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Energy drinks induce adverse histopathological changes in gastric and duodenal mucosae of rats

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ABSTRACT

Health disorders associated with energy drinks have been reported. Previous studies included the effects of energy drinks on several organs such as liver, kidney, and brain. However, histopathological studies on stomach and duodenum are relatively scarce. This study was performed to investigate the histopathological effects of prolonged excessive consumption of Red Bull, one of the most consumed energy drinks, on the mucosae of stomach and duodenum of adult Wistar rats. Material and Methods: Thirty male Wistar rats, with average body weight of 150-200 g, were randomly allocated into two groups; an experimental group that received Red Bull with a daily oral dose of 1.071 ml/100 g of body weight for three months and a control group that received an equivalent dose of distilled water for the same duration. At the end of the experiment, all the animals were euthanized. Stomach and duodenum were dissected and processed for histopathological and immunohistochemical studies. The gastric and duodenal mucosae of Red Bull group showed marked mucosal atrophy, loss of intestinal villi and crypts, mononuclear inflammatory cellular infiltration, and increased apoptosis. The present study demonstrated the adverse histopathological changes in the mucosae of stomach and duodenum produced by prolonged excessive consumption of Red Bull in rats.

1. Introduction

Energy drink consumption has continued to gain popularity all over the world. These drinks are marketed for young people as natural alternatives that increase fun and improve physical and cognitive performance such as concentration, attention, and alertness (Kaminer, 2010).

Energy drinks are non-alcoholic, often lightly carbonated beverages that are designed to give the consumer a burst of energy by the addition of a number of energy-enhancing ingredients, most notably caffeine (Akande and Banjoko, 2011). They are usually formulated to give the consumer an energy jolt by using a combination of methylxanthines, B vitamins complex, and exotic herbal ingredients. They commonly include caffeine, other plant-based stimulants (guarana, ephedrine, yerba mate), simple sugars (glucose, fructose), amino acids (taurine, carnitine, creatine), herbs (various forms of ginseng, ginkobiloba), maltodextrin, inositol and glucuronolactone (a naturally occurring glucose metabolite) (Alford et al., 2001).

Red Bull is considered the most popular energy drink. The company's claim that Red Bull ''gives you wings'' suggests that consumption of their product will provide the consumer with more energy and enhanced performance, both mentally and physically (Ragsdale et al., 2010). The most common ingredient in Red Bull is caffeine, which is often combined with taurine, glucoronolactone, guarana and B vitamins (Reissig et al., 2009).

As the popularity of energy drinks continues to rise, it is important to consider their potential adverse effects. There are few studies on the effects of the energy drinks on various systems of the body. Alterations in the cardiovascular and hemopoietic

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systems (Higgins et al., 2010; Ragsdale et al., 2010; Worthley et al., 2010; Khayyat et al., 2012) as well as neurologic complications (Babu et al., 2011; Wolk et al., 2012) have been reported following energy drinks consumption. Regarding Red Bull, one study reported enhancement of the mood state, as well as physical and psychomotor performance (motor reaction, concentration, work, memory, and subjective sensation of alertness and vigor) after Red Bull consumption (Alford et al., 2001). Changes in the structure and functions of secretory glands (Mubarak, 2012) were also reported. To the best of the authors' knowledge, previous studies about the histopathological effects of Red Bull on the stomach and duodenum are relatively scarce in the literature. Therefore, the aim of the present study was to investigate the histopathological effects of Red Bull consumption on the mucosae of stomach and duodenum of male rats.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats (supplied from the Animal Care Centre, College of Medicine, King Saud University), weighing 150-200 g, were used in this study. They were housed under standard environmental conditions with free access to standard pelleted rat chow and tap water ad libitum. The study was conducted in accordance with the research ethics standards established by the guide for the care and use of laboratory animals of the College of Medicine Research Centre (CMRC) at King Saud University. All animals were housed in suitable plastic cages, at a controlled temperature (25 ± 2 C°) with fixed 12-hour light-dark cycles for one week before the start of the experiment for acclimatization.

2.2. Experimental groups

Thirty animals were randomly allocated into 2 groups of 15 rats each:

Group I (control group): Animals were given distilled water, using oropharyngeal metallic curved tube, with a daily dose of 1.071 ml/100 g of body weight (B.W.) for three months.

Group II (Red Bull group): Animals were given Red Bull, using oropharyngeal metallic curved tube, with a daily dose of 1.071 ml/100 g B.W. for three months (Ferreira et al., 2004).

Each can of Red Bull (Red Bull; Taurine drink, filled in Switzerland by Rauch Trading AG for Red Bull GmbH, Austria) contains 250 ml of fluid. Ingredients include water, glucose, sucrose, sodium citrate, carbon dioxide, taurine (0.4%), caffeine (0.03%), glucuronolactone (0.24%), inositol (0.02%), B vitamins (B3, B5, B6, B12), and flavors.

At the end of the experiment, all animals were euthanized. The stomach and duodenum were excised and processed for histopathological and immunohistochemical studies.

2.3. Histopathology

The excised stomach was opened, cleaned, and photographed to show macroscopic changes of its wall. The duodenum was flushed with normal saline to clean its interior. Stomach and duodenum were fixed in 10% buffered formalin at 4°C for 48 hours and processed to prepare 5-µm-thick paraffin sections. These sections were stained with hematoxylin and eosin (HandE), and Periodic Acid Schiff (PAS).

2.4. Immunohistochemistry

Stomach and duodenum 5-µm-thick paraffin sections were immunostained with cleaved caspase-3 (Asp 175, IHC Detection Kit, apoptosis marker, Cell Signaling Technology, product number: 8120, Danvers, MA, USA) (Ohira et al., 2014). These sections were deparaffinized and rehydrated. For antigen unmasking, the sections were immersed in boiling 0.01 M sodium citrate buffer solution (pH 6.0). The endogenous peroxidase activity was quenched by peroxidase quench, and then the sections were incubated in the diluted primary antibody for one hour at room temperature, while the negative control sections were incubated in the washing buffer only. Then, they were incubated in biotinylated secondary antibody followed by avidin-biotin (AB) reagent. To demonstrate the antigen-antibody reaction, the sections were incubated in substrate chromogen, diaminobenzidine (DAB). The sections were counterstained with Harris hematoxylin, then dehydrated, cleared and mounted.

2.5. Image analysis

High-resolution whole-slide digital scans of all stained histological sections were created with Aperio ScanScope scanner (Leica Microsystems, Germany). The digital slide images were viewed and analyzed using Aperio ImageScope software (Leica Microsystems, Germany). Using the linear measurement tool, mucosal thickness was measured at five randomly chosen points on each slide image. Goblet cells (in PAS-stained sections) and caspase positive cells (in caspase-immunostained sections) were counted in 5 randomly chosen fixed areas of 0.49 mm^2 using the Manual Tag feature of Image-Pro Plus software (Media Cybernetics, Inc.). The analysis output results were then exported to Excel sheets and subjected to statistical analysis.

2.6. Statistical analysis

Data collected were subjected to statistical analysis using IBM SPSS Statistics version 22 software. The homogeneity of the obtained numerical data was first checked with Levene test and the homogeneity of variance assumption has been met. Independent samples T-test was used for comparison between the two studied groups.

Differences were considered significant when P was equal to or less than 0.05. A 95% confidence level was used to calculate a confidence interval (CI), which is a range of values around the mean where the "true" (population) mean, can be expected to be located, with 95% certainty.

3. Results

3.1. Body weight

The gain of the body weight in Red Bull group was non-significantly retarded (82.25 ±12.26%) compared to control animals (103.73 ± 9.59%) (P = 0.21) (Table 1).

Table 1: Body weight gain percentage in the studied groups

Groups	Mean ± SE	95% CI	P value
Control	103.73±9.59	77.10 to 130.36	0.21 (NS)
Test	82.25±12.26	48.22 to 116.28	

CI: confidence interval; NS: nonsignificant (P>0.05)

3.2. Stomach

The wall of the dissected opened stomach of the control group showed normal appearance, while that of Red Bull group revealed obvious shrinkage with intense congestion of the mucosa (Fig. 1).

HandE-stained sections of the fundic mucosa of stomach from control group showed the normal mucosal thickness (1053.28 ± 39.06 µm) and the normal histological architecture of surface epithelium, gastric pits, fundic glands, lamina propria, and muscularis mucosae (Figs. 2A and 2B). These sections demonstrated numerous parietal cells in the superficial part of the fundic glands and numerous peptic cells in their deeper part. However, sections from Red Bull group showed significant mucosal atrophy (561.30 ± 16.10 µm) (P = 0.00) (Table 2) and massive loss of the histological architecture of the fundic mucosa. Numerous cells of surface epithelium and fundic glands were exfoliated while others revealed marked necrosis with pyknotic nuclei. The lamina propria was infiltrated with many mononuclear inflammatory cells (Figs. 2C and 2D).

Table 2: Gastric mucosal thickness (µm) in the studied groups

Groups	Mean ± SE	95% CI	P value
Control	1053.28±39.06	964.91 to 1141.65	0.000*
Test	561.30±16.10	524.89 to 597.71	

* significant (P≤0.05)

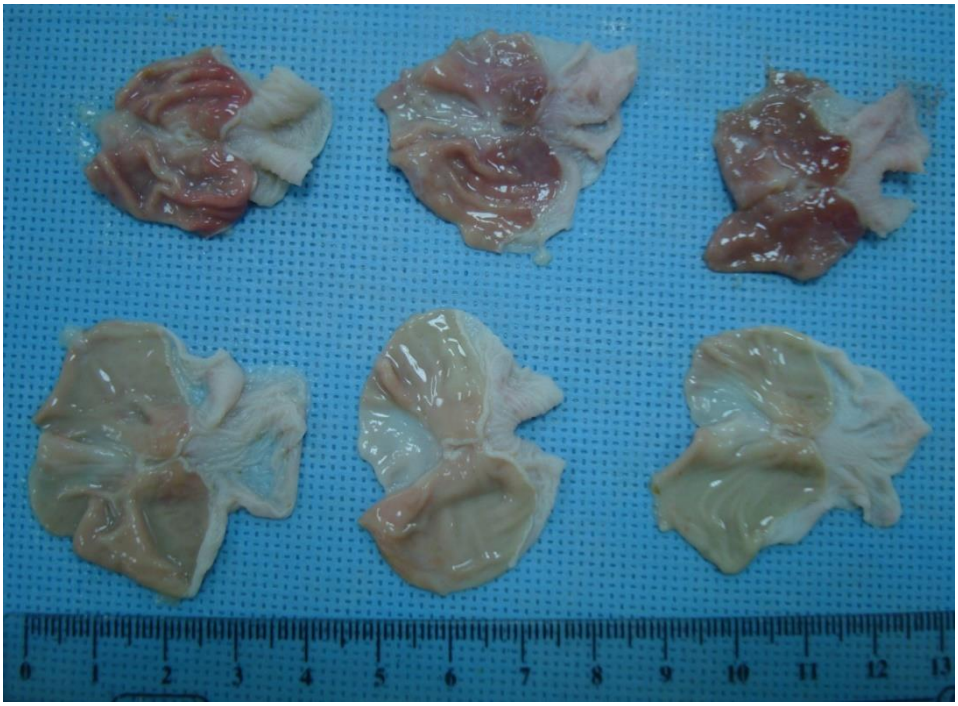

Fig. 1: Opened stomach from Red Bull group (upper row) and control group (lower row). The mucosa of stomach from Red Bull group is swollen and congested

PAS-stained sections of the mucosa from control group showed positive reaction in the mucus-secreting cells and the basement membrane of fundic glands (Figs. 3A and 3B). Sections from Red Bull group showed marked depletion of PAS-positive reaction (Figs. 3C and 3D).

Caspase-immunopostive cells in the mucosa from control group were few (51.14 ± 8.43) and located mainly in its superficial part (Figs. 4A and 4B). However, these cells in Red Bull group were significantly more numerous (120.14 ± 14.69) (P = 0.002) (Table 3), more strongly-stained, and more widely distributed all over the mucosa (Figs. 4C and 4D).

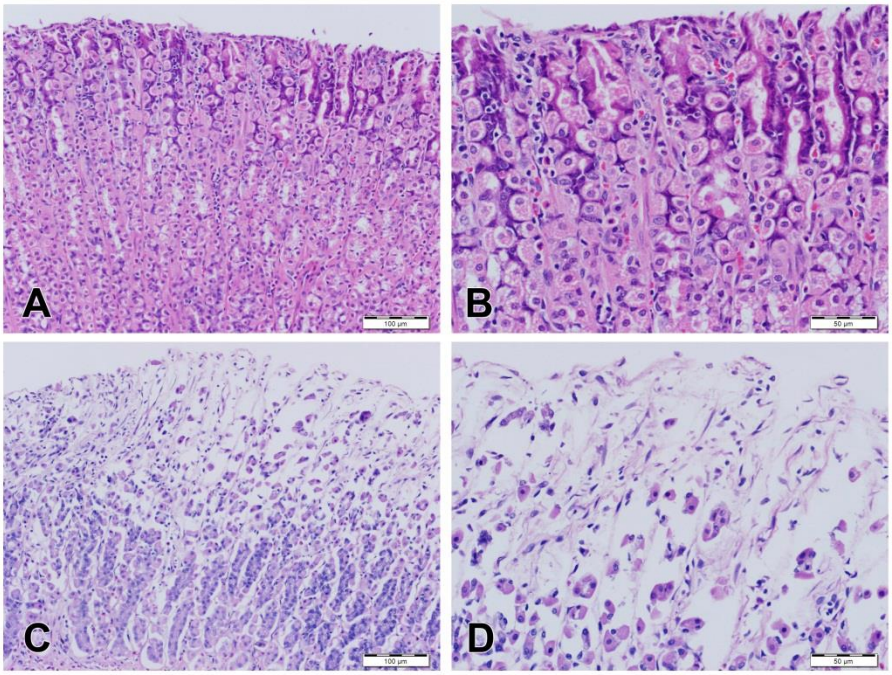

Fig. 2: H&E-stained sections of fundic mucosa of the stomach. (A) and (B) represent control group, (C) and (D) represent Red Bull group and show marked loss of fundic glands

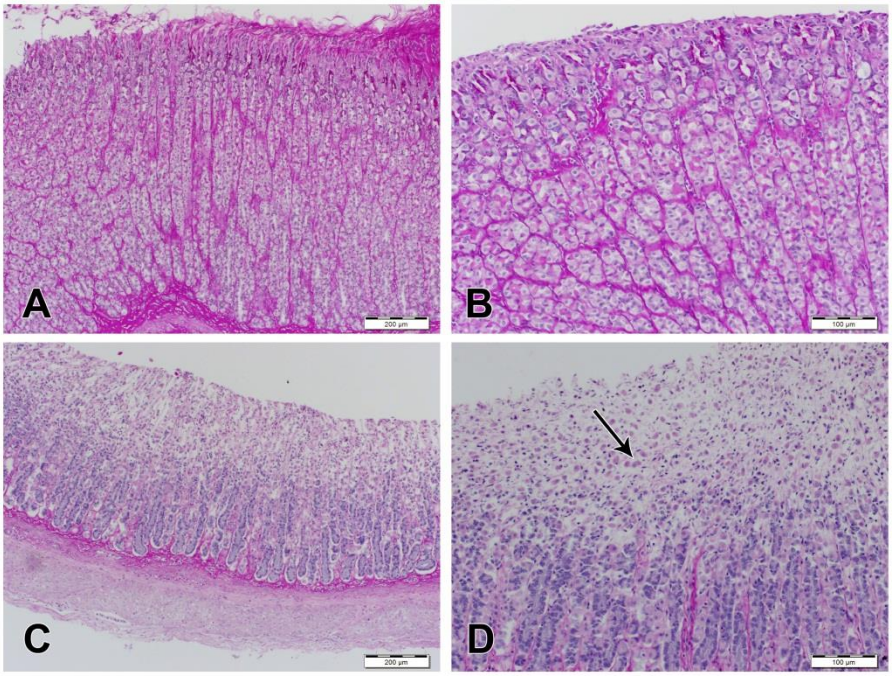

Fig. 3: PAS-stained sections of fundic mucosa of the stomach. (A) and (B) represent control group, (C) and (D) represent Red Bull group and show marked reduction of PAS-positive material especially in mucus-neck cells (arrow)

Table 3: Gastric mucosal caspase expression (area %) in the studied groups

Groups	Mean ± SE	95% CI	P value
Control	51.14±8.43	30.51 to 71.78	0.002*
Test	120.14±14.69	84.21 to 156.07	

* significant (P≤0.05)

3.3. Duodenum

HandE-stained sections of the duodenum from control group showed the normal histological architecture of duodenal mucosa with normal thickness (929.16 ± 7.65 µm). These sections revealed the intestinal villi consisting of a core of connective tissue covered by simple columnar epithelium with goblet cells (Figs. 5A and 5B). However, sections from Red Bull group showed significant mucosal atrophy (461.49 ± 18.50 µm) (*P* = 0.00) (Table 4) and marked destruction and loss of the intestinal villi and crypts with sloughing of their epithelial cells. The lamina propria revealed marked infiltration of numerous mononuclear inflammatory cells (Figs. 5C and 5D).

PAS-stained sections of the duodenum from control group showed intense positive reaction in

the apical part of numerous goblet cells (242.86 ± 8.74) (Figs. 6A and 6B). However, sections from Red Bull group showed PAS-positive reaction in significantly fewer goblet cells (78.14 ± 15.28) (P = 0.00) (Table 5) (Figs. 6C and 6D).

Table 4: Duodenal mucosal thickness (µm) in the studied groups

Groups	Mean ± SE	95% CI	P value
Control	929.16±7.64760	911.86 to 946.46	0.000*
Test	461.49±18.50332	419.63 to 503.35	

* significant (P≤0.05)

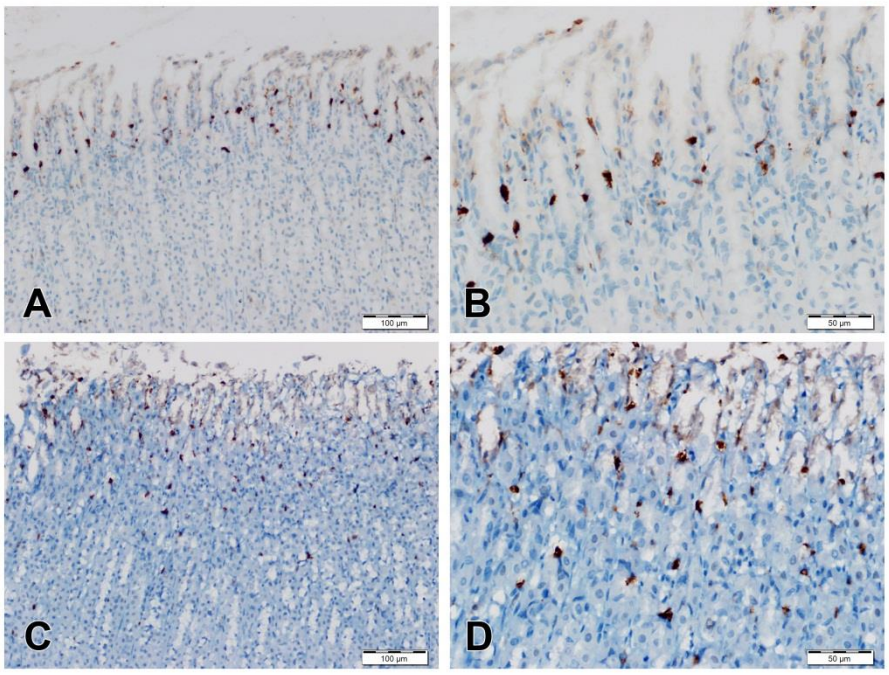

Fig. 4: Caspase-immunostained sections of fundic mucosa of the stomach, (A) and (B) represent control group and show few immunopositive cells, (C) and (D) represent Red Bull group and show more immunopositive cells

Table 5: Duodenal mucosal goblet cell number in the studied groups

Groups	Mean ± SE	95% CI	P value
Control	242.86±8.74	221.48 to 264.23	0.000*
Test	78.14±15.28	40.76 to 115.53	

* significant (P≤0.05)

Caspase-immunopositive cells in the mucosa of duodenum from control group were few (16.00 ± 2.24), limited mainly to the villi, and with weak intensity of immune reactivity (Figs. 7A and 7B). However, sections from Red Bull group showed significant increase of caspase-immunopositive cells (107.71 ± 6.68) (P = 0.00) (Table 6) that were widely distributed in the mucosa (Figs. 7C and 7D). Compared to control sections, the intensity of immune reactivity was very strong.

Table 6: Duodenal mucosal caspase expression (area %) in the studied groups

Groups	Mean ± SE	95% CI	P value
Control	16.00±2.24	10.59 to 21.47	0.000*
Test	107.71±6.69	91.34 to 124.09	

* significant (P≤0.05)

4. Discussion

Several warnings have been issued regarding the potential advers eeffects of energy drinks (Baum and Weiss, 2001; Berger and Alford, 2009; Akande and Banjoko, 2011). Red bull is one of the most consumed energy drinks. Its main ingredients include caffeine, taurine, glucuronolactone, and B vitamins (Reissig et al., 2009).

The results of the present study revealed non-significant retardation of body weight gain in the Red Bull group compared to the control group. This non-significant retardation of body weight gain was not in accordance with the massive histopathological lesions observed in the mucosae of both stomach and duodenum in Red Bull group. It might be that retardation of body weight gain was not significant because absorption was still possible through the distal intestinal mucosa, supposed to be intact. Similarly, Ebuehi et al. (2011), Ayuob and ElBeshbeishy (2016) reported no significant change in the body weight of animals that ingested energy drinks.

Macroscopically, the results revealed shrinkage and congestion of the stomach wall in Red Bull group indicating severe mucosal damage. Similarly, a previous study recorded hemorrhagic areas in submandibular salivary glands in rats treated with Red Bull (Mubarak, 2012).

In the present study, ingestion of Red Bull energy drink had adversely affected the histological structure of the rats' mucosae of stomach and duodenum. The observed effects were in the form of mucosal atrophy with loss of intestinal villi and crypts and exfoliation of epithelial cells. The mucosal atrophy and histopathological lesions of stomach and duodenum detected in the present study could be attributed to caffeine (Ayuob and ElBeshbeishy, 2016) and taurine (Huxtable, 1992) contents of Red Bull.

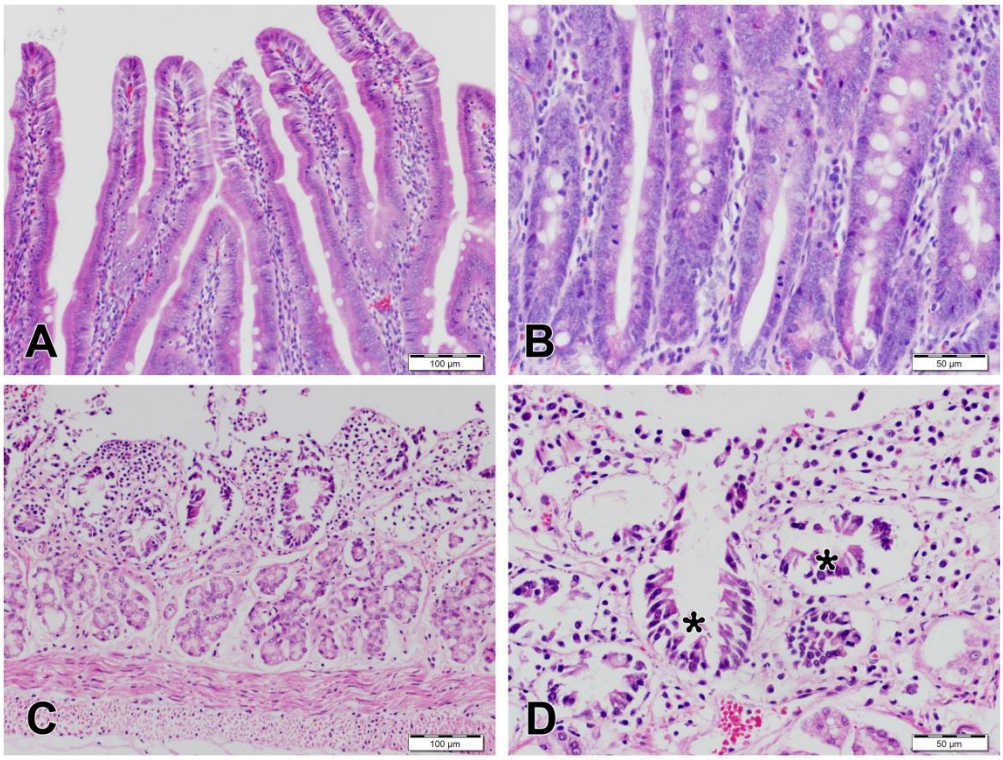

Fig. 5: H&E-stained sections of duodenal mucosa. (A) and (B) represent control group, (C) and (D) represent Red Bull group and show exfoliation of most of the cells of intestinal villi and crypts

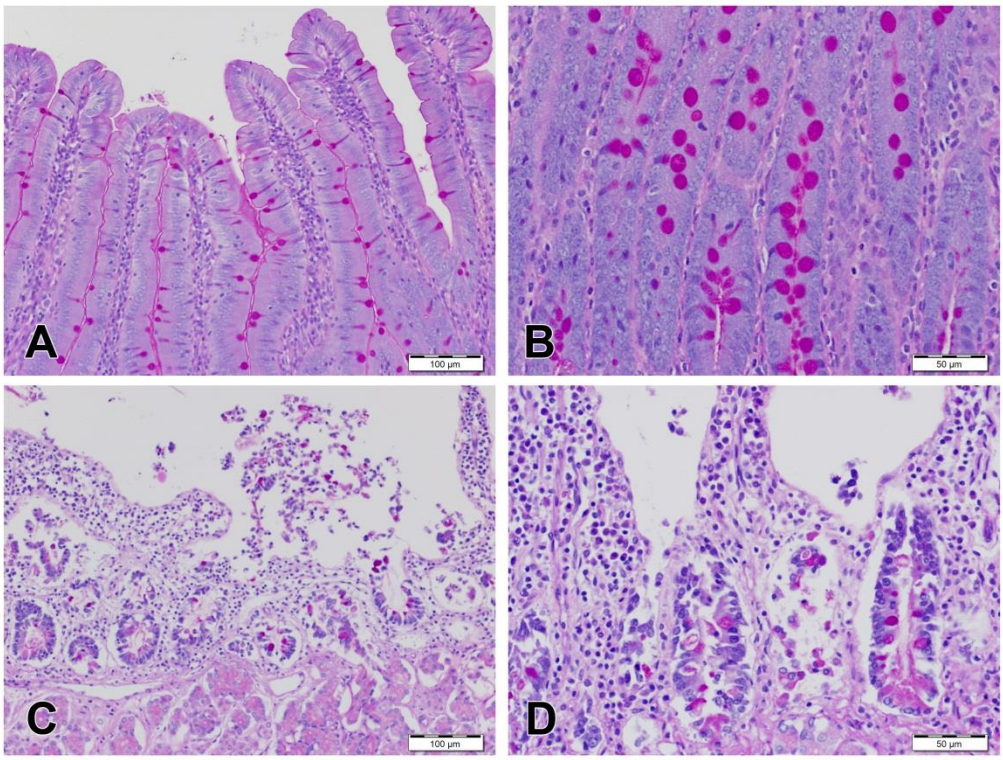

Fig. 6: PAS-stained sections of duodenal mucosa. (A) and (B) represent control group, (C) and (D) represent Red Bull group and show almost complete depletion of PAS reaction

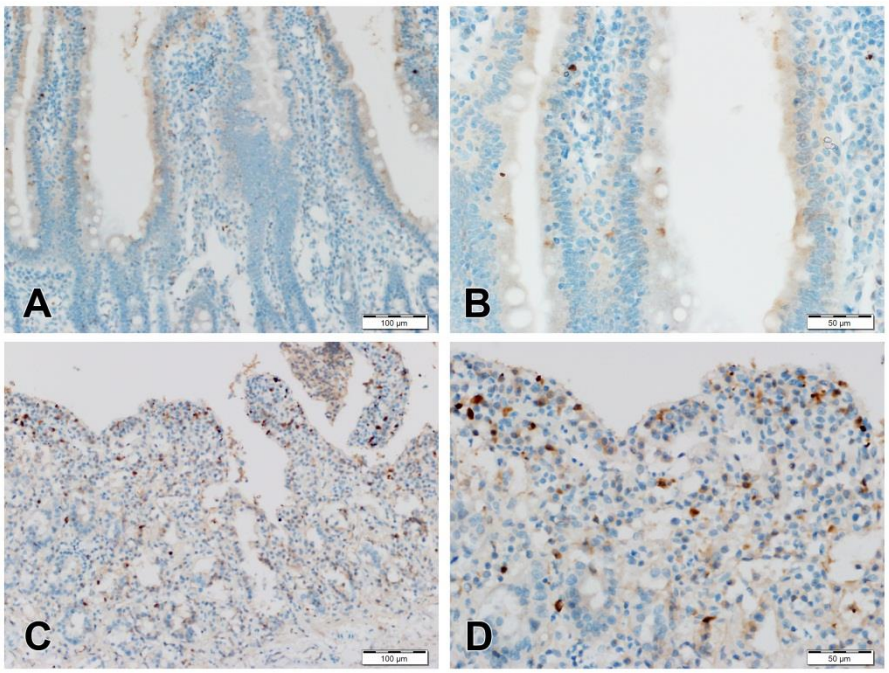

Fig. 7: (Caspase-immunostained sections of duodenal mucosa. (A) and (B) represent control group and show few immunopositive cells, (C) and (D) represent Red Bull group and show more immunopositive cells

Previous studies demonstrated histopathological lesions in different organs including pancreas and fundus of stomach (Ayuob and ElBeshbeishy, 2016), liver (Khayyat et al., 2012), submandibular salivary gland (Mubarak, 2012), and testis (Dias et al., 2015). Recently, Ayuob and ElBeshbeishy (2016) assessed the effect of chronic consumption of Power Horse, one of the energy drinks, for 4 weeks on the structure of pancreas and fundic mucosa of stomach in adult male albino rats. Their results showed degenerative histopathological changes in the fundic gastric mucosa with reduced numbers of parietal cells. They attributed these changes to the caffeine-induced elevation of tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS), which created an oxidant/antioxidant imbalance in these tissues toward increased oxidant stress.

Another study revealed that caffeine administered for 3 weeks significantly potentiated the gastric mucosal damage induced by aspirin in rats (Parmar et al., 1985). Caffeine is considered as a moderately strong stimulant of gastric acid secretion in dog and man (Cooke, 1976). It stimulates gastrin release and gastric acid secretion. It also prolongs the adaptive relaxation of the proximal stomach that might slow gastric emptying (Boekema et al., 1999). This, in turn, might prolong the duration of direct contact of Red Bull with mucosa of stomach causing the changes detected macroscopically and microscopically in such mucosa.

Khayyat et al. (2012) studied the effects of different kinds of energy drinks on rat liver. They attributed such effects to different reaction of taurine associated with caffeine. Similarly, excessive ingestion of combination of caffeine-and-taurine-containing energy drinks has been reported to produce myocardial ischemia by inducing coronary vasospasm (Berger and Alford, 2009).

Mucosae of stomach and duodenum of Red Bull group showed mononuclear inflammatory cellular infiltration. Akande and Banjoko (2011) reported an increase in urea concentration in rats following excessive energy drink consumption. They attributed such increase to caffeine through inhibition of A_2 Adenosine receptors, which, in turn, accelerates the development of interstitial inflammation, augments proteinuria and changes renal function and structure. Tofovic et al. (2002) reported that caffeine produced severe tubulo-interstitial damage in rats including tubular atrophy, presence of proteinaceous material, tubular dilatation, interstitial inflammation and interstitial fibrosis, as well as increased glomerulosclerosis.

In the present study, marked depletion of mucus secretion in the mucosae of stomach and duodenum of Red Bull group occurred, as evidenced by marked depletion of PAS-positive material. This could be attributed to the massive mucosal damage and exfoliation of epithelial cells. Our results were in accordance with Nawrot et al. (2003), who also reported that the inhibitory effect of caffeine on gastric mucosal mucus secretion may be one of the important factors of the gastric mucosal injury.

The results of the present study demonstrated a significant increase of apoptotic cells as evident by the significant increase of caspase-3-immunopositive cells in the mucosae of stomach and duodenum of the Red Bull group. Previous studies demonstrated oxidative stress-induced apoptosis of gastric epithelial cells (Schubert, 2009; Ayuob and ElBeshbeishy, 2016) and pancreatic acinar cells (Ayuob and ElBeshbeishy, 2016). Huxtable (1992) suggested an additive effect of taurine to that of

caffeine on the gastric and duodenal mucosae. He reported that taurine conjugates with bile acids and aids digestion of lipids including lipids of cell membrane. This might explain the marked cellular apoptosis observed in the mucosae of both stomach and duodenum. In addition, there were health concerns about other ingredients in energy drinks. Sodium benzoate had been claimed to be the cause of rat cellular necrosis following Red Bull excessive consumption (Mubarak, 2012). Shimizu et al. (1996) ascribed renal tubular and glomerular necrosis as a consequence of administration of energy drinks to the depletion of ATP, which finally leads to the death of the cells.

Although Red Bull energy drink produced all these unexpected histopathological changes, the consumption of energy drinks continued to gain popularity. Energy drinks could produce different effects due to the different mixture of their ingredients. Therefore, more researches on different types of energy drinks and their possible specific adverse histopathological effects on different tissues and organs are recommended.

Regarding the limitations of the study, the authors did not test the reversibility of the histopathological effects of Red Bull on gastric and duodenal mucosae and did not test the effect of Red Bull on the serum level of gastrointestinal hormones.

In conclusion, data of the present study indicated that the massive histopathological changes in the mucosae of stomach and duodenum were the results of excessive consumption of Red Bull energy drink for a long duration. Caffeine was the most blamed ingredient to produce such effects with the possibility of involvement of other ingredients such as taurine, sodium benzoate and ascorbic acid. The present results would stimulate further studies to investigate the histopathologic effect of energy drinks on jejunum, ileum and large intestine. Investigation of the optimal dosage of consumption, as well as of the reversibility of effects in case of excessive consumption would be also essential.

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Disclosure

The authors report no conflicts of interest in this work.

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