POTENTIAL CYTOTOXIC EFFECT OF CAFFEINATED AND NON-CAFFEINATED CARBONATED SOFT DRINKS ON LIVER AND PANCREASOF ADULT MALE ALBINO RATS; (BIOCHEMICAL, HISTOLOGICAL AND DNA STUDY)

BY

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ABSTRACT

Objective: The carbonated soft drinks (CSDs) consumption has markedly increased worldwide in the last decades, for both the number of servings/given time and the quantity per serving. Variable health problems have been linked to CSDs consumption. This aroused suspicion about a genotoxic effect in addition to the cytotoxicity investigated. This experimental study aimed to evaluate the possible harmful effects of caffeinated versus non-caffeinated SDs on DNA and cellular integrity. **Material and Methods**: forty adult Wistar albino rats were allowed for free intake of two groups of CSDs (caffeinated and non-caffeinated) separately for 3months, while observing their body weights. After scarification, blood biochemistry, histopathological examination, comet analysis and morphometry were done to detect statistical significance of the findings. Results; caffeinated CSDs proved statistically to have a biochemical, genotoxic, and cytotoxic effects worse than the non-caffeinated CSDs. **Conclusion**: the deleterious genotoxic and cytotoxic effects of CSDs necessitates precise measures to control their free high consumption levels.

Key words: *Domestic beverages, genotoxicity, cytotoxicity, comet assay.*

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INTRODUCTION

Carbonated soft drinks (CSDs) represent the second most worldwide popular beverages after tea ***(Düsman, et al., 2013; Nielsen and Popkin 2004).***

The CSDs are non-alcoholic, sugar-sweetened beverages (SSB) that are formed mainly of water, phosphoric acid, carbon dioxide (0.3-0.6% w/v), antioxidants (<100 ppm), acidulants (0.05- 0.3% w/v), sweeteners (8-12%, w/v); either sugar or other substitutes (of about 150 calories/ 12 fl oz), colorings (0-70 ppm), flavorings (0.1- 0.5% w/v), in addition to some chemical preservatives (lawful limits), or even foaming agents (as saponins, up to 200 mg/mL) ***(Kregiel, 2015; Nielsen and Popkin 2004***) and many of them are caffeinated (around 35mg/12 fl oz) ***(Chou and Bell 2007; Foster 2021).***

Variable health problems have been linked to CSDs consumption, including increased risk of obesity, type 2 diabetes, metabolic syndrome, cardiovascular disease, hepatic lipogenesis, hypertension ***(Alkhedaide, Soliman and Ibrahim 2016; Ambrosini, et al., 2013; Hector, et al., 2009)***, impaired bone health ***(Mahmood, et al., 2008; Tucker, et al., 2006)***, renal impairment ***(Adjene, et al., 2010)*** dental erosion, mutagenic activity and hyperactivity ***(Düsman, et al., 2013).***

Numerous studies have shown that health hazards are due to the components used among various brands and their high consumption levels ***(Gaby 2005; Hofmann, et al., 2002; Vlassara, et al., 2002).***

It seems that high-fructose, like sucrose; raises the dietary glycemic load causing insulin resistance, impairs the function of b-cell, increases lipid peroxidation, and induces fatty liver in rats as well as it activates inflammation. ***(Ambrosini, et al., 2013; Davail, et al., 2005).***

Chronic caffeine consumption did not prove to have major negative health consequences ***(Iyadurai and Chung 2007)*** although of its mildly addictive psycho-activity through which brands benefits by inducing a physiologic and psychologic desire to consume the drink ***(Keast and Riddell 2007).*** Other components of the CSDs seem to be harmful to the genetic material ***(Choudhury and Palo 2004; Rayes 2008).***

Aim of the work:

Considering that few studies evaluated the cytotoxic and mutagenic potential of soft drinks, one of the aims of this study was to investigate the effect on DNA integrity as well as examining the presence of a significant varying effects, both histologically and biochemically, of the chronic

consumption of caffeinated CSDs versus non-caffeinated beverages on the largest two accessory digestive organs (liver and pancreas).

MATERIAL AND METHODS

- **Experimental Animals:**

Forty adult male Wister albino rats were bred for three months in Animal house and laboratories of Faculty of Medicine, Zagazig University following IACUC instructions with approval number (ZU-IACUC/3/F/79/2021). They were supplied from Zagazig Scientific and Medical Research Center (ZSMRC), Zagazig University, Al-Sharkia, Egypt.

- **Test Chemicals**

Commercially available CSDs

- **Experimental groups**

At the start of the experiment, rats' body weights were 200 ± 20g. Rats were allowed freely to access food (standard rodent food pellets) and fluids throughout the experiment. After acclimatization for 3 days, the animals were divided into three groups randomly as follows.

Control group (ten rats) was allowed for free intake of food and water. **Caffeinated CSDs Group** (of fifteen rats) were allowed for free intake of food and caffeinated CSDs.

Non- caffeinated CSDs Group (of fifteen rats) was allowed for free food intake and non- caffeinated CSDs.

- **Sampling**

All rats were weighed twice/ month. During the last day of the experiment, animals were deprived of food overnight then anaesthetized by thiopental 1%30 mg/kg BW iv.

Blood samples were taken from the retro-orbital plexus using a capillary tube.

Later, the pancreas and liver were dissected out of all rats. A snip of about one gram was taken from each liver and preserved in liquid nitrogen for further comet assay analysis. The rest of the liver and pancreas were processed to obtain slides for histopathological (H&E) and immunohistochemical examination (caspase for liver samples and anti-insulin for pancreatic samples).

- **Microscopic examination and imaging:**

All the slides (H&E and immune-stained) were examined with a light microscope (LEICA DM500, Switzerland) attached with a five megapixels Leica digital camera (ICC50 W, Switzerland) available at Image Analysis Unit of Human Anatomy and Embryology Department, Faculty of Human Medicine, Zagazig University.

- Histopathologic liver scoring**:**

According to ***(Kleiner, et al., 2005),*** the histological scoring was calculated from a group of features, including: steatosis, inflammation, and ballooning (hepatocellular injury). Concerning steatosis, score 0 = <5% affection, score 1= 5-33% affection, score 2= >33-66% affection, and score 3= >66% affection. Concerning inflammation, score 0 = no foci, score 1= < 2 foci/200X, score 2= 2-4 foci/200X and score 3= > 4 foci/200X. Concerning ballooning, score 0 = none, score 1= few, score 2= prominent.

- **Morpho-histometric study:**

The optic densities of the Caspase immunostained liver slides were calculated using image-J software to compare the intensity of protein expression among the three groups. For image analysis, the RGB images of 2 different fields of vision for 2 consecutive cuts in each specimen with high resolution saved as jpeg were obtained by light microscope (LEICA DM500, Switzerland) attached with a 5 megapixels Leica digital camera (ICC50 W, Switzerland) available at Image Analysis Unit of Human Anatomy and Embryology Department, Faculty of Human Medicine, Zagazig University at magnification 400× for each sample.

The quantification process was performed for the area of all islets of Langerhans detected in the whole field for three consecutive cuts in each specimen, 4µm thickness each, and their mean was considered as one variable. The analysis of images and the quantification of parameters were done using the image-processing software (Leica Q Win plus Image Analysis System, Leica Micros Imaging Solutions Ltd, Cambridge, UK). The results automatically appeared on the monitor as a collective report in square microns (μm^2) for the measured area.

- **Comet assay analysis:**

To assess the extent of DNA damage in the preprocessed cells by measuring the length of DNA migration and the percentage of

migrated DNA. A comet five image analysis software developed by Kinetic Imaging; Ltd. (Liverpool, UK) linked to a CCD camera was used. The analysis was done at animal reproduction research institute (ARRI), Cairo.

- **Biochemical investigation:**

To evaluate the function of both endocrinal pancreas and liver through detecting the level of random glucose, ALT, AST, ALP, bilirubin, and proteins. All investigations were done at the Clinical Pathology Department, Faculty of Medicine, Zagazig University, using Cobas c702/8000 autoanalyzer (Roche diagnostic, Mannheim, Germany) and its specified chemicals.

- **Statistical analysis:**

Statistics were conducted for body weights, histopathologic liver scoring, morpho-histometric results, comet, and biochemical investigation. Data were presented as mean ± standard error. ANOVA test was conducted to detect the significant difference among groups where the *p*-values were represented as 0.05 (*), 0.01 (**) or 0.001 (***) respectively.

RESULTS

A-Body weight:

By the end of the experiment, the amount of BW gain among groups showed highly significant increase in both caffeinated CSDs and non-caffeinated CSDs groups when compared to the control group. Furthermore, caffeinated CSDs group showed a significant increase of the body weight when compared to the non-caffeinated CSDs group **(Fig. 1).**

B-Histopathological examination:

Hematoxylin and Eosin slides:

Liver specimens:

The control group showed a normal configuration of hepatocytes arranged in sheets around the normal central vein and portal triad. The portal triad showed intact portal vein and bile duct. Sinusoids show normal Kupfer cells. **Figs. (2, Ca & Cb)**

The non-caffeinated CSDs group showed intact portal vein and bile duct. The hepatocytes were lightly stained with vacuolated cytoplasm. The hepatocytes were arranged in sheets around slightly dilated sinusoids. Dispersed minimal fatty infiltration was noticed peripherally among hepatocytes. The central vein appeared congested and surrounded by dilated sinusoids with clear Kupfer cells. **Figs. (2, NCa & NCb).**

The caffeinated CSDs group showed the portal triad with dilated portal vein. The bile duct showed pronounced proliferation. Inflammatory infiltration was detected in the portal area. The hepatocytes with lightly stained cytoplasm showed areas of fatty infiltrations in-between. Sinusoids were dilated and congested. The central vein was congested. **Fig. (2, CC)**

Table (1) showing the histopathological scoring results of the liver specimens of the three groups:

Item	Def	Score	Control (SS=10)	Caffeinated CSDS (SS=15)	Non-caffeinated CSDS (SS=15)
Steatosis***	<5%	0	8		
	5-33%	1	2	3	13
	> 33-66%	2		12	2
	> 66%	3			
Inflammation Lobular	No foci	0	0	0	0
	<2 foci/200X	1			
	2-4 foci/200X	2			
	>4 foci/200X	3			
Portal***,[1]	≤minimal	0	10	3	12
	>minimal	1		12	3
Ballooning***	None	0	10		3
	Few	1		3	10
	Prominent	2		12	2

*Ss; sample size. ***; p value < 0.001 with the other two groups. [1]; no statistical significance between the non-caffeinated and control groups.*

Table (2) showing the mean values and significance of the results of comet and optic density of the three groups

Group	Control	Caffeinated CSDs	Non-Caffeinated CSDs
Comet	1.292	2.378***	2.312***
optic density	0.3903	0.42***	0.4383*** x***

**** indicates high significant difference with the control group.*
X indicates significant difference between the two treated groups.*

Table (3) showing the mean values and significance of biochemical results of the three groups

Group	control	Caffeinated CSDs	Non-Caffeinated CSDs
Total protein (g/dl)	7.26	7.29	7.053
Albumin (g/dl)	4.372	3.973^{**}	4.061^{*}
AlP (u/l)	91.67	182^{***} x^{***}	96.67
AST (u/l)	98.97	116.63^{**} x^{***}	95.63
ALT (u/l)	41.25	57.74^{***} x^{**}	50.3^{***}
Total bilirubin (mg/dl)	0.037	0.047	0.023^{*} x^{***}
Serum glucose (mg/dl)	128.67	122.7	148.3^{**} x^{***}

****, **, * indicates high, moderate and mild significant difference with the control group.*
X indicates significant difference between the two treated groups.*

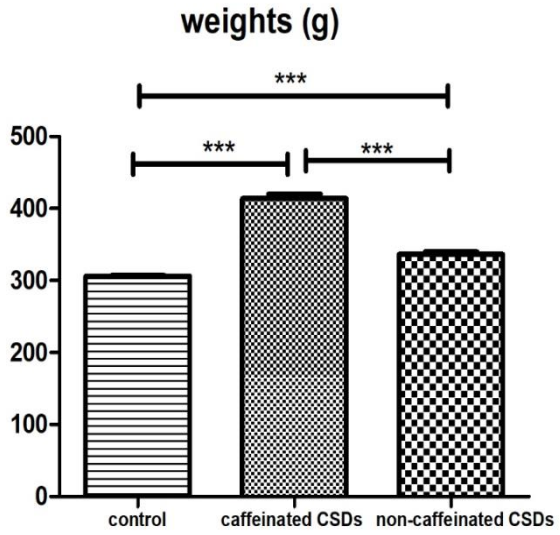

Fig. (1): *Comparison of the mean values of the body weight (in grams) of the three groups (the control, caffeinated CSDs and non-caffeinated CSDs groups) (SS of 40 albino rats) and the statistical significance of their change, showing a high statistically significant difference between the marked groups. (***) indicates a p-value less than 0.001.*

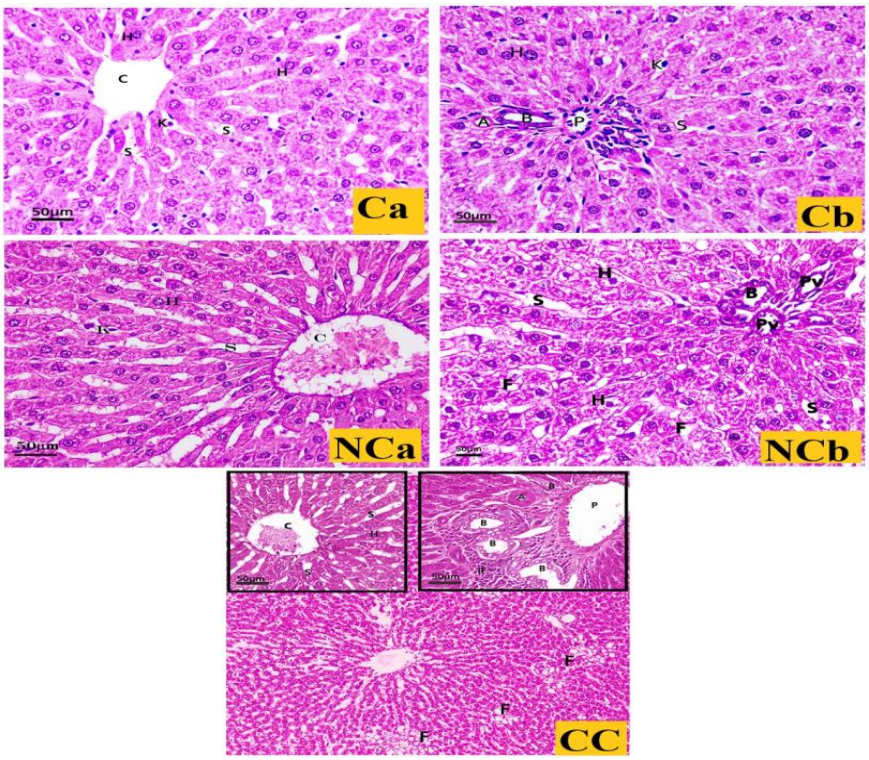

Fig. (2): *A photomicrographs of Hematoxylin and Eosin (H&E) stained liver plate representative of the histopathology of the three groups showing hepatocytes' cytoplasmic and nuclear reaction (SS of 40 albino rats. Ca and Cb x400; control group showing the portal triad and central vein (C) respectively, portal vein (P), bile duct (B), hepatic artery (A), hepatocytes (H) in arranged sheets, sinusoids (S) show Kupfer cells (K). NCa x400; non-caffeinated CSDs group showing congested central vein (C) and dilated sinusoids (S). NCb x400; non-caffeinated CSDs group showing the portal triad with their portal vessels (Pv), hepatocytes (H) with lightly stained vacuolated cytoplasm, and dispersed ballooning cells with fatty infiltration (F). CC; caffeinated CSDs group x100 showing wide areas of ballooning hepatocytes with fatty infiltration (F) and lightly stained cytoplasm, the two insets for the portal area and the central vein at x400 show the portal triad having dilated portal vein (P), bile duct (B) with pronounced proliferation, hepatic artery (A), inflammatory infiltration (IF), hepatocytes (H) some show darkly stained nucleus, dilated congested sinusoids (S), and congested central vein (C).*

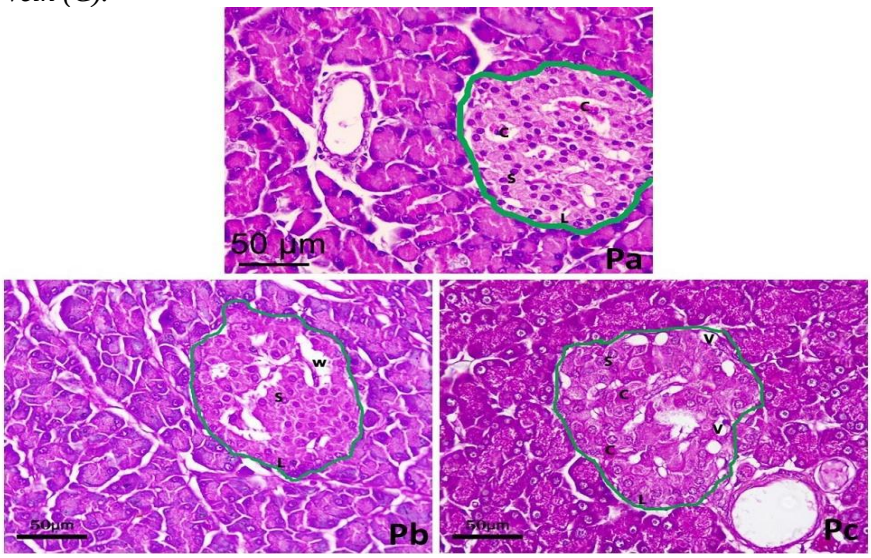

Fig. (3): *A photomicrographs of Hematoxylin and Eosin (H&E) stained pancreas plate of the three groups, (SS of 40 albino rats), showing the changes of islets of Langerhans (L) of the pancreas of the three groups. Pa; control group having normal clusters of islet cells with vesicular nuclei (S) and in-between capillaries (C). Pb; non-caffeinated CSDs group having clusters of islet cells with vesicular nuclei (S) separated by wide spaces (W) in-between. Pc; caffeinated CSDs group having clusters of islet cells with vesicular nuclei (S) and vacuolations (V). x400*

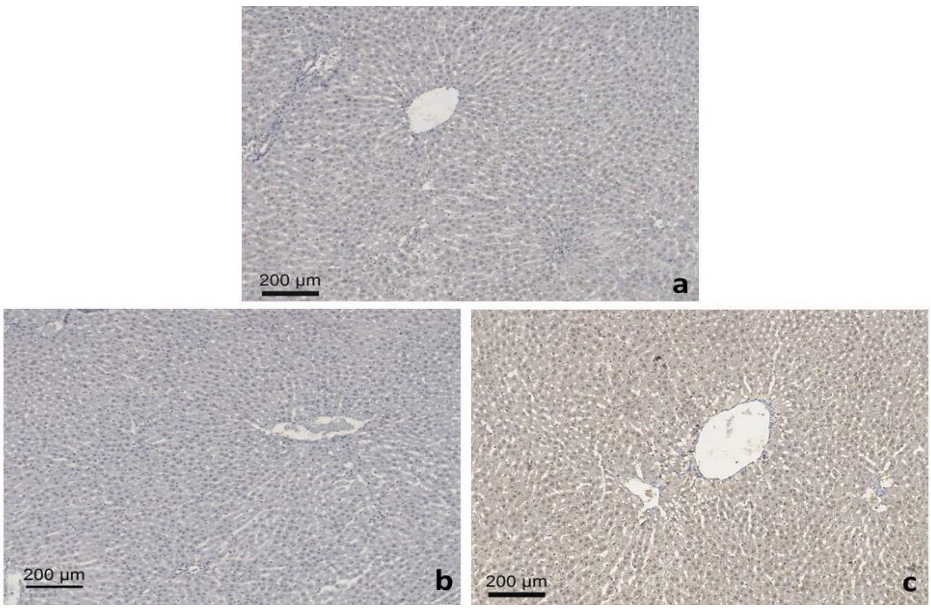

Fig. (4): *A photomicrographs of Caspase immunostaining liver plate of the three groups, showing sample of histopathological changes of the liver (SS of 40 albino rats). a; control group showing negative hepatocytes' cytoplasmic reaction. b; non- caffeinated CSDs group showing negative hepatocytes' cytoplasmic reaction, c; caffeinated CSDs group showing positive hepatocytes' cytoplasmic reaction. (Caspase immunoreactivity x100).*

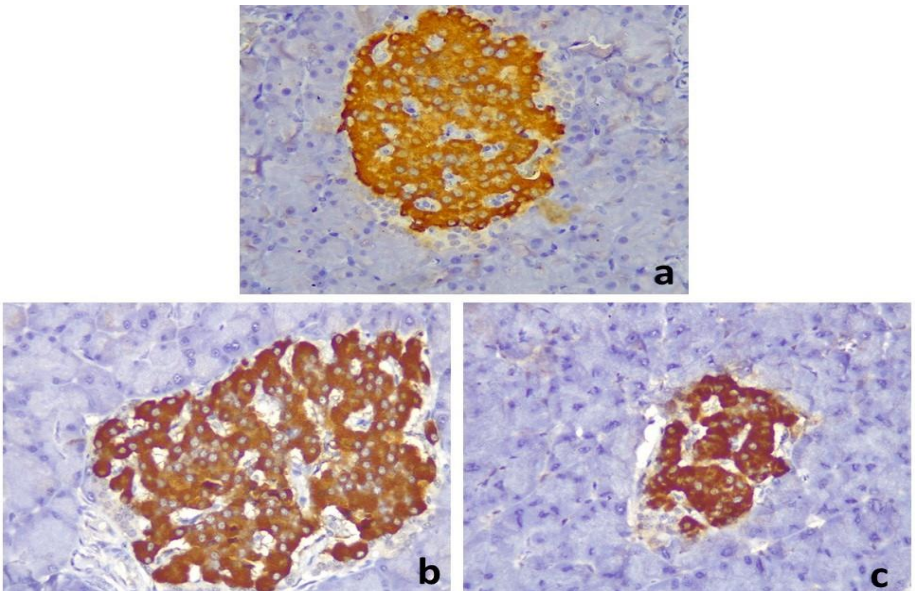

Fig. (5): *A photomicrographs of Anti-insulin immunostaining plate of the islets of Langerhans of the pancreas of the three groups (a; control, b; non- caffeinated CSDs group, c; caffeinated CSDs group), showing B cells of islets of Langerhans with strong positive reaction for the anti-insulin antibodies seen as deep, brown-stained granules in the cytoplasm (SS of 40 albino rats. The cytoplasm of non-B-cells of the islets show no reaction in any group (Anti-insulin immunoreactivity x400).*

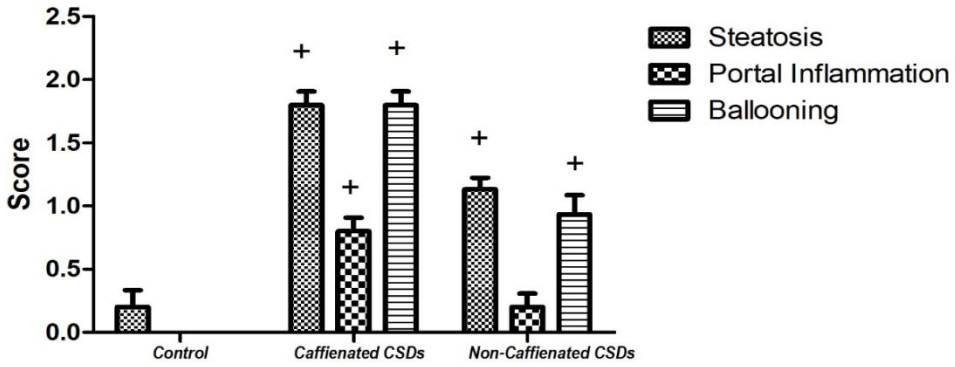

Fig. (6): *Histopathological scoring of steatosis, inflammation, and ballooning of liver specimens of the three groups, showing the mean values, standard error and the significant difference of the three groups (SS of 40 albino rats). Mean values and standard error of the histopathological scoring results showing marked differences among the three groups concerning both steatosis and ballooning, while portal inflammation shows significant difference between the caffeinated CSDs group and the other two groups, but no significance is detected between the non-caffeinated CSDs group and the control one + indicates ($p < 0.001$) with the other two groups.*

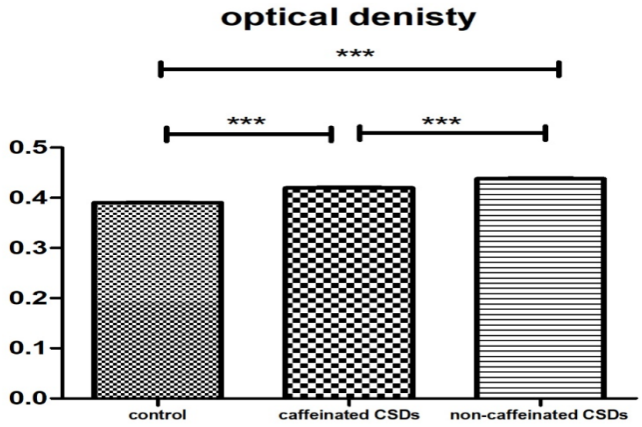

Fig. (7): *Comparison between the optical densities of the three groups (the control, caffeinated CSDs and non-caffeinated CSDs groups), regarding the mean values, standard error and the significant difference of the optical density (SS of 40 albino rats), showing a statistically significant difference between the treated groups and the control group as well as between the two treated groups. (***) indicates a p-value less than 0.001*

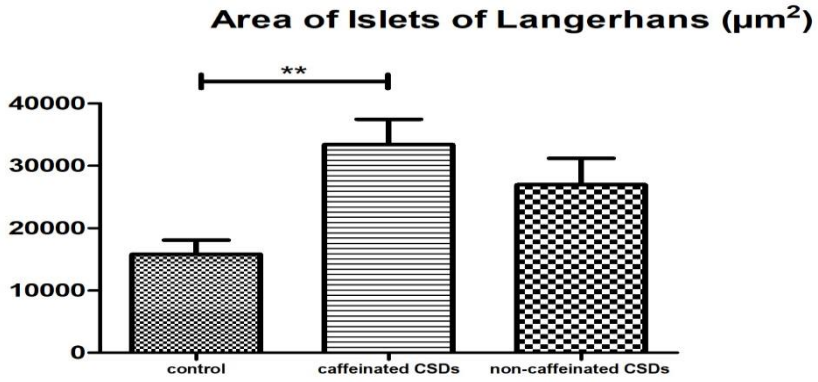

Fig. (8): *Comparison between the areas of the islets of Langerhans of the pancreas of the three groups (the control, caffeinated CSDs and non-caffeinated CSDs groups) (SS of 40 albino rats), regarding the mean values, standard error and the statistical difference among the three groups. Mean values and standard error of the surface area of Islets of Langerhans (in µm2) showing a statistically significant difference with the control group. (**) indicates a p-value less than 0.01*

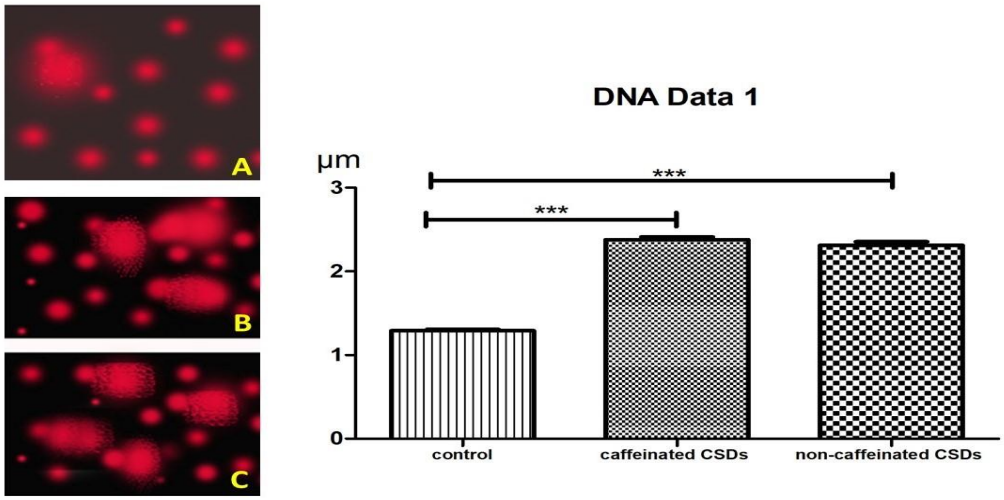

Fig. (9): *Comparison between the length of DNA migration and the percentage of migrated DNA of the liver specimens of the three groups, showing the length of DNA migration and the percentage of migrated DNA; pic. A; sample of control group, pic.B; sample of caffeinated CSDs group and pic.C; sample of non-caffeinated CSDs group. The data is represented staistically by the mean values and standard error of DNA index. There is a high statistically significant difference between the control group and both caffeinated CSDs and non-caffeinated CSDs groups, respectively. While no statistical significance is detected between caffeinated CSDs and non-caffeinated CSDs groups. (***) indicates a p-value less than 0.001.*

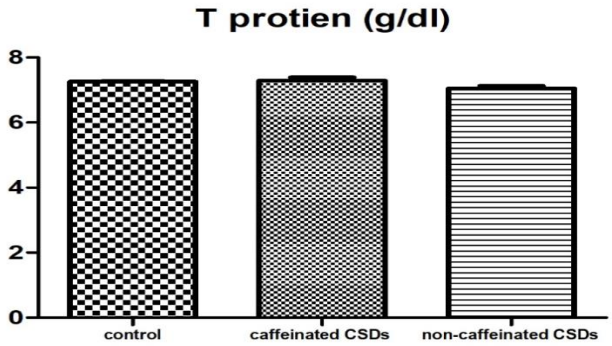

Fig. (10): *Comparison between the total protein levels of the three groups (the control, caffinated CSDs and non-caffinated CSDs groups)(SS of 40 albino rats), showing the mean values, standard error of total protein level (g/dl) and the statistical difference between the three groups wth no statistically significant difference between groups.*

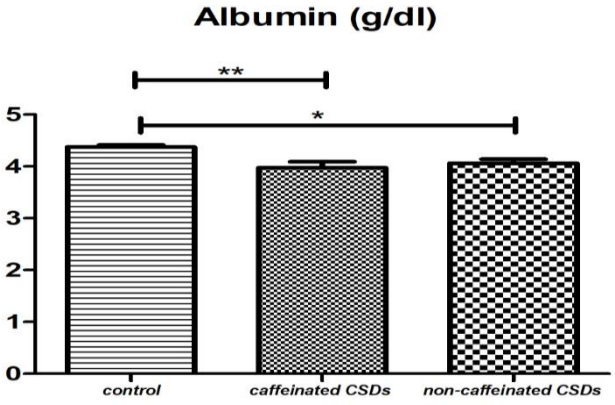

Fig. (11): *Comparison between the albumin levels of the three groups (the control, caffinated CSDs and non-caffinated CSDs groups)(SS of 40 albino rats), showing the mean values, standard error of albumin level (g/dl) and the statistical difference between the groups with a statistically significant difference between the marked groups. (**) indicates a p-value less than 0.01, (*) indicates a p-value less than 0.05.*

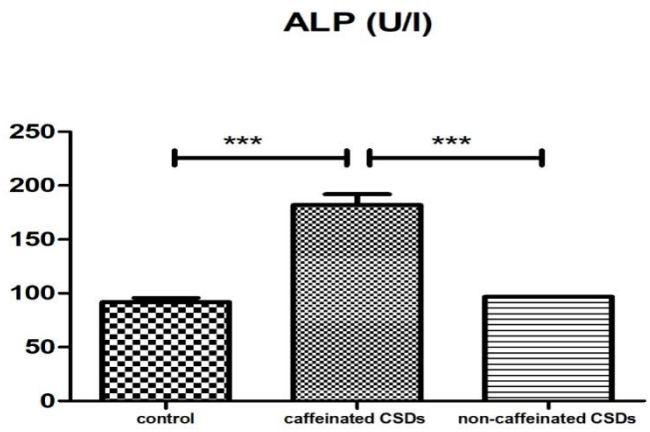

Fig. (12): *Comparison between the ALP levels of the three groups (the control, caffinated CSDs and non-caffinated CSDs groups) (SS of 40 albino rats), showing the mean values, standard error of ALP level (U/L) and the statistical difference between the groups, with a high statistically significant difference between the control group and the other two groups. (***) says a p-value less than 0.001.*

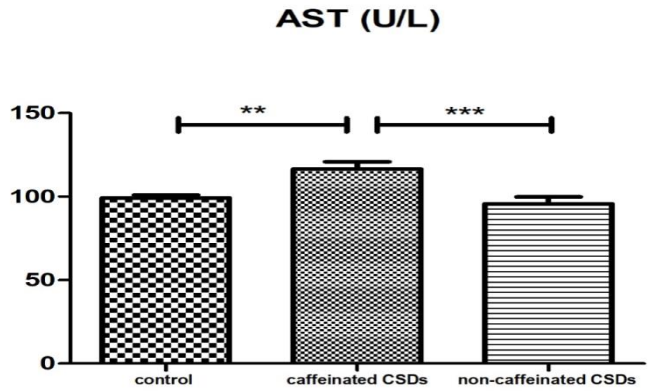

Fig. (13): *Comparison between the AST levels of the three groups, showing the mean values, standard error of AST level and the statistical difference between the groups (the control, caffinated CSDs and non-caffinated CSDs groups) (SS of 40 albino rats), showing a statistically significant difference between control group and the other two groups. (***) indicates a p-value less than 0.001. (**) indicates a p-value less than 0.01.*

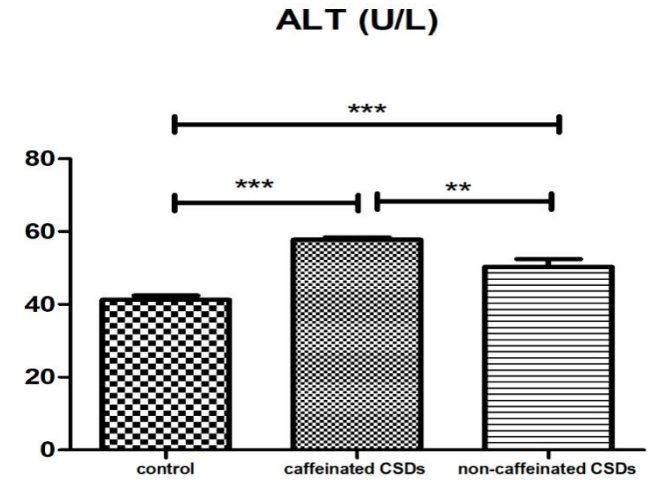

Fig. (14): *Comparison between the ALT levels of the three groups (the control, C and N groups) (SS of 40 albino rats), showing the mean values, standard error of ALT level and the statistical difference between the groups, with a statistically significant difference between control group and the other two groups on one hand and between both Caffeinated and non-caffeinated CSDs groups on the other hand. (***) indicates a p-value less than 0.001. (**) indicates a p-value less than 0.01.*

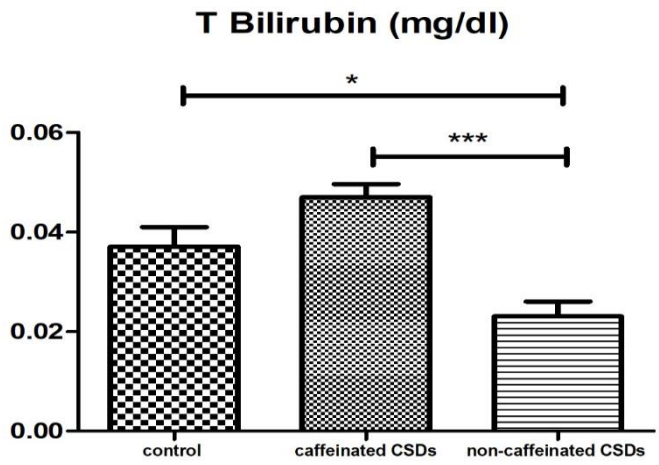

Fig. (15): *Comparison between the total bilirubin levels of the three groups (the control, caffinated CSDs and non-caffinated CSDs groups) (SS of 40 albino rats), showing the mean values, standard error of total bilirubin level and the statistical difference between the groups, with a statistically significant difference between the non-caffeinated CSDs group and each of the other two groups. (***) indicates a p-value less than 0.001. (*) indicates a p-value less than 0.05.*

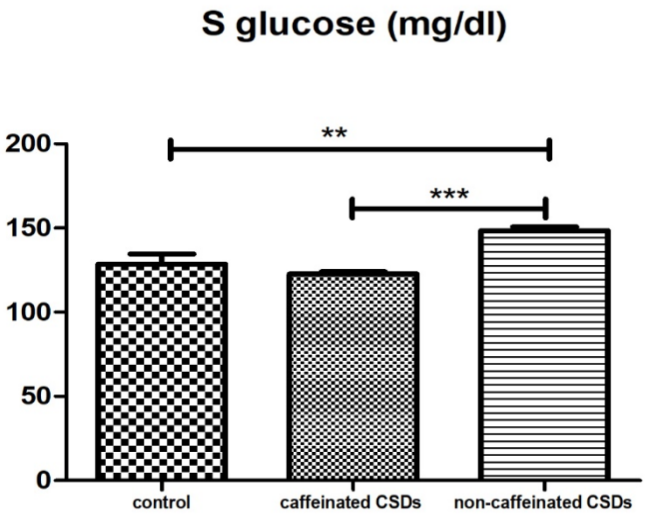

Fig. (16): *Comparison between the serum glucose levels of the three groups (the control, caffinated CSDs and non-caffinated CSDs groups) (SS of 40 albino rats), showing the mean values, standard error of serum glucose level and the statistical difference between the groups), with a statistically significant difference between the groups. (***) indicates a p-value less than 0.001. (**) indicates a p-value less than 0.01.*

Pancreas specimens:

The islets of Langerhans (L) of the control group showed normal clusters of islet cells with vesicular nuclei and in-between capillaries **(fig. 3, Pa)**. The non-caffeinated CSDs group showed clusters of islet cells with vesicular nuclei separated by wide spaces in-between **(fig. 3, Pb).**While the caffeinated CSDs group showed vacuolations within the clusters of islet cells. **(Fig. 3, Pc)**.

Immunohistochemical slides:

Liver specimens:

Diffuse positive hepatocytes' cytoplasmic and nuclear reaction of Caspase immunoreactivity was obvious in the caffeinated CSDs group (**Fig. 4, C)** and to a less extent in the non-caffeinated CSDs group **(Fig. 4, b).** The control group showed a negative reaction **(Fig. 4, a)**.

Pancreas specimens:

The islets of Langerhans of the pancreas of the three groups showed a strong positive reaction for anti-insulin antibodies immunostaining of B cells. The cytoplasm of non-B-cells of the Islets showed no reaction in any group **(Fig. 5).**

C-Histological scoring:

Statistical analysis of the scoring results showed high significant difference among the three groups concerning both steatosis and ballooning ($p<0.001$), while portal inflammation showed significant difference between the caffeinated CSDs group and the other two groups ($p<0.001$), but no significance was detected between the non-caffeinated CSDs group and the control one **(Table 1, fig. 6).**

D-Morpho-histometric study:

The optical density of the Caspase immunoreactivity was compared among liver slides using the ANOVA test. This showed a highly significant difference among the three groups. The intensity of protein expression of Caspase 3 staining showed highly statistically significant increase in the caffeinated CSDs group when compared to the control group. Also, there was a highly statistically significant increase in the non-caffeinated CSDs group when compared to the other groups **(fig. 7)**.

The mean surface area of islets of Langerhans of each group was compared to other groups using the ANOVA test. The caffeinated CSDs showed a significant increase compared with the control group, but no significance was detected on comparing other groups **(fig. 8).**

E-Comet assay analysis:

The data of comet shows a high statistically significant increase of the rate of DNA damage in both the caffeinated CSDs and non-caffeinated CSDs groups when each compared to the control group. In contrast, no statistical significance was detected between the caffeinated CSDs and non-caffeinated CSDs groups **(fig. 9).**

On comparing the statistical data of both optical density (indicator of cytoplasmic damage) and comet assay (indicator of genotoxicity and DNA damage), it was obvious that CSDs has deleterious cytotoxic and genotoxic effects **(Table 2).**

F-Biochemical investigation: (Table 3)

Concerning the liver tests.

The total protein level did not show any statistically significant difference whereby the albumin level decreased significantly in both caffeinated CSDs and non-caffeinated CSDs groups when compared to the control group **(fig 10, 11).**

The alkaline phosphatase level showed a statistically significant increase in the caffeinated CSDs group when compared to both the non-caffeinated CSDs and the control groups. Also, a statistically significant increase of AST and ALT levels was detected in the caffeinated CSDs group when compared to the other two groups. Concerning the ALT level of the non-caffeinated CSDs group, there was a statistically significant increase when compared to the control group **(fig. 12-14).**

Percentage change for caffeinated CSDs group concerning the three variables (ALP, AST, ALT) was 98.9%, 17.8%, 40.16% respectively. Percentage change for the non-caffeinated CSDs group concerning the three variables (ALP, AST, ALT) was 5.45%, -3.36%, 22.07% respectively. These levels of the "Percentage change" showed a higher ALP level change in the caffeinated CSDs group.

The total bilirubin level also showed a statistically significant increase in the caffeinated CSDs group when compared to the non-caffeinated CSDs group. While there was a statistically significant decrease in the non-caffeinated CSDs group when compared to the control group **(fig. 15).**

Concerning the serum glucose, a statistically significant increase of glucose levels was detected in the non-caffeinated CSDs group when compared to both the control and thecaffeinated CSDs groups. Whereby the caffeinated CSDs group showed no significant difference when compared with the control group **(fig. 16).**

DISCUSSION

In the present study, CSDs groups proved statistically to have harmful health effects. There was remarkable increase in BW in both CSDs groups when compared to the control one. Previous work of ***(Ebbeling, et al. 2012; Malik et al., 2006; Qi, et al., 2012)*** recorded

similar results of increased body weight related to consumption of soft drinks. Also in a systematic review, carried out by ***(Gitanjali, et al., 2015)***, and through the meta-analysis of number of cohort studies, it was concluded that soft drinks' ingestion for long times led to an increase in BMI for each serving per day.

Concerning the non-caffeinated CSDs group, the increased BW was associated with significant hyperglycemia while islets of Langerhans were mostly normal except for few islets showing spacing between cells. On the contrary, the caffeinated CSDs group was euglycemic although they showed increased BW and significant hyperplasia of islets of Langerhans. Minority of the islets showed degenerative changes in the form of cellular vacuolation. These findings suggest prediabetic changes and development of obesity as detected by ***Jones et al., (2010)*** that associates high consumption of soft drinks**.** Also such results were potentiated by number of previous studies ***(Imamura, et al., 2015; Ma, et al., 2016; Moon, et al., 2022).***

On the contrary, ***DenBiggelaar, et al. 2020*** claimed such diabetic changes to the artificially sweetened soft drinks rather than the sugar sweetened ones.

Concerning the hepatic changes, the present study showed disturbed biochemical liver tests, in which there was much higher level of Percentage change of the ALP when compared to AST and ALT in the caffeinated CSDs groups. (Giannini, Testa and Savar 2005)**,** (Carey 2022) and ***(Lala et al., 2022)*** reported that the diseases that majorly affect the secretion of hepatocyte cause predominant elevations of ALP. This was supported by the increased levels of bilirubin as well as the histological findings in the present study that showed marked affection in the biliary system of the caffeinated CSDs group. The portal triad showed proliferative bile ductular reactions with obvious cellular infiltration denoting an active compensatory regenerative process according to ***(López, 2016).***

There was a significant decrease in albumin level in the caffeinated CSDs group. This is related, directly, to the evidence of histopathological affection of the hepatocytes. Yet, the total protein levels showed non-significant change, most probably due to the compensatory increase in globulin production according to the work of ***(Al-Joudi and Wahab 2004.)***

Accumulation of ballooning, fatty cells was a marked finding in the peripheral areas of the liver in the caffeinated CSDs group than the non- caffeinated. ***(Murali and Carey 2014)*** refered the finding of fat accumulation in the liver in the absence of causes of secondary fat accumulation to be a case of Non-alcoholic fatty liver disease (NAFLD) and added that "Patients with NAFLD are non-alcoholic, usually obese, have a high BMI and may have DM" and that was obvious in the previously mentioned results of the current study.

These findings were supported by ***(Assy et al., 2008; Chen, et al., 2019; Leung and Tapper 2022; Ma, et al., 2015; Park, et al.; 2021)*** who reported an increased risk of (NAFLD) with increased consumption of soft drinks especially in obese individuals.

According to ***(Brunt, et al., 2004; Kleiner, et al., 2005),*** the present histopathological scoring results strongly supports development of an active NAFLD which was detected with both types of experimentaly tested SDs.

Interference with genetic material was one of the suspected risks that had aroused due to the strong evidence from number of meta-analysis on clinical trials and outcomes assessing the link between the increased incidence of chronic diseases in last decades, and even the higher risks of all-cause mortality, and the surging levels of SSB consumption according to ***(Malik, et al., 2006; Palmer, et al., 2008; Singh, et al., 2015; Weed, et al., 2011; Zhang, et al., 2021).*** In the present study, DNA destruction was detected by COMET analysis in liver speciemens of CSDs groups indicating a direct gentoxic stress affecting the cells. This finding was also supported by the reults of measuring the optical denisty of caspase immunostained liver specimens, where both findings proved to be significant when comparing the CSDs groups with the control group. ***(Hannah, et al., 2010)*** who studied plant roots (*Allium cepa)* for short periods of 2, 24, 48hrs concluded the occurrence *of a* mitotic inhibitory effect. Also in the study of (***Düsman, et al., 2013)*** testing the effect of CSDs on bone marrow samples for a limited

period of 24hrs, they reported a statistical significant chromosomal break off. Furthermore, ***(El Terras, et al. 2016)*** in their study on the brain of wistar rats after free consumption of carbonated soft drinks, they reported an altered genes expression, especially those genes associated with the brain activity.

But in an in vivo and in vitro study of ***(Mateo-Fernández, et al., 2016)*** no genotoxicity effects were reported with the used concentrations, doses and period of time. In another study carried by ***(Qi, et al., 2012)***, they pointed to a pronounced genetic association of adiposity with the increased consumption of sugar-sweetened beverages.

CONCLUSION

The results of this study showed that the high consumption of CSDs caused an overwhelming health hazards at various levels; biochemically, histologicaly and even at the DNA level. Furthermore the caffinated CSDs effect is worse than the non-caffinated ones

RECOMMENDATION

The rising risks of morbidity in the last decades in relation to the massive increase of soft drinks (SDs) consumption necessitates precise measures to control this free SDs intake.

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