were among the factors that were evaluated. Regarding the degree of

congestion, the number of congested vessels was counted in the whole examined thyroid tissue for each rat; thyroids with only 1 or 2 congested vessels were graded as 1, those with 3or 4 congested vessels were graded as 2 and those with 5 or more congested vessels were graded as 3. As for the rest of examined items, they were graded into three categories in a comparative semi-quantitatively fashion.

**Immunohistochemical examination**

An immunohistochemical analysis was conducted using caspase-3 and ki-67. From each paraffin block, two extra 4 µm thick sections were produced on positively charged slides for immunohistochemistry staining.

For caspase 3 immunostaining, a polyclonal anti-caspase 3 antibody (cat. no. A11319; ABclonal Biotech) and a dako automated immunostainer were employed. Caspase 3 positivity was only evaluated in the follicular cells' cytoplasm; However, due to the observed heterogeneous staining pattern, an H-score was applied using the formula [(0 X negative staining) + (1 X weak staining) + (2 X moderate staining) + (3 X strong staining)] ***(Pu et al., 2017)***.

For Ki-67 immunostaining, the monoclonal anti-Ki-67 antibody (clone 30-9, cat. no. 790-4286, Ventana) and the Ventana Benchmark ultra-automated immunostainer were used. Each rat was given four fields, or hot spots, to count the nuclei of follicular cells that tested positive for Ki-67 ***(Gubina-Vakulyck et al., 2020)***.

Using an Olympus light microscope (model BX53F2), every slide was examined. A digital Olympus high-definition camera (model EP50) was attached to the identical microscope used to capture the images.

**STATISTICAL ANALYSIS:**

The data was coded and entered using the statistical program for the social sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). The mean, standard deviation, and frequencies (number of cases) and relative frequencies (percentages) for categorical variables, and the median and interquartile range for quantitative variables that were not normally distributed, were used to summarize the data. To compare groups for regularly distributed quantitative variables, analysis of variance (ANOVA) with multiple comparisons post hoc test was utilized; for non-normally distributed quantitative variables, non-parametric Kruskal-Wallis test and Mann-Whitney test were utilized ***(Chan, 2003a)***. Categorical data were compared using the Chi square (2) test. An exact test was used in its place when the estimated frequency was less than five ***(Chan, 2003b)***. Correlations between quantitative variables were performed using the Spearman correlation coefficient ***(Chan, 2003c)***. P-value was considered statistically significant at the 0.05 threshold. Post hoc Bonferroni corrected was used for pairwise comparison adjusted for type 1 statistical error Correlations between quantitative variables were performed using the Spearman correlation coefficient.

## RESULTS

Free T3 and T4 and TSH mean values revealed statistically significant higher values in the LD group (3.31) and (2.63), HD group (2.97) and (2.33), and control group (2.66) and (1.28) with significant P values of 0.009 and <0.001, respectively. The control group had the highest mean TSH value (0.86), which was followed by the means of the LD and HD groups (0.50 and 0.38, respectively) as illustrated in **table (1)**.

Histopathological analysis showed that the HD group had more congestion than the low dosage and control groups; 62.5% of the thyroid glands in the HD groups were rated as 3, compared to just 12.5% in the control and LD groups. The HD group also had the highest amount of intra-follicular colloid; in the LD, HD, and control groups, the amount of colloid found on histological examination was classified as 3, with percentages of 62.5%, 50%, and 37.5%, respectively.

The follicular epithelium was found to be more flattened in the low dosage and control groups (37.5% and 50% of them, respectively, were rated as 3), while the thyroid gland follicular cells in the HD group showed higher hyperplastic alterations. Furthermore, the thyroid glands of the LD and HD groups showed higher levels of cytoplasmic vacuolization of follicular cells compared to the control group. The control, low dose, and high dosage groups did not exhibit any

obvious signs of inflammation or fibrosis. All of the pathological results in the control, LD, and HD groups did not differ statistically from one another **(Table 2 and Figure 1)**.

As shown in **table (3)** and **figure (2)**, In terms of the ki-67 score, the HD group's mean score was the highest at 5.25, followed by the means of the control group at 2.50, and the LD group at 0.38. Regarding caspase 3, the mean caspase H score indicated the greatest value in LD group (55.13) followed by the control group mean (32.63) and finally the HD group mean (25.63). While there was no significant difference (p=0.051) for caspase 3, there was a significant difference (p=0.046) for ki-67 between the three assessed groups. There was no a significant difference in TSH in the three study groups **(Table 4 and Figure 3)**.

As shown in **table (5)**, A posthoc pairwise analysis of ki-67 between the study's various groups showed that there was a significant difference (P values = 0.024 and 0.043, respectively) between the LD and HD groups, but no significant difference (P value = 0.810) between the control and HD groups.

As shown in **table (6)**, in the studied groups, a pairwise posthoc analysis between free T3 and free T4 was performed. Regarding free T3, there was a significant difference between (Control and LD) with significant P value 0.007 and a non-significant difference between (Control and HD) and (LD and HD), with non-significant p values of 0.346 and 0.251, respectively. Regarding free T4, there was a significant difference (P value <0.001) between (Control and LD) and (Control and HD), and a non-significant difference (p value 0.459) between (LD and HD).

**Table (7)** indicates that the pathological findings and the other parameters studied (ki-67, caspase 3, TSH, free T3, and free T4) showed non-significant correlations, with the exception of cytoplasmic vacuolization, which exhibited a significant positive correlation with free T3 (r=0.32 and p value 0.035) as illustrated in **figure (4)**.

TSH, free T3, and free T4, and ki-67 and caspase 3 revealed non-significant associations, with the exception of caspase 3, which had a significant positive correlation with free T3 (r=0.409 and p value 0.047) as illustrated in **table (8)** and **figure (5)**.

**Table (1): Comparison between the control, LD, HD groups regarding biochemical markers (Free T3, Free T4).**

| Variable | Control | | Low dose | | High dose | | |
|---|---|---|---|---|---|---|---|
| | Mean | Standard Deviation | Mean | Standard Deviation | Mean | Standard Deviation | P value |
| **Free T3** | 2.66 | 0.46 | 3.31 | 0.27 | 2.97 | 0.37 | 0.009 |
| **Free T4** | 1.28 | 0.26 | 2.63 | 0.39 | 2.33 | 0.53 | <0.001 |

**Table (2): Comparison between the control, LD, HD groups regarding the pathological findings.**

| Pathological findings | Grade | Control | | Low dose | | High dose | | P value |
|---|---|---|---|---|---|---|---|---|
| | | Count | % | Count | % | Count | % | |
| **Congestion** | **Grade 1** | 3 | 37.5% | 6 | 75.0% | 1 | 12.5% | 0.059 |
| | **Grade 2** | 4 | 50.0% | 1 | 12.5% | 2 | 25.0% | |
| | **Grade 3** | 1 | 12.5% | 1 | 12.5% | 5 | 62.5% | |
| **Colloid content of follicles** | **Grade 1** | 3 | 37.5% | 2 | 25.0% | 2 | 25.0% | 0.931 |
| | **Grade 2** | 2 | 25.0% | 2 | 25.0% | 1 | 12.5% | |
| | **Grade 3** | 3 | 37.5% | 4 | 50.0% | 5 | 62.5% | |
| **Flattening of follicular epithelium** | **Grade 1** | 2 | 25.0% | 3 | 37.5% | 3 | 37.5% | 0.160 |
| | **Grade 2** | 3 | 37.5% | 1 | 12.5% | 5 | 62.5% | |
| | **Grade 3** | 3 | 37.5% | 4 | 50.0% | 0 | 0.0% | |
| **Cytoplasmic vacuolization** | **Grade 1** | 8 | 100.0% | 4 | 50.0% | 5 | 62.5% | 0.126 |
| | **Grade 2** | 0 | 0.0% | 3 | 37.5% | 1 | 12.5% | |
| | **Grade 3** | 0 | 0.0% | 1 | 12.5% | 2 | 25.0% | |

**Table (3): Description of non-normal data regarding Ki-67, Caspase 3, and TSH in the studied groups.**

| Variable | Mean | Standard Deviation | Mean | Standard Deviation | Mean | Standard Deviation |
|---|---|---|---|---|---|---|
| **Ki-67** | 2.50 | 2.39 | 0.38 | 0.52 | 5.25 | 10.12 |
| **Caspase-3** | 32.63 | 24.02 | 55.13 | 24.68 | 25.63 | 14.67 |
| **TSH** | 0.86 | 1.07 | 0.50 | 0.38 | 0.38 | 0.22 |

**Table (4): Median, 1st and 3rd Quartile regarding ki-67, caspase-3 and TSH in the 3 studied groups.**

| Variable | Control | | | Low dose | | | High dose | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Median | 1st quartile | 3rd quartile | Median | 1st quartile | 3rd quartile | Median | 1st quartile | 3rd quartile | P value |
| **Ki-67** | 1.00 | 1.00 | 5.00 | 0.00 | 0.00 | 1.00 | 1.50 | 0.50 | 4.00 | 0.046 |
| **Caspase 3** | 26.00 | 23.50 | 35.00 | 55.00 | 39.00 | 75.50 | 22.50 | 18.50 | 37.50 | 0.051 |
| **TSH** | 0.19 | 0.13 | 1.76 | 0.57 | 0.17 | 0.64 | 0.41 | 0.20 | 0.51 | 0.912 |

**Table (5): Post hoc pairwise comparison of Ki-67 in different studied groups.**

| Variable | Control versus Low dose | Control versus High dose | Low dose versus High dose |
|---|---|---|---|
| **Ki-67** | 0.024 | 0.810 | 0.043 |

**Table (6): Post hoc pairwise comparison of Free T3, and T4 in different studied groups.**

| Variable | Control versus Low dose | Control versus High dose | Low dose versus High dose |
|---|---|---|---|
| **Free T3** | 0.007 | 0.346 | 0.251 |
| **Free T4** | < 0.001 | < 0.001 | 0.459 |

**Table (7): Correlation between different pathological findings and Ki-67, Caspase 3, TSH, FreeT3, and Free T4.**

| Pathological finding | | Ki-67 | Caspase 3 | TSH | Free T3 | Free T4 |
|---|---|---|---|---|---|---|
| **Congestion** | Correlation Coefficient | -0.122- | -0.209- | 0.048 | -0.060- | -0.143- |
| | P value | 0.570 | 0.327 | 0.825 | 0.780 | 0.504 |
| | N | 24 | 24 | 24 | 24 | 24 |
| **Colloid content of follicles** | Correlation Coefficient | 0.068 | -0.132- | -0.289- | -0.036- | 0.293 |
| | P value | 0.753 | 0.538 | 0.170 | 0.867 | 0.165 |
| | N | 24 | 24 | 24 | 24 | 24 |
| **Flattening of follicular epithelium** | Correlation Coefficient | 0.232 | 0.052 | -0.127- | -0.051- | 0.081 |
| | P value | 0.275 | 0.808 | 0.553 | 0.811 | 0.707 |
| | N | 24 | 24 | 24 | 24 | 24 |
| **Cytoplasmic vacuolization** | Correlation Coefficient | -0.383- | 0.366 | 0.385 | 0.432 | 0.324 |
| | P value | 0.065 | 0.079 | 0.064 | 0.035 | 0.123 |
| | N | 24 | 24 | 24 | 24 | 24 |

**Table (8): Correlation between TSH, FreeT3 and Free T4 and Ki-67, Caspase 3.**

| Variable | | Ki-67 | Caspase 3 |
|---|---|---|---|
| **TSH** | Correlation Coefficient | -0.273- | 0.101 |
| | P value | 0.197 | 0.640 |
| | N | 24 | 24 |
| **Free T3** | Correlation Coefficient | -0.399- | 0.409 |
| | P value | 0.053 | 0.047 |
| | N | 24 | 24 |
| **Free T4** | Correlation Coefficient | -0.316- | 0.291 |
| | P value | 0.132 | 0.168 |
| | N | 24 | 24 |

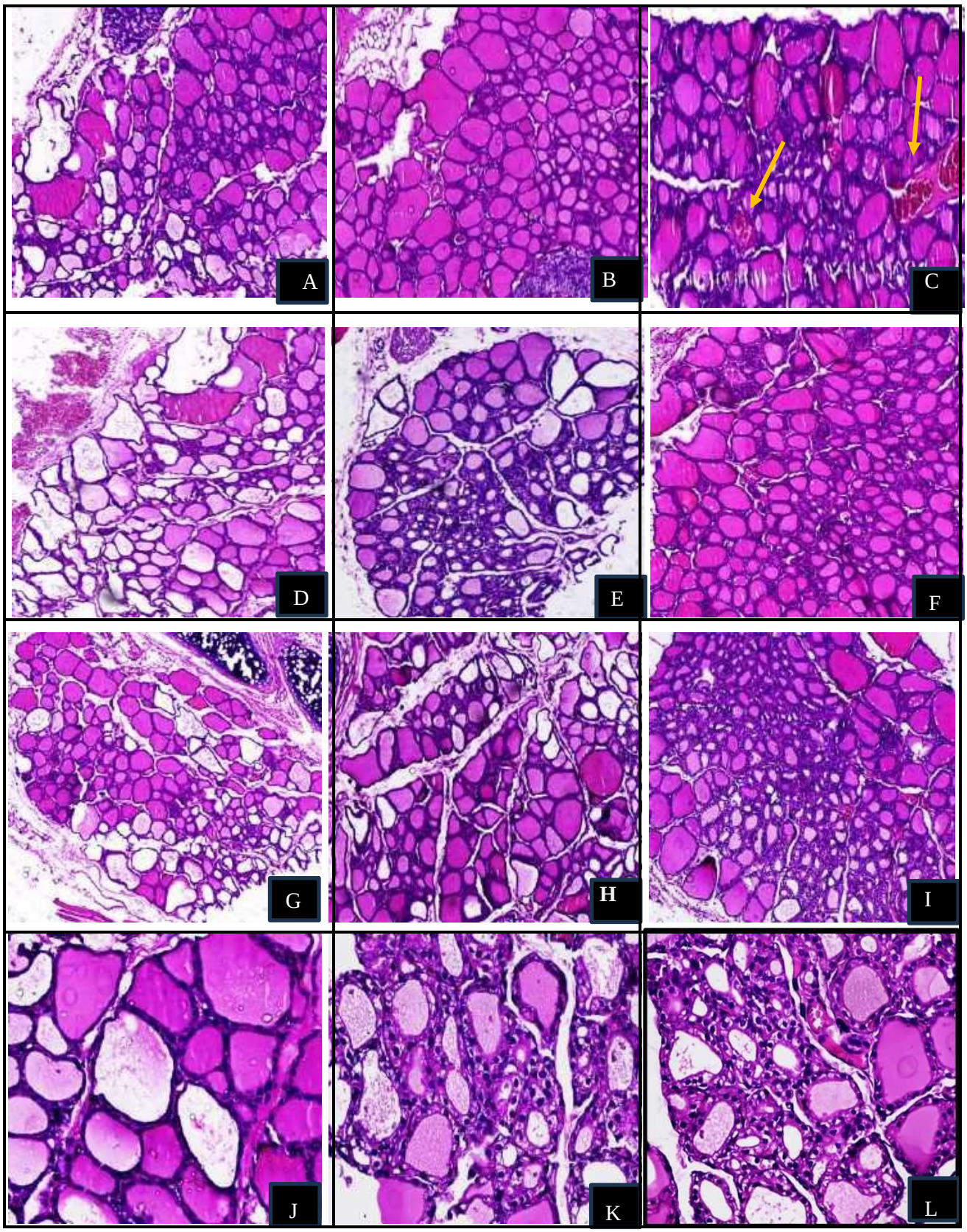


***Figure (1): Representative H&E-stained sections of the examined thyroid glands. The highest degree of congested vessels was detected in the HD groups (arrows, fig. C x200 original magnification) compared to control (fig. A x200 original magnification) and LD (fig. B x200 original magnification). The greatest amount of colloid was also detected in the HD groups (fig. F x100 original magnification) compared to control (fig. D x100 original magnification) and LD (fig. E x100 original magnification). The follicular cells showed higher degree of proliferation in the HD groups (fig. I x 100 original magnification), while more flattening was detected in the control (fig. G x100 original magnification) and LD (fig. H x100 original magnification) groups. The cytoplasmic vacuolization was more prominent in the HD (fig. L x 400 original magnification) and LD (fig. K x400 original magnification) compared to the control group (fig. J x400 original magnification).***

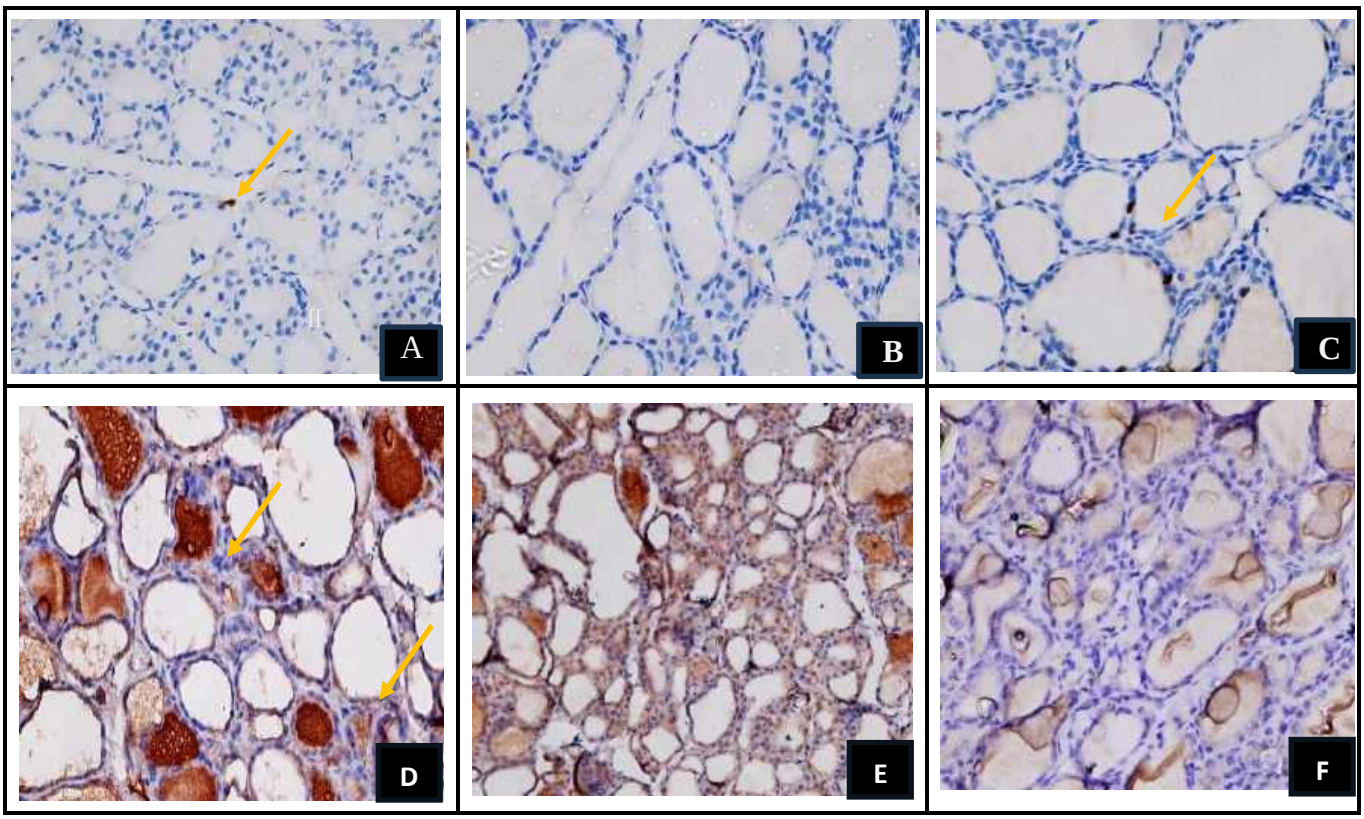


***Figure (2): Representative immunohistochemical stained sections of the examined thyroid glands: The highest Ki-67 score was detected in the HD group (arrow, Fig. C x400 original magnification), followed by the control group (arrow, Fig. A x400 original magnification) and finally the low dose group (Fig. B x400 original magnification). Conversely, the highest Caspase 3 H score was detected in the low group (Fig. E x200 original magnification), followed by the control group (arrows, Fig. D x200 original magnification) and finally the high dose group (Fig. F x200 original magnification).***

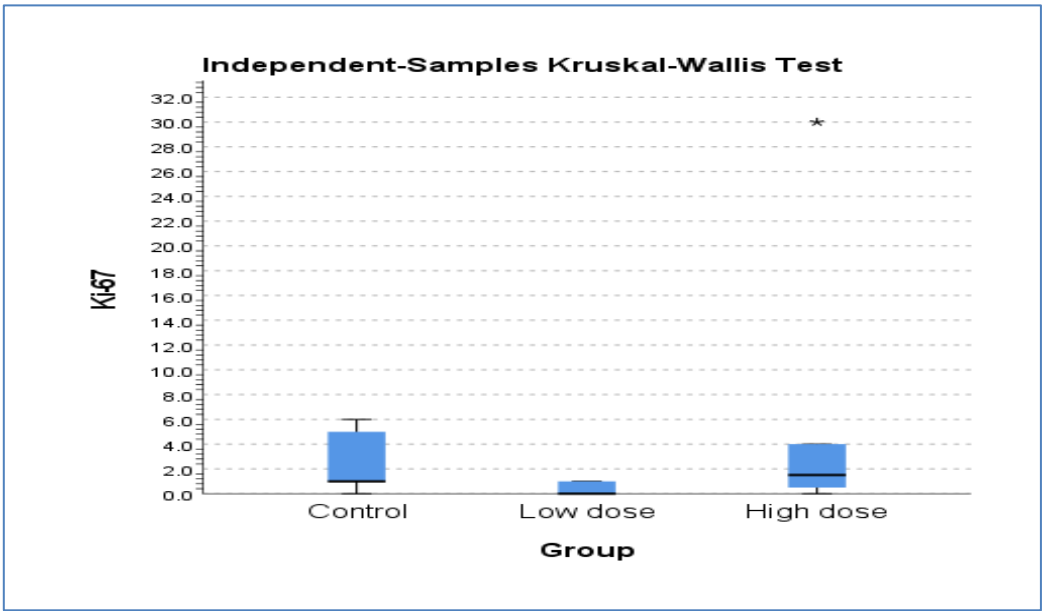


***Figure (3): Independent samples Kruskal- Wallis test showing Ki-67 in the 3 studied groups***

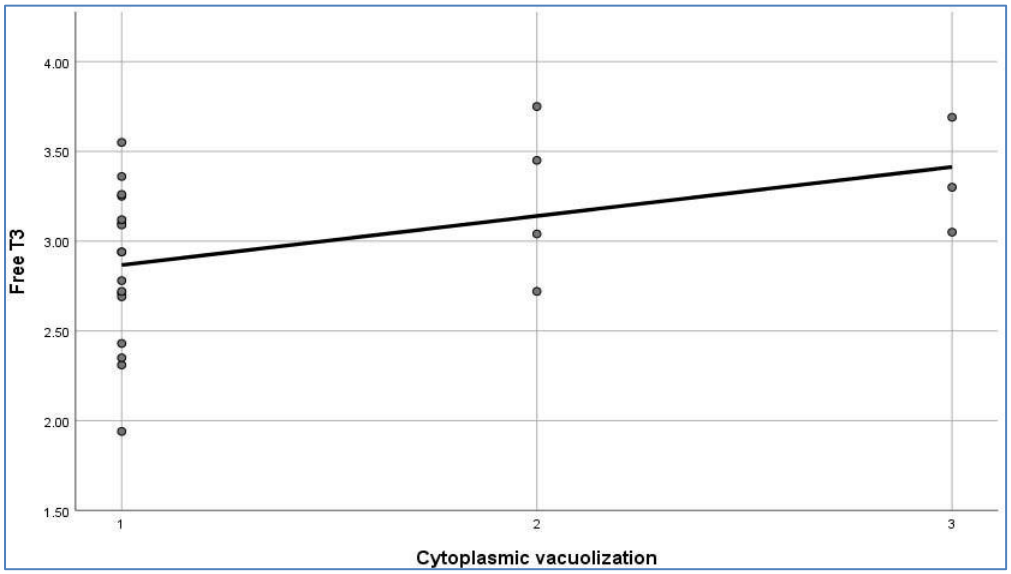


***Figure (4): Significant positive correlation regarding cytoplasmic vacuolization and FreeT3 with r = 0.32 and significant p value 0.035.***

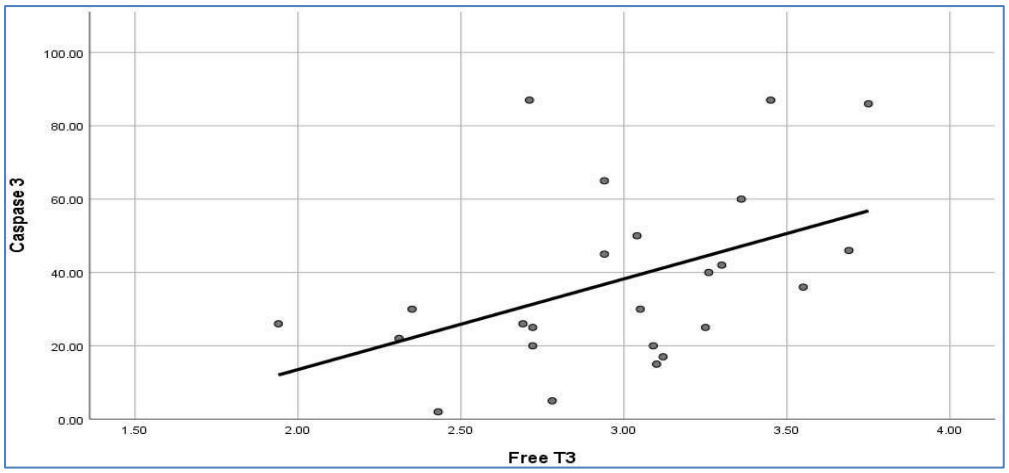


***Figure (5): Significant positive correlation regarding Caspase 3 and Free T3with r = 0.409 and significant p value 0.047.***

## DISCUSSION

Caffeine is the most often found component of ED, and Taurine, glucuronolactone, guarana, and B-group vitamins are often used in combination with it ***(Piccioni et al., 2021)***. It has long been believed that the majority of the adverse consequences linked to ED usage are caused by caffeine. The other chemicals in ED, however, may be more dangerous because a randomized study revealed that the hemodynamic and electrophysiological changes associated with ED intake were more profound and long-lasting than those induced by caffeine alone ***(Fletcher et al., 2017)***.

The goals of our research were to determine the effects of caffeinated energy beverages on albino rats' thyroid glands using biochemical, histological, and immunohistochemical analyses as well as by looking for indicators of the rate of apoptosis and regeneration. Three groups of twenty-four adult albino rats were created; group 1 was fed a basic diet and distilled water, while groups two and three received energy drinks for a period of fourteen days at two different dosages. The rats in group 3 received a daily high dose of caffeinated energy drinks (20 mg/kg), while the rats in group 2 received a daily low dose (10 mg/kg). Ultimately, histology, immunohistochemistry using ki-67 and caspase-3, and measurements of T3, T4, and TSH were carried out.

Regarding biochemical analysis, our findings demonstrated a significant increase in T3 and T4 in the low-dose group. Additionally, statistically significant higher values were found for free T3 and T4 and TSH mean values in the HD group (2.97) and (2.33), LD group (3.31) and (2.63), and control group (2.66) and (1.28) with significant P values of 0.009 and <0.001, respectively. The LD and HD groups' means were 0.50 and 0.38, respectively, while the control group's mean TSH value was the highest at 0.86.

In accordance with our study, ***Zhao et al., (2023)*** reported that caffeine released hypothalamic growth inhibitory hormone, which suppressed TSH secretion. Additionally, it has been suggested that caffeine may lower TSH, which may then lower T3 and T4 ***(de Silva et al., 2017)***.

***He et al. (2003)*** also stated that large doses of caffeine directly cause cellular death; however, due to the adverse effects of high doses, this finding has not been able to be clinically verified.

According to a recent study, which involved participants who drank more than 500 mg of caffeine per day for three months, exposure to caffeine at doses greater than 200 mg per day for longer than six months results in a significant difference in the amount of free T3 hormone, but has no effect on the amount of thyroid-stimulating hormone (TSH), which remains within normal physiological ranges regardless of the daily dosage ***(Upadrasta, 2024)***.

***Ibrahim (2011)*** also demonstrated that both groups (C1 and C2) of male albino rats treated with caffeine at doses of 7.5 mg/kg and 14 mg/kg of body weight, respectively, experienced significant increases in TSH and T3 and T4 levels in addition to a non-significant increase in TSH when compared to the control group.

***Zheng et al. (2023)*** found, in contrast to our research, that moderate consumption of caffeine (9.97–264.97 mg/d) suggested a negative connection (p=0.001) with serum TSH, while intake of less than 9.97 mg/d was positively linked with the hormone. Additionally, among those with metabolic problems, there was a nonlinear connection between serum TSH and caffeine use. Caffeine intake of less than 9.97 mg/d was positively associated with serum TSH however, moderate caffeine consumption (9.97–264.97 mg/d) indicated a negative association ($p = 0.001$, standardized $\beta = -0.152$). We suggested that the sources of caffeine in Americans' diets may be connected to this variety. Soft drinks and energy drinks accounted for 17% of the total quantity of caffeine used by adults, according to data from the NHANES 2011–2012. These beverages are also heavy in calories, sugar, and fat and may contribute to excessive weight gain. ***(Drewnowski and Rehm, 2016)***.

As regards immunohistochemical analysis, our results indicated that the HD group had the highest mean ki-67 score (5.25), followed by the averages of the control group (2.50) and the LD group (0.38).

In terms of caspase 3, the LD group's mean caspase H score was highest (55.13), followed by the means of the control group (32.63) and the HD group (25.63). For Ki-67, there was a significant difference (p=0.046) across the three examined groups, while there was no significant difference (p=0.051) for caspase 3.

In accordance with our study, the quantity of ki-67+ were significantly higher in rats given ED as compared to the control group. Oral ED exposure for two weeks induces hepatocyte regrowth, apoptosis, and liver damage. Hepatocyte apoptosis is known to be triggered by oxidative stress, which is linked to an increased reactive oxygen species ***(Liu et al., 2017; Cao et al., 2016; Wang et al., 2016)***.

***Ayuob and ElBeshbeishy's (2016)*** indicate that when rats were given one of the energy drinks, Power Horse, there was a significant caspase-3 reaction. According to a study by ***Tkachenko et al. (2018)*** at Kharkiv University in Ukraine on male albino rats, oral exposure to caffeinated energy drinks for an extended period of time promotes ki-67 overexpression, which is consistent with our study.

Contrary to our findings, ***Abonar et al. (2022)*** reported that after giving energy drinks to rats for four weeks, the immunohistochemistry analysis revealed a substantial increase in caspase-3. As per the research conducted by ***Gubina-Vakulyck et al. (2020)*** at Kharkiv

National Medical University's vivarium for two weeks, rats in the experimental group were given 12 ml /kg of ED orally. In contrast to our findings, chromatin margination also indicates the initiation of apoptosis, as indicated by a statistically significant rise in caspase-3 levels.

Regarding the histopathological analyses and the apoptotic/regenerative signs on the adult albino rats' thyroid gland, our findings showed that the HD group had greater thyroid gland congestion than the low dosage and control groups; 62.5% of the thyroid glands in the HD groups were rated as 3, compared to only 12.5% in the LD and control groups.

The amount of intra-follicular colloid was similarly largest in the HD group; on histological analysis, the amount of colloid was classified as 3 in the LD, HD, and control groups, with percentages of 62.5%, 50%, and 37.5%, respectively. The low dosage and control groups had more flattened follicular epithelium (graded as 3 in 37.5% and 50% of them, respectively), whereas the HD group's thyroid gland follicular cells displayed greater hyperplastic changes.

In addition, compared to the control group, the thyroid glands of the LD and HD groups displayed greater levels of cytoplasmic vacuolization of follicular cells. There were no overt indications of inflammation or fibrosis in the control, low dose, or high dosage groups. The pathological outcomes in the LD, HD, and control groups did not show any statistically significant differences from one another. This effect is regarded as an entirely novel area of study. The harmful effects of caffeinated ED on other organs have been studied in the past. To the best of our knowledge, we are the first to conduct research into how caffeine affects the thyroid gland.

## CONCLUSION

Oral consumption of ED was associated with significantly overexpression of Ki-67 in thyroid gland suggesting a proliferative response to thyroid gland injury and significant increase in free T3 and T4 in LD group. The results of this study support the idea that energy-boosting beverage use has many harmful impacts on the thyroid's normal structure.

## RECOMMENDATIONS

- More researches are needed to examine the long-term impacts of energy drinks and how they might reverse over time.
- ED and all other caffeinated beverages should be consumed with cautiously or avoided entirely.
- These drinks should only be sold in limited quantities; the government ought to monitor their free availability.
- Programs for raising awareness are required in regards to the amount of caffeine in energy drinks and their psychological dependence.
- The government should enact laws prohibiting the selling of energy drinks, particularly to minors.

### Authors' contributions

Each author contributes to the material collecting, writing, and editing of the manuscript.

### Availability of data and materials

The corresponding author can provide the datasets created and/or analyzed during the current work upon reasonable request.

### Declaration of interests

The authors have disclosed no relevant financial or non-financial interests.

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### Ethical approval

The study was carried out with the ethical approval number **CUF-III-66-23** issued by the Cairo University, Faculty of Medicine's ethical committee.

### Abbreviations

*CED:Caffeinated energy drinks*
*ED: Energy drinks*
*HD: High dose LD: Low dose*
*T3: Triiodothyronine*
*T4: Thyroxine*

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