Carbonated soft drinks induce oxidative stress and alter the expression of certain genes in the brains of Wistar rats

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Abstract. In Saudi Arabia, the consumption of carbonated soft drinks is common and often occurs with each meal. Carbonated soft drink consumption has been shown to exhibit effects on the liver, kidney and bone. However, the effects of these soft drinks on brain activity have not been widely examined, particularly at the gene level. Therefore, the current study was conducted with the aim of evaluating the effects of chronic carbonated soft drink consumption on oxidative stress, brain gene biomarkers associated with aggression and brain histology. In total, 40 male Wistar rats were divided into four groups: Group 1 served as a control and was provided access to food and water ad libitum; and groups 2-4 were given free access to food and carbonated soft drinks only (Cola for group 2, Pepsi for group 3 and 7-UP for group 4). Animals were maintained on these diets for 3 consecutive months. Upon completion of the experimental period, animals were sacrificed and serological and histopathological analyses were performed on blood and tissues samples. Reverse transcription-polymerase chain reaction was used to analyze alterations in gene expression levels. Results revealed that carbonated soft drinks increased the serum levels of malondialdehyde (MDA). Carbonated soft drinks were also observed to downregulate the expression of antioxidants glutathione reductase (GR), catalase and glutathione peroxidase (GPx) in the brain when compared with that in the control rats. Rats administered carbonated soft drinks also exhibited decreased monoamine oxidase A (MAO-A) and acetylcholine esterase (AChE) serum and mRNA levels in the brain. In addition, soft drink consumption upregulated mRNA expression of dopamine D2 receptor (DD2R), while 5-hydroxytryptamine transporter (5-HTT) expression was decreased. However, following histological examination, all rats had a normal brain structure. The results of this study demonstrated that that carbonated soft drinks induced oxidative stress and altered the expression of certain genes that are associated with the brain activity and thus should be consumed with caution.

Introduction

Consumption of carbonated soft drinks is high in Saudi Arabia, particularly in middle-aged individuals aged between 35-50 years old. The effects of these products on health are unclear, although epidemiological studies have suggested their association with obesity, kidney disease, liver disease and osteoporosis (1,2). Carbonated soft drinks contain several compounds including caffeine; which is the most widely consumed behaviorally active substance worldwide. Almost all caffeine comes from dietary sources (4). Acute and chronic caffeine intake appear to have only minor negative consequences on health (5). For this reason and because few...
Caffeine users report loss of control over their caffeine intake, governmental regulatory agencies impose no restrictions on its use. In the majority of carbonated beverages, caffeine is deliberately added to make it addictive. However, caffeine in carbonated drinks is more readily absorbed than that from other non-carbonated beverages. The majority of carbonated soft drinks also contain phosphoric acid, caffeine, sugar or aspartame or saccharin, caramel coloring, carbon dioxide, and aluminum. Each of which have been demonstrated to have negative effects on human health (5).

Caffeine is known to be an addictive drug that has the ability to stimulate mental alertness, overcome fatigue and enhance endurance. Caffeine acts by blocking adenosine (neurotransmitter) receptor sites in the central nervous system, and adenosine generally exhibits a depressant action in the brain, heart and kidneys. The resultant stimulation is accompanied by constriction of the cerebral arteries, elevated heartbeat, high blood pressure and excessive excretion of urine. Cases of caffeine-associated fatalities and seizures have previously been identified (4,5) due to a combination of excess caffeine intake and cardiovascular disorders. Moreover, several studies have reported a weaker compensatory response after consumption of caloric liquids (6,7). Previous studies have examined the effect of energy intake on brain histology and activity (6-9). It has been reported that soda exhibited an adverse effect on the cerebellum, whereas non-diet soda exhibited harmful effects (10).

In the Middle East, particularly in Saudi Arabia, it is common for individuals to consume carbonated soft drinks 3 times per day with each meal. Therefore, the current study was conducted to examine the effect of chronic consumption of three common drinks in Saudi Arabia (Cola, Pepsi and 7-UP) on oxidative stress, antioxidant levels, aggression markers, and histopathology of the brain to outline their potential effects on the brains of Wistar rats. In addition, the effect of soft beverages on the expression and activity of certain genes associated with anxiety, violence and/or aggression, such as monoamine oxidase (MAO) and dopamine D2 receptors (DD2R) were examined.

Materials and methods

Chemicals and kits. Ethidium bromide, agarose, Mayer's hematoxylin and eosin (H&E) and Tris-Borate-EDTA (TBE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Wistar albino rats were purchased from the King Fahd Center for Scientific Research, King Abdel-Aziz University (Jeddah, Saudi Arabia). Serologic kits for catalase, malondialdehyde (MDA), glutathione reductase (GR) and glutathione peroxidase (GPx) were purchased from Bio-diagnostic Co., (Giza, Egypt). Cola (Atlanta, GA, Pepsi (PepsiCo, Purchase, NY, USA) and 7-UP (Dr Pepper Snapple Group, Inc., Plano, TX, USA) were used. DNA 100 bp ladder was purchased from MBI, Fermentas, Thermo Fisher Scientific. Inc. (Waltham, MA, USA). Qiazol for RNA extraction and oligo dT primers were purchased from Qiagen, Inc., (Valencia, CA, USA).

Animals, experimental design and sampling. All animal procedures were approved by the Ethical Committee Office of Taif University (Taif, Saudi Arabia). Forty male Wistar rats (age, 3 months; weight, 200-280 g) were used for this study. For acclimatization, animals were handled daily and kept under observation for 1 week prior to the onset of the experiment. The animals were kept under a 12-h light-dark cycle and had ad libitum access to food and water. Animals were divided into the following 4 groups: Control group (CNT) without any treatment; Cola group; Pepsi group and 7-UP group. Groups 2-4 received free access to food and only carbonated soft drinks for 3 consecutive months. At the end of the 3 months, all rats were anesthetized using diethyl ether inhalation and sacrificed via decapitation. Blood was collected in vacutainer tubes from retro-orbital venous plexuses following anesthetization. Brain tissues from the right hemisphere were harvested for gene expression and left hemisphere tissues were used for histopathological analyses. Serum was extracted after blood centrifugation for 10 min at 4,000 x g. For gene expression analysis, brain tissues were kept in QIAzol reagent at -80°C for RNA extraction and in 10% neutral buffered formalin (NBF) at room temperature for 24 h for histopathological and immunohistochemical analysis.

Serum chemistry assays. Catalase, GR, GP and MDA were measured using commercial spectrophotometric analysis kits (Bio-Diagnostic Company, Giza, Egypt). MAO and acetylcholine esterase (AChE) levels were measured using commercial enzyme-linked immunosorbent assay kits obtained from MyBioSource, Co. (San Diego, CA, USA). All procedures were conducted according to the manufacturer's protocol. mRNA expression levels of glutathione-S-transferase (GST) and 5-hydroxytryptamine transporter (5-HTT) were assessed using reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Gene expression analysis. Total RNA was extracted from the brain tissue samples as previously described (11). RNA concentration and purity were determined spectrophotometrically after measuring the optical density at 260 and 280 nm using a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). The RNA integrity was confirmed after running in 1.5% denatured agarose gel stained with ethidium bromide. A mixture of 3 µg total RNA and 0.5 ng oligo dT primer (Qiagen Inc., Valencia, CA, USA) were used for cDNA synthesis in a total volume of 11 µl sterilized diethylpyrocarbonate (DEPC) water and was incubated in the Bio-Rad T100 Thermal cycler (Bio-Rad) at 65°C for 10 min for denaturation. Then, 2 µl of 10X RT-buffer, 2 µl of 10 mM dNTPs and 100 units Moloney Murine Leukemia Virus Reverse Transcriptase (SibEnzyme. Ak, Novosibirsk, Russia) were added and made up to a total volume of 20 µl with DEPC water. The mixture was then re-incubated in the thermal cycler at 37°C for 1 h, then at 90°C for 10 min to inactivate the enzyme. For semi-quantitative RT-PCR analysis, specific primers for examined genes (Table 1) were designed using the Oligo-4 computer program (version 7; Molecular Biology Insights, Colorado Springs, CO, USA) and synthesized by Macrogen (Macrogen Inc., Gasa-dong, Korea). PCR was conducted in a final volume of 25 µl consisting of 1 µl cDNA, 1 µl of 10 pM of each primer (forward and reverse), and 12.5 µl PCR master mix (Promega Corporation, Madison, WI, USA), the volume was made up to 25/µl using sterilized deionized water. PCR was conducted using the Bio-Rad T100
Thermal Cycler with the following cycle sequence: 94°C for 5 min for one cycle, followed by 27-31 cycles (Table I) each of which consisted of denaturation at 94°C for 1 min, annealing at the specific temperature corresponding to each primer (Table I) and extension at 72°C for 1 min with an additional final extension at 72°C for 7 min. As a reference, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was examined (Table I). PCR products were visualized under UV light after electrophoresis on 1.5% agarose (Bio Basic Int., Markham, ON, Canada) gel stained with ethidium bromide in TBE buffer. PCR products were confirmed using a 100 bp DNA ladder and were subsequently photographed using an InGenius 3.0 gel documentation system (Syngene, Frederick, MD, USA). The intensities of

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product size (bp)</th>
<th>Annealing</th>
<th>Direction</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
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<tr>
<td>GST</td>
<td>570</td>
<td>55</td>
<td>Forward</td>
<td>GCTGGAGTGGAGTTTGAAGAA</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTCCTGACCACGTCAACATAG</td>
</tr>
<tr>
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<td>406</td>
<td>57</td>
<td>Forward</td>
<td>AAGGTGCTGCTCATTTGAAGATG</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
<td>CGTCTGGACCTACCAGGAACTTT</td>
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<tr>
<td>MAOA</td>
<td>492</td>
<td>60</td>
<td>Forward</td>
<td>ATGGATGAAATGGAAAAAGAGAT</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
<td>TAATTGGTCTCTCAGGTTGA</td>
</tr>
<tr>
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<td>Forward</td>
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<tr>
<td></td>
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<td></td>
<td>Reverse</td>
<td>CGGCTGATGAGAGATTCTTG</td>
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<tr>
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<td>488</td>
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<td>Forward</td>
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<tr>
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<td>Forward</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCCCTCAAGATGTCAGCAA</td>
</tr>
</tbody>
</table>

GST, glutathione-S-transferase; GPx, glutathione peroxidase; MAOA, monoamine oxidase A; AChE, acetylcholine esterase; DDR2, dopamine D2 receptor; 5-HTT, 5-hydroxytryptamine transporter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 1. Serum changes in MDA and antioxidant levels in Wistar rats after chronic consumption of soft drinks for 3 months. Serum was assayed using colorimetric kits to determine the levels of (A) MDA, (B) GR, (C) GPx and (D) catalase. Values are presented as the mean ± standard error of the mean for 10 rats per treatment. *P<0.05, compared with control. MDA, malondialdehyde; GR, glutathione reductase; GPx, glutathione peroxidase.
the bands were quantified densitometrically using Image J software version 1.47 (http://imagej.en.softonic.com/).

**Brain histopathology.** Brain was removed following diethyl ether inhalation and sacrifice of the rats and fixed overnight in a 10% NBF solution. Fixed brain tissues were processed routinely, washed and preserved in 70% ethanol, dehydrated in ascending grades of ethanol solution, cleared in xylene, embedded in paraffin wax, pressed and cut into 5-µm sections. Subsequently, the sections were placed on top of glass slides. The slides were stained with Mayer's H&E (12). Tissue slides were visualized using a Wolfe S9-0982 microscope (Carolina Biological Supply Co., Burlington, NC, USA) and photos were captured using a Canon Power-Shot SX500 IS digital camera (Canon, Tokyo, Japan).

**Statistical analysis.** Results are presented as the mean ± standard error of mean. Data were analyzed using analysis of variance and Fisher post hoc descriptive tests using SPSS software version 11.5 (SPSS, Inc., Chicago, IL, USA). Regression analysis was performed using the same software. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Effect of carbonated soft drink consumption for 3 months on serum levels of MDA, GR, GPx and catalase in Wistar rats.** Consumption of Cola, Pepsi and 7-UP for 3 months showed a significant increase in MDA levels (Fig. 1A; P<0.05) with the greatest increase in the rats from the Pepsi group. In parallel, the levels of antioxidants GR, GPx and catalase in the rats were decreased significantly in all groups administered carbonated soft drinks compared with the control. Notably, the greatest changes were observed in the rats from the Pepsi group (Fig. 1B-D).

**Effect of carbonated soft drink consumption for 3 months on mRNA expression of GST and GPx in the brain tissues of Wistar rats.** As shown in Fig. 2, carbonated soft drink...
Consumption for 3 months downregulated the mRNA expressions of GST and GPx. Expression levels were significantly decreased by 50 and 40% in the Cola and Pepsi groups for GST and GPx, respectively (P<0.05). Although still significantly reduced when compared with the control (P<0.05), rats in the 7-UP group demonstrated increased levels of GST and GPx, as compared with the Cola and Pepsi groups.

**Effect of carbonated soft drink consumption for 3 months on serum levels of MAO-A and AChE in Wistar rats.** Next, the changes in MAO-A and AChE levels (Fig. 3) were examined. Cola, Pepsi and 7-UP consumption for 3 months resulted in a significant decrease in MAO and AChE levels (P<0.05). Rats in the Pepsi group exhibited the greatest decreases in MAO-A and AChE levels, as compared with the other groups.

**Effect of carbonated soft drink consumption for 3 months on mRNA expression of MAO-A and AChE in brain tissues of Wistar rats.** Fig. 4 shows that, consistent with serum changes of MAO-A and AChE, the mRNA expression of MAO-A and AChE was significantly downregulated in the brain tissues of rats administered carbonated soft drinks (P<0.05). Rats in the Cola group exhibited the greatest decrease in MAO-A and AChE expression, followed by Pepsi and 7-UP, respectively. The decrease was not identified to be significantly different between the Pepsi and 7-UP groups (Fig. 4).

**Effect of carbonated soft drink consumption for 3 months on mRNA expression of DD2R and 5-HTT in brain tissues of Wistar rats.** The effects of carbonated soft drink consumption on the expression of certain genes that have been shown to be associated with aggression were investigated. As shown in Fig. 5A, expression of DD2R was significantly upregulated in rats in the Cola, Pepsi and 7-UP groups (P<0.05). By contrast, the rats in these groups exhibited significant downregulation of 5-HTT mRNA expression (Fig. 5B; P<0.05). Furthermore, rats in the Cola and 7-UP groups exhibited significantly decreased expression of 5-HTT, as compared with rats in the Pepsi group (P<0.05).

**Effect of carbonated soft drink consumption for 3 months on brain histopathology.** Although changes induced by carbonated soft drinks were observed at the biochemical and molecular levels, brain histopathology analysis showed normal brain architecture in all groups (Fig. 6). The gray matter of the rats appeared normal with its well-organized regularly...
arranged six layers and different size and shape nerve cells. The normal pattern of the white matter is formed of homogeneously stained nerve fibers running down the cortex was also identified.

**Discussion**

The results of current study confirmed that chronic consumption of carbonated soft drinks induced oxidative stress and changes in antioxidant expression in the brains of Wistar rats. Moreover, soft drink consumption decreased the serum levels and mRNA expression of MAO and AChE in the brain. Notably, it was also demonstrated that the levels of DD2R were downregulated and the levels of 5-HTT expression were upregulated in the brain, while brain histopathology remained unaffected.

Oxidative stress has been associated with the etiopathogenesis of several chronic diseases and exhibits a key role in the aging process (13). One of the consequences of uncontrolled oxidative stress (imbalance between the prooxidant and antioxidant levels in favor of prooxidants) is injury to cells, tissues and organs by oxidative damage. It has long been recognized that high levels of free radicals or reactive oxygen species (ROS) can directly damage lipids. The primary sources of endogenous ROS production are the mitochondria, plasma membrane, endoplasmic reticulum and peroxisomes (14). ROS are produced through a variety of mechanisms, including enzymatic reactions and/or auto-oxidation of several compounds, such as catecholamines and hydroquinone. In this study, chronic carbonated soft drink consumption induced oxidative stress in the brain as indicated by the increase in MDA levels in addition to the decrease in the expression of antioxidants, GR, GPx and catalase.

Carbonated soft drinks contain caffeine and phosphoric acid. Caffeine causes the release of adrenaline and an accompanying increase in blood sugar levels to produce the required energy. Caffeine reaches its peak level in the blood within 1 h of consumption and remains in the body for 4-6 h (4,5). Caffeine in soft drinks causes an increase in the release of acid into the stomach, which may lead to an upset stomach or heartburn. Moreover, caffeine has been reported that chronic exposure to the various components of energy and soft drinks may result in significant alterations in the cardiovascular system and brain activity (15,16). The results of the present study demonstrated an alteration in MAO-A and AChE at the serum and mRNA levels. MAO has 2 isozymes, A and B. MAO-A in humans is encoded by the MAOA gene (17,18). It preferentially deaminates norepinephrine, epinephrine, serotonin and dopamine (all are equally deaminated by MAO-A and MAO-B). Inhibition of both MAO-A and MAO-B using MAO inhibitor is used in the treatment of clinical depression, erectile dysfunction and anxiety. MAO-A has been shown to be increased in patients with depression (19) and an association has been demonstrated between low-activity forms of the MAO-A gene and autism (20). A dysfunctional MAO-A gene has also been correlated with increased aggression levels in mice (21,22), as well as with heightened levels of aggression in humans (23). The results of the present study demonstrated that the consumption of carbonated soft drinks decreased MAO-A gene expression and were associated with oversensitivity, as MAO-A decrease is correlated with aggression and violence, this may suggest consumption of carbonated soft drinks is correlated with increased aggression and violence but this requires further investigation.

It is well-established that cholinergic neurons are involved in several neuropsychic functions, such as learning, memory
and sleep. Acetylcholine exhibits a key role in modulating these functions (24). A central cholinergic deficit is strongly associated with certain neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (25). Evidence of autism due to dysfunction of the cholinergic system has recently been reported (26). AChE is a specific cholinergic marker protein for the functional state of cholinergic neurons. It is key in the maintenance of acetylcholine levels at the cholinergic neurons (27) as it is responsible for degradation of acetylcholine to acetate and choline in the synaptic cleft. Notably, AChE was observed to be decreased in the serum and AChE mRNA expression was observed to be decreased in the brain. It has previously been suggested that acetylcholine disruption may be a primary cause of depression and/or aggression (28).

Dopamine is a neurotransmitter of the catecholamine and phenethyamine families that exhibits a number of roles in the human brain and body. There are 5 isoforms of the dopamine receptor, dopamine D1-5 receptors. D2R is the most common receptor in the mammalian brain. D2DR antagonists have been used for decades to treat aggressive behavior in psychotic patients (29). In addition, in a preclinical study the role of dopamine D1, D2 and D3 receptors in the modulation of aggression has been documented (30). Several studies have indicated that the mesocorticolimbic dopamine system is involved in the preparation, execution and consequences of aggressive acts (31-33). Pharmacologically induced dopamine increases are associated with increased aggressive behavior under certain conditions (32,33). The results of the present study demonstrated that D2DR levels increased following chronic carbonated soft drink consumption. This was probably due to the increase in dopamine levels resulting from the downregulation of MAO-A expression.

Two major enzymes are responsible for catecholamine catabolism in the brain: Catechol-O-methyltransferase (COMT) and monoamine oxidase A (MAO-A). If aggressive behavior is enhanced by catecholaminergic activity, then decreased activity of COMT and MAO-A should indirectly increase levels of aggression (31,34). 5-HTT expression was shown to be downregulated following chronic carbonated soft drink consumption. A number of studies have shown that elevated serotonin levels lead to decreased aggression in a number of species (35), including humans (36). Blocking serotonin transporter molecules is effective in reducing and preventing aggressive behavior in humans and other animals, presumably due to increased brain 5-HT levels (35). Clinically, blocking 5-HTT with the administration of selective serotonin reuptake inhibitors (SSRIs), reduces aggressive outbursts and violent behavior in psychiatric patients (37-39). In addition, in animal models, acute and chronic treatment with SSRIs can dose-dependently reduce aggressive behavior (40). Acute administration of several SSRIs reduced aggression in different contexts and species, including rodents and non-human primates (40,41). 5-HTT is a type of monoamine transporter protein that transports serotonin from the synaptic cleft to the pre-synaptic neuron. This transport of serotonin by the 5-HTT protein terminates the action of serotonin and recycles it in a sodium-dependent manner. This protein is the target of numerous antidepressant agents, including those of the SSRI class (42). A repeat length polymorphism in the promoter of this gene has been shown to affect the rate of serotonin uptake and may exhibit a role in sudden infant death syndrome, aggressive behavior in Alzheimer disease patients, post-traumatic stress disorder and depression-susceptibility in individuals experiencing emotional trauma (43). It has also been suggested that alterations in 5-HTT expression levels following the consumption of carbonated soft drinks may be a predisposing factor for depression. Histological examination of the brain revealed that the brain exhibited normal histology and cell distribution following chronic carbonated soft drink consumption. This finding was also reported in another study (10) for regular soft drinks; however, previously diet soft drinks were shown to exhibit adverse effects on the cerebellum of albino rats (10).

In conclusion, chronic term carbonated soft drink consumption induced oxidative stress and alterations in antioxidants and the expression levels of certain genes associated with brain function. Therefore, the results of the present study suggested that the consumption of carbonated soft drinks may induce adverse effects, thus these drinks must be consumed with caution.

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References

10. Eluwa MA, Iyadurai S, Chung SS: Soft drink consumption induced oxidative stress and alterations in antioxidants and the expression levels of certain genes associated with brain function. Therefore, the results of the present study suggested that the consumption of carbonated soft drinks may induce adverse effects, thus these drinks must be consumed with caution. 5-HTT expression was shown to be downregulated following chronic carbonated soft drink consumption. The authors would like to thank Al-Saedan Research Chair for Genetic Behavioral Disorders, Taif University, Kingdom of Saudi Arabia, for their financial support.

References


