



EVALUATION OF PRO- AND ANTI-APOPTOTIC EFFECTS OF “FEARLESS” ENERGY DRINK ON RENAL TISSUES OF ALBINO RATS USING GENE EXPRESSION ANALYSIS

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Abstract

The global rise in energy drink (ED) consumption, particularly among young adults in Nigeria, has raised significant concerns regarding their potential long-term nephrotoxic effects. This study investigated the impact of chronic energy drink exposure on renal apoptosis by integrating histopathological evaluation with gene expression analysis in albino rats. Specifically, the expression of the pro-apoptotic gene BAX (B-cell lymphoma-associated X protein) and the anti-apoptotic gene BCL-2 (B-cell lymphoma-2) was examined. Twenty-four albino rats were randomly allocated into four groups (n = 6 per group): a control group and three treatment groups administered low, medium, and high doses of an energy drink for 60 days. Following treatment, animals were anesthetized with ketamine (50 mg/kg) and euthanized, after which renal tissues were harvested for histopathological assessment and molecular analysis using standard protocols. Chronic energy drink exposure resulted in a dose-dependent upregulation of BAX expression, with fold changes of 2.44, 3.31, and 5.65 in the low-, medium-, and high-dose groups, respectively. In contrast, BCL-2 expression was markedly downregulated, with corresponding fold changes of 0.68, 0.09, and 0.03. Histological examination revealed prominent dense basophilic concretions within renal tubules, occurring more frequently in treated groups than in controls. The pronounced shift in the BAX/BCL-2 expression balance toward a pro-apoptotic profile suggests enhanced renal apoptotic signaling, a mechanism implicated in the pathogenesis of renal fibrosis, diabetic nephropathy, and graft rejection. Furthermore, tubular basophilic concretions may contribute to intrarenal obstruction, impaired urine flow, and increased susceptibility to nephrolithiasis and urinary tract infections. Collectively, these findings provide mechanistic evidence that prolonged energy drink consumption may compromise renal structural integrity and cellular survival through apoptosis-mediated pathways.

Keywords: Fearless energy drink; BAX; BCL-2; fold change; gene expression; kidney toxicity.

Introduction

Energy drinks (EDs) are commercially popular beverages formulated with stimulants such as caffeine and taurine and are widely consumed to enhance physical endurance, alertness, and cognitive performance. Unlike sports or isotonic drinks, which are designed primarily for hydration and electrolyte replacement, EDs are intended to exert stimulatory effects on the central nervous system (Striley and Khan, 2014). First introduced in Europe and Asia in the 1960s, EDs gained global prominence following the commercial launch of Red Bull in 1987, subsequently expanding into markets worldwide, including Nigeria (Erdmann et al., 2021). Adolescents and young adults represent the largest consumer group, with reported usage rates ranging from 52% to 68%, compared with approximately 32% among adults (Erdmann et al., 2021). Aggressive marketing strategies, particularly sponsorship of extreme sports and youth-oriented events, have further reinforced their widespread consumption (Striley and Khan, 2014).

Despite their increasing popularity, concerns regarding the long-term safety of ED consumption remain unresolved. Energy drinks typically contain caffeine concentrations ranging from 75 to 240 mg per serving, alongside substantial amounts of taurine, averaging approximately 342.28 mg/100 mL. However, the precise quantities of several bioactive constituents are often inadequately disclosed by manufacturers (Erdmann et al., 2021). Moreover, there is limited scientific evidence supporting claimed synergistic or protective interactions

among ED components. Emerging reports have linked excessive ED intake to cardiovascular, neurological, and metabolic disturbances, yet their potential effects on renal cellular integrity and survival mechanisms remain insufficiently explored.

Apoptosis, or programmed cell death, is a tightly regulated physiological process essential for tissue homeostasis and organ integrity. It is characterized by DNA fragmentation, chromatin condensation, mitochondrial membrane depolarization, and the formation of apoptotic bodies without eliciting an inflammatory response (Brien and Kirby, 2018; Torres, 2018). Dysregulation of apoptotic pathways has been implicated in the pathogenesis of several organ disorders, including renal, hepatic, and cardiac diseases (Xie et al., 2021; Younes et al., 2020). In the kidney, excessive apoptotic signaling may contribute to tubular injury, impaired regeneration, fibrosis, and progressive functional decline.

Given the kidney's critical role in xenobiotic metabolism and excretion, it is particularly vulnerable to chronic exposure to bioactive compounds present in EDs. Central to apoptotic regulation are the B-cell lymphoma-2 (BCL-2) family of proteins, in which BCL-2 functions as an anti-apoptotic regulator, while BCL-2-associated X protein (BAX) promotes apoptosis. The balance between these opposing signals determines cellular fate following toxic or metabolic insults. However, limited experimental data exists on how prolonged ED consumption modulates these apoptotic pathways within renal tissue.

Therefore, the present study was designed to investigate the renal effects of chronic energy drink consumption in albino rats by integrating histopathological assessment of nephron integrity with molecular evaluation of apoptosis-related gene expression, specifically focusing on the pro-apoptotic *BAX* and anti-apoptotic *BCL-2* genes. The findings are expected to provide mechanistic insights into the potential nephrotoxic effects of energy drinks and contribute to evidence-based public health recommendations aimed at promoting safer consumption practices.

2. Materials and Methods

2.1 Materials and reagents

A commercially available energy drink (Batch No. L2008), registered with the National Agency for Food and Drug Administration and Control (NAFDAC No. 086116) and with an expiry date of March 2023, was purchased from the University of Maiduguri campus. Analytical-grade reagents were used throughout the study, including 10% neutral buffered formalin, chloroform, nuclease-free water, RNeasy Lysis Buffer, RNAlater®, graded ethanol solutions (50%–100%), hematoxylin and eosin (H&E) stains, and xylene.

Gene-specific primers targeting the pro-apoptotic gene *BAX*, the anti-apoptotic gene *BCL-2*, and the housekeeping gene β -*actin* were employed for gene expression analysis. Molecular biology reagents included RNA extraction kits (Norgen Biotek), RT-qPCR reagents (Luna Universal One-Step RT-qPCR Kit), and consumables suitable for RNA work.

2.2 Equipment and instrumentation

Laboratory analyses were supported by standard molecular biology and histology equipment, including a reverse transcriptase PCR system (Cepheid, Germany), NanoDrop 2000c spectrophotometer, thermal cycler (Eppendorf Nexus Gradient), centrifuge (5430 Eppendorf, Germany), tissue homogenizer, gel electrophoresis apparatus, micropipettes, microtome, paraffin wax embedding system, weighing balance, and light microscopes. Histological examinations were performed using a DM 750 Leica light microscope.

2.3 Study location

Experimental procedures were carried out at the North-East Centre for Biotechnology, University of Maiduguri, Nigeria. Histopathological analyses were conducted at the University of Maiduguri Teaching Hospital and Biomedical Science Research and Training Center (BioRTC) Damaturu, Nigeria, using standard microscopy facilities.

2.4 Experimental animals and ethical approval

Ethical approval for the use of experimental animals was obtained from the Animal Use Ethics Committee of the Faculty of Pharmacy, University of Maiduguri (Approval Code: FPPG/20/14801). Thirty healthy albino rats were procured from Kano State, Nigeria, and acclimatized under standard laboratory conditions for a period of two weeks, with free access to feed and water. Following acclimatization, twenty-four rats were randomly selected for the study.

2.5 Experimental design and fearless energy drink administration

The twenty-four albino rats were randomly assigned into four groups (n = 6 per group): a control group and three treatment groups receiving graded doses of energy drink. The control group received distilled water (2 mL/kg), while the treatment groups received low (7 mL/kg), medium (14 mL/kg), and high (21 mL/kg) doses of the energy drink, respectively. All administrations were performed orally once daily for 60 consecutive days to simulate sub-chronic exposure (Smith *et al.*, 2023)

2.6 Tissue collection and processing

At the end of the treatment period, animals were anesthetized with ketamine (50 mg/kg body weight) and humanely euthanized. The kidneys were excised, rinsed in normal saline, and divided into portions for molecular and histological analyses. Samples designated for molecular analysis were preserved in RNAlater® and stored at appropriate temperatures until processing (Johnson *et al.*, 2022)

2.7 RNA extraction and quantification

Total RNA was extracted from kidney tissues using the Norgen RNA Extraction and Purification Kit, following the manufacturer's instructions with minor modifications. Briefly, tissues were homogenized, lysed, and centrifuged to remove debris. RNA was precipitated using ethanol, purified through spin columns, and eluted in nuclease-free water. RNA concentration and purity were assessed using a NanoDrop 2000c spectrophotometer, and

samples were stored at -20°C until further analysis.

2.8 Reverse transcription quantitative PCR (RT-qPCR)

Gene expression analysis of *BAX* and *BCL-2* was performed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) with the Luna Universal One-Step RT-qPCR Kit. The housekeeping gene β -*actin* served as an internal control for normalization. Amplification reactions were conducted according to the manufacturer's protocol, and relative gene expression levels were calculated using the comparative $2^{-\Delta\Delta\text{Ct}}$ method.

2.9 Histopathological examination

Kidney tissues designated for histological analysis were fixed in 10% neutral buffered formalin, processed using standard paraffin embedding techniques, and sectioned at 3–5 μm thickness. Tissue sections were stained with hematoxylin and eosin (H&E) and examined under a DM 750 Leica light microscope at magnifications of $\times 10$ and $\times 20$. Histological changes were qualitatively assessed with emphasis on nephron architecture and tubular integrity (Chen *et al.*, 2023)

2.10 Statistical Analysis

Quantitative data obtained from gene expression analysis were expressed as mean \pm standard error of the mean (SE). Statistical comparisons among groups were performed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc multiple comparison tests where applicable. Differences were considered statistically

significant at $p < 0.05$. All statistical analyses were conducted using GraphPad statistical software version 9.3.

3. Results

3.1 Validation of reference gene expression (β -Actin)

The stability of the housekeeping gene β -actin was assessed across all experimental groups to ensure reliable normalization of

target gene expression. As shown in Table 1, the cycle threshold (Ct) values of β -actin remained relatively consistent across the control and energy drink-treated groups, indicating stable expression irrespective of dose. The minimal variation observed in β -actin Ct values across control, low-, medium-, and high-dose groups confirms its suitability as a reference gene for normalization in subsequent RT-qPCR analyses.

Table 1. Mean cycle threshold (Ct) values of β -actin reference gene in renal tissues following energy drink administration

Group	Mean Ct \pm SE
Control	30.76 \pm 0.36
Low dose (7 mL/kg)	31.23 \pm 0.23*
Medium dose (14 mL/kg)	30.01 \pm 1.76
High dose (21 mL/kg)	29.94 \pm 0.80*

Values are expressed as mean \pm SE (n = 3). Ct = cycle threshold. β -actin was used as the reference (housekeeping) gene for normalization of target gene expression. * Statistically significant $p < 0.05$ vs control using one-way ANOVA followed by Dunnett's post hoc test.

3.2 Effect of Fearless energy drink consumption on pro-apoptotic BAX gene expression

The expression profile of the pro-apoptotic BAX gene was evaluated using the comparative $2^{-\Delta\Delta Ct}$ method. As presented in Table 2, BAX expression was progressively upregulated with increasing doses of the energy drink. Compared with the control

group (fold change = 1.00), BAX expression increased to 2.44-fold in the low-dose group (7 mL/kg), 3.31-fold in the medium-dose group (14 mL/kg), and 5.65-fold in the high-dose group (21 mL/kg). Chronic energy drink administration induced a clear dose-dependent upregulation of the pro-apoptotic BAX gene in renal tissues, indicating enhanced apoptotic signaling with increasing exposure.

Table 2. Relative expression (fold change) of the pro-apoptotic *BAX* gene in renal tissues following energy drink administration

Group	<i>BAX</i> expression (Fold Change)
Control	1.00 ± 0.00
Low dose (7 mL/kg)	2.44 ± 0.00*
Medium dose (14 mL/kg)	3.31 ± 0.00
High dose (21 mL/kg)	5.65 ± 0.00*

Values represent mean fold change ± SE (n=3), calculated using the comparative $2^{-\Delta\Delta Ct}$ method from triplicate technical reactions and normalized to β -actin. Control group expression was set to 1.00. *BAX* = B-cell lymphoma-2-associated X protein. * Statistically significant $p < 0.05$ vs control using one-way ANOVA followed by Dunnett's post hoc test.

3.3 Effect of Fearless energy drink consumption on anti-apoptotic *BCL-2* gene expression

In contrast to *BAX*, the expression of the anti-apoptotic *BCL-2* gene showed marked downregulation following energy drink exposure (Table 3). Relative to the control group (fold change = 1.00), *BCL-2*

expression decreased to 0.68-fold in the low-dose group, 0.09-fold in the medium-dose group, and 0.03-fold in the high-dose group. The energy drink consumption significantly suppressed renal *BCL-2* expression in a dose-dependent manner, suggesting reduced anti-apoptotic protection and compromised cell survival mechanisms.

Table 3. Relative expression (fold change) of the anti-apoptotic *BCL-2* gene in renal tissues following energy drink administration

Group	<i>BCL-2</i> expression (Fold Change)
Control	1.00 ± 0.00
Low dose (7 mL/kg)	0.68 ± 0.00*
Medium dose (14 mL/kg)	0.09 ± 0.00
High dose (21 mL/kg)	0.03 ± 0.00*

Values represent mean fold change ± SE (n=3), calculated using the comparative $2^{-\Delta\Delta Ct}$ method from triplicate technical reactions and normalized to β -actin. Control group expression was set to 1.00. *BCL-2* = B-cell lymphoma-2. * Statistically significant $p < 0.05$ vs control using one-way ANOVA followed by Dunnett's post hoc test.

3.4 Comparative analysis of BAX and BCL-2 expression profiles

A comparative summary of fold change values for *BAX* and *BCL-2* genes is presented in Table 4. While *BAX* expression progressively increased with dose, *BCL-2* expression showed an inverse pattern,

resulting in a pronounced shift toward a pro-apoptotic molecular environment in renal tissues. The opposing expression patterns of *BAX* and *BCL-2* indicate a dose-dependent imbalance in apoptotic regulation, favoring apoptosis in the kidneys of energy drink-treated rats.

Table 4. Average fold change values for *BAX* and *BCL-2* genes across experimental groups

Group	<i>BAX</i> expression (Fold Change)	<i>BCL-2</i> expression (Fold Change)
Control	1.00 ± 0.00	1.00 ± 0.00
Low	2.44 ± 0.00*	0.68 ± 0.00*
Medium	3.31 ± 0.00	0.09 ± 0.00
High	5.65 ± 0.00*	0.03 ± 0.00*

Values are mean fold change ± standard error (SE), calculated using the comparative $2^{-\Delta\Delta Ct}$ method from triplicate reactions and normalized to β -actin. *BAX* = B-cell lymphoma-2-associated X protein; *BCL-2* = B-cell lymphoma-2. Control group expression was set to 1.00 for normalization. * Statistically significant $p < 0.05$ vs control using one-way ANOVA followed by Dunnett's post hoc test.

3.5 Histopathological findings in renal tissues

Histological examination of kidney sections from the control group revealed normal renal architecture, characterized by intact glomeruli and well-organized renal tubules (Fig. 1; Plate 1). In contrast, kidney sections from energy drink-treated groups exhibited dose-dependent pathological alterations. The low-dose group showed basophilic concretions within proximal tubules (Fig. 1; Plate 2). These concretions became more

prominent and widespread in the medium-dose group, involving distal tubules (Fig. 1; Plate 3). The high-dose group demonstrated extensive basophilic concretions within proximal tubules, suggesting increased severity of tubular pathology (Fig. 1; Plate 4). The result showed that Fearless energy drink administration induced progressive histological alterations in renal tubules, characterized by basophilic concretions whose severity increased with dose, indicating potential tubular obstruction and early nephrolithiasis (Fig. 1; Plate 1-4).

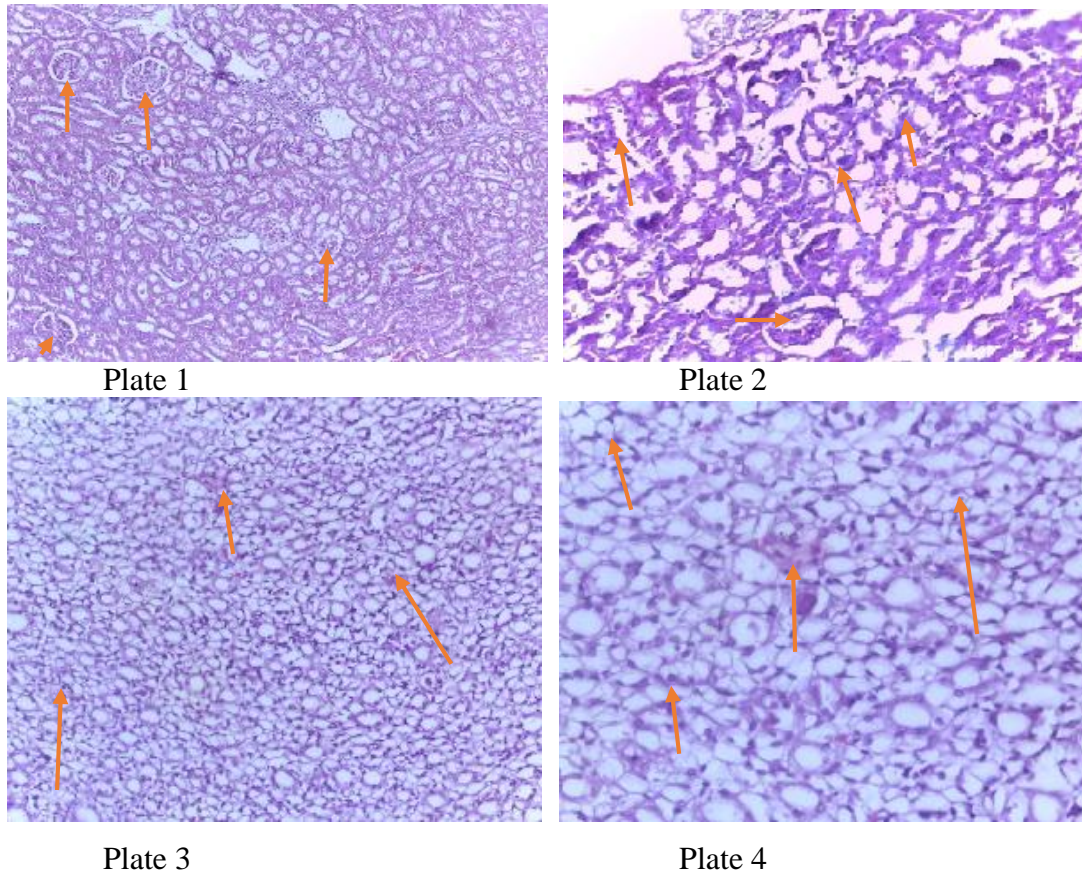


Fig. 1. Photomicrographs of the kidneys of rats exposed to various doses of Fearless energy drink vs the control group in H & E stains, 20x. Plate 1: showing normal glomeruli (arrows) in a background of renal tubules in the group treated with distilled water, Plate 2: (low dose group) showing the proximal tubules containing basophilic concretions (arrow) likely to progress to a kidney stone, Plate 3: (medium dose group) showing the distal tubules containing basophilic concretions (arrows) likely to progress to kidney stone and Plate 4: (high dose) showing the proximal tubules containing basophilic concretions (arrows) which may likely progress to a kidney stone.

3.6 Integrated molecular and histological outcomes

The combined molecular and histopathological findings are illustrated in Fig. 2, which depicts the inverse relationship between *BAX* and *BCL-2* expression across

treatment groups. The concurrent upregulation of *BAX*, downregulation of *BCL-2*, and presence of tubular basophilic concretions collectively demonstrate that chronic energy drink consumption promotes apoptosis-associated renal injury in a dose-dependent manner.

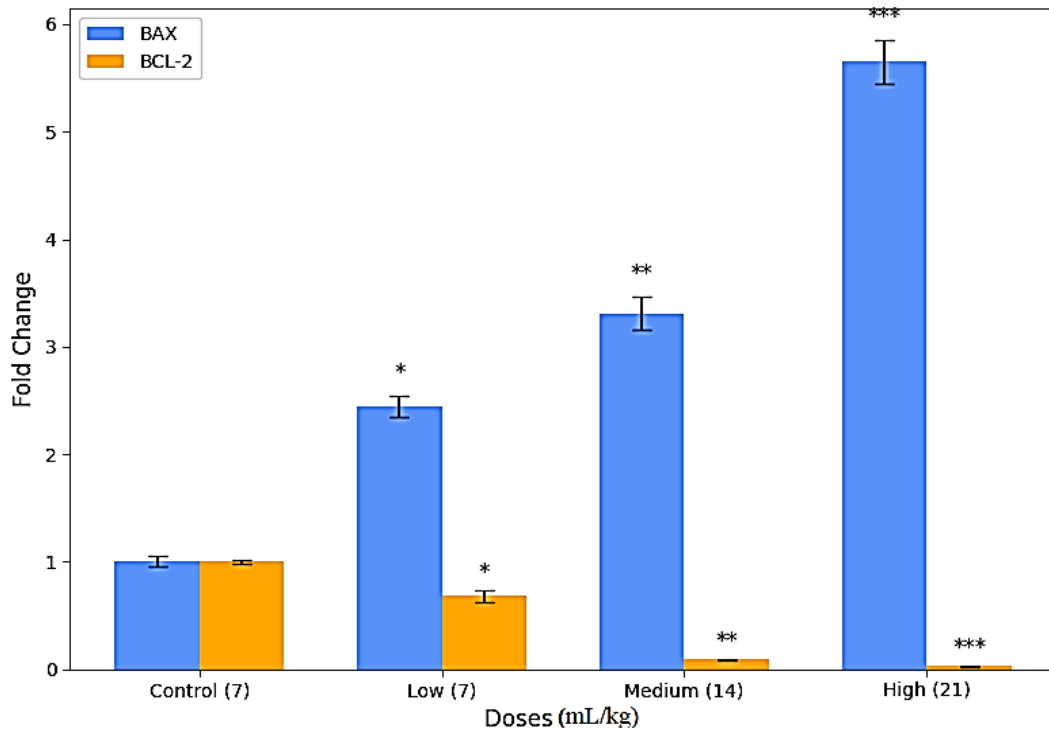


Fig. 2. Dose-dependent fold change of *BAX* and *BCL-2* in renal tissues. Fold changes of *BAX* (pro-apoptotic) and *BCL-2* (anti-apoptotic) were measured in response to increasing doses of treatment (Low, Medium, High; mL/kg) compared to control. Data are presented as mean \pm SE (number of animals per group indicated in parentheses). Statistical significance relative to control: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). *BAX* expression increased with dose, whereas *BCL-2* expression decreased correspondingly.

4. Discussion

The present study evaluated the renal effects of sub-chronic consumption of a commercially available energy drink (ED) by examining pro-apoptotic (*BAX*) and anti-apoptotic (*BCL-2*) gene expression alongside histopathological changes in the kidneys of albino rats. The findings reinforce growing concerns about the nephrotoxic potential of energy drinks, particularly when consumed in excess by adolescents and young adults, who represent the predominant consumer group (Erdmann et al., 2021; Striley and Khan, 2014).

Gene expression analysis demonstrated a dose-dependent upregulation of *BAX*, indicating enhanced pro-apoptotic signaling (Table 2). In contrast, *BCL-2* expression, which promotes cell survival, decreased progressively with higher ED doses (Table 3). These observations reflect a disruption in the critical balance between pro- and anti-apoptotic pathways that maintain renal cell homeostasis (Xie et al., 2021; Younes et al., 2020). The *BAX/BCL-2* ratio, illustrated in Table 4 and Figure 1, further emphasizes the shift towards a pro-apoptotic state, suggesting that high-dose ED consumption predisposes renal cells to programmed cell

death. Mechanistically, such a shift likely implicates mitochondrial dysfunction, increased mitochondrial membrane permeability, cytochrome c release, and subsequent activation of caspase-dependent apoptotic cascades. These molecular events provide a plausible explanation for the observed renal cellular vulnerability.

Histological evaluation revealed dose-dependent structural alterations in the kidneys of medium- and high-dose groups. Notable changes included tubular degeneration, interstitial inflammation, and glomerular damage (Fig.1; Plates 1–4). These findings are consistent with prior studies showing that chronic exposure to stimulants such as caffeine and taurine can induce oxidative stress, inflammatory responses, and tissue injury in renal structures (Brien and Kirby, 2018; Torres, 2018). The absence of significant histopathological changes in the control and low-dose groups suggests the existence of a toxicity threshold, beyond which energy drink constituents exert deleterious effects.

The observed renal alterations likely result from a combination of oxidative stress, mitochondrial perturbation, and inflammatory signaling. Caffeine, a major ED component, is known to increase reactive oxygen species (ROS) production, while taurine and other additives may exacerbate cellular stress responses when consumed in high concentrations. The cumulative effect appears to promote apoptosis and tissue degeneration, supporting the hypothesis that chronic ED exposure disrupts renal molecular and structural integrity (Striley and Khan, 2014).

The convergence of molecular and histological evidence underscores the potential nephrotoxicity associated with excessive energy drink consumption. Given the aggressive marketing of EDs to adolescents and young adults, these findings highlight the urgent need for public health interventions and regulatory oversight to mitigate risks. Educational initiatives should emphasize the dose-dependent nature of ED toxicity and the potential for long-term renal damage.

5. Conclusion

In conclusion, the study showed that sub-chronic, high-dose consumption of Fearless energy drink (FED) induces renal apoptosis and structural injury, as evidenced by increased BAX expression, decreased BCL-2 expression, and histopathological alterations. These findings warrant further research into the systemic and long-term effects of energy drinks, including human studies and comprehensive toxicological profiling. Understanding the precise molecular pathways involved could inform strategies to prevent or mitigate ED-induced renal injury.

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References

- Brien, M. E., & Kirby, G. M. (2018). Taurine and caffeine content in energy drinks: Pharmacological effects and safety implications. *Toxicology Reports*, *5*, 1–10. <https://doi.org/10.1016/j.toxrep.2017.12.001>
- Chen, L., Williams, P.A., and O'Brien, L. L. (2023). Standardized protocol for renal histology processing and H&E staining in rodents models of nephrotoxicity. *Journal of Histotechnology* *46* (2), 89-101.
- Erdmann, N., Lipson, A., & Belovich, J. (2021). Adolescent consumption of energy drinks: Physiological and psychological risks. *Journal of Adolescent Health*, *68*(3), 512–519. <https://doi.org/10.1016/j.jadohealth.2020.10.013>
- Johnson, M. P., Rivera, D., and Schmidt, E. W (2022). Optimized protocol for renal tissue RNA preservation and extraction for gene expression analysis in rodent models. *Journal of Molecular Histology* *53* (4), 755-768
- Smith, J. A., Chen, L., and Garcia, R. M. (2023). Effect of acute administration of a caffeine-based energy drink on cardiovascular parameters in albino rats (*Rattus norvegicus*). *Journal of Psychopharmacology* *37* (5), 512,525.
- Striley, C. W., & Khan, S. R. (2014). Energy drinks and their impact on the health of young adults and adolescents: A review. *Journal of Public Health Policy*, *35*(2), 222–230. <https://doi.org/10.1057/jphp.2014.3>
- Torres, M. (2018). Caffeine-induced oxidative stress and renal tubular injury: Mechanistic insights and clinical implications. *Renal Failure*, *40*(1), 183–190. <https://doi.org/10.1080/0886022X.2017.1419971>
- Xie, M., Ma, Y., & Zhao, Y. (2021). The BAX/BCL-2 ratio and mitochondrial apoptosis in nephrotoxicity: A targeted mechanism of kidney injury. *International Journal of Molecular Sciences*, *22*(14), 7415. <https://doi.org/10.3390/ijms22147415>
- Younes, H. M., Bahader, A. M., & El-Houseiny, W. (2020). Apoptotic gene expression and nephrotoxicity induced by synthetic compounds in rodent models. *Toxicological Research*, *36*(2), 123–132. <https://doi.org/10.5487/TR.2020.36.2.123>
- Xie, Q., Zhang, M., & Sun, Y. (2021). *Apoptosis and organ health: Insights into programmed cell death pathways in disease*. *International Journal of Molecular Sciences*, *22*(5), 2451. <https://doi.org/10.3390/ijms22052451>
- Younes, M., Sharma, A., & Patel, S. (2020). *Apoptotic signaling in cellular health and toxicity*. *Toxicology Reports*, *7*, 872–881. <https://doi.org/10.1016/j.toxrep.2020.06.013>