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Prevention of Oxidative Damage of Liver, Kidney and Serum Proteins with Apoptosis of above Tissues in Guinea Pigs Fed on Carbonated Soft Drink by Vitamin C

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ABSTRACT

Damages of liver, kidney and serum proteins with apoptosis of these tissues in guinea pigs fed on carbonated soft drink was reported earlier. The present study establish the fact that if antioxidant supplementation is made along with carbonated soft drink such damages could be prevented. Vitamin C is a potent antioxidant and guinea pigs cannot synthesise ascorbic acid like mice. Excess sugar in carbonated soft drink increases production of destructive free radicals in animal. Ascorbic acid was supplemented orally in a measured dose along with carbonated soft drink, also in a measured volume to guinea pigs during the experimental period. Though oxyblot assay, fluorescence activated cell sorting assay and other biochemical findings, this paper reports that oxidative damages of the tissues along with apoptosis under the condition of carbonated soft drink feeding to guinea pigs could be prevented with supplementation of ascorbic acid.

Key words: Oxidative damage, apoptosis of tissues, carbonated soft drink, ascorbic acid

INTRODUCTION

Ascorbic acid is a potent antioxidant as reported in different toxic conditions in biological system (Flora, 2009). Mice can elicit significant antioxidant response as they can synthesize vitamin C in response to oxidative stress (Wolfer *et al.*, 2002). Carbonated soft drink contains excess of sugar. Excess sugar consumption increases production of destructive free radicals and lower level of key antioxidants as reported by Mohanty *et al.* (2000). High sugar consumption is also associated with an increase in free radical generation, leading to damage of fats, protein and DNA (Tappy and Le, 2010). High fructose feeding in rats showed increased level of oxidative stress and increased reactive oxygen species in circulatory leukocytes (Al-Awwadi *et al.*, 2005).

Oxidative damage of liver, kidney and serum proteins with apoptosis of above tissues in guinea pigs fed on carbonated soft drink have been reported from this laboratory earlier (Banerjee *et al.*, 2013). When similar exposure was made with mice, cytotoxic damage in the liver tissue of mice is very low even at relatively high doses of oral administration of carbonated soft drink. This some what led to speculation that vitamin C may play a pivotal role in prevention of potential oxidative damage induced by the consumption of carbonated soft drink *in vivo*.

In order to elucidate whether the nature of damage induced by carbonated soft drink is oxidative in nature (Banerjee *et al.*, 2013), vitamin C was used as a potential antioxidant to explore possible prevention of such damage. In this present study vitamin C was orally administered along with carbonated soft drink to guinea pigs in therapeutic levels, along with a group that was administered subclinical levels of vitamin C (to avoid scurvy), beside the control group. Guinea pigs were chosen as model animal as they, like humans are incapable of synthesizing vitamin C and many of its metabolic activities are like that of human.

MATERIALS AND METODS

Carbonated soft drink: Carbonated soft drink, available in Indian market in 600-1200 mL bottle, was used to feed the animal. The volume of carbonated soft drink which was administered orally to guinea pigs varied from 6-8 mL depending on body weight of the animal. Here volume of the carbonated soft drink was determined proportionately assuming average body weight of guinea pigs 500 g compared to 600-1200 mL soft drink consumption per day by an individual of average 60 kg b.wt in India.

Vitamin C: Vitamin C was added directly to carbonated soft drink in a dose of 20 mg for each guinea pig. Here dose of vitamin C was determined proportionately assuming average body weight of an individual adult 60 kg and his supplementary needs of vitamin C per day.

Chemicals and reagents: Different chemicals and regents used here have been discussed in details earlier (Banerjee *et al.*, 2013). Ascorbic acid (Vitamin C), Serum aspartate aminotransferase (AST substrate), aspartate amino transferase (ALT substrate), p-nitrophenyl phosphate (pNPP substrate), thiobarbituric acid (TBA), haematoxylin, eosin, used were of analytical grade and were purchased from the Sigma Chemical Company, E. Merck Ltd. and SRL(India).

Animals: All animal treatment procedures have been discussed in details earlier as in Banerjee *et al.* (2013). All animal treatment procedures meet the NIH guidelines (NRC, 1985) and were conducted with institutional animal ethics committee approval.

Different assay procedures of methods and techniques used here have been discussed in details by Banerjee *et al.* (2013).

Assay of Serum Glutamic Oxaloacetic Transaminase (SGOT): Serum glutamic oxaloacetic transaminase was measured by reaction with serum aspartate aminotransferase (AST substrate) (Henry, 1974; Steinberg *et al.*, 1956). After incubation of the liver microsomal extract with AST substrate for 1 h at 37°C in water bath, the reaction mixture was allowed to react with 2, 4-dinitrophenyl hydrazine (DNPH) followed by another 30 min incubation at room temperature. Finally the reaction was stopped 1(N) NaOH. The absorbance was measured at 510 nm using a double beam Shimadzu spectrophotometer model UV-2450 against a reagent blank. The results were expressed in IU/L. For the *in vivo* experiments the protein concentration was approximately maintained at 40 μg μL^{-1} of microsomal suspension according to requirement.

Assay of Serum Glutamic Pyruvic Transaminase (SGPT): Serum glutamic pyruvic transaminase was measured by reaction with aspartate amino transferase (ALT substrate) (Wilkinson *et al.*, 1972). After incubation of the liver microsomal extract with ALT substrate for 1 h at 37°C in water bath, the reaction mixture was allowed to react with 2, 4-dinitrophenyl

hydrazine (DNPH) followed by another 30 min incubation at room temperature. Finally the reaction was stopped 1(N) NaOH. The absorbance was measured at 510 nm using a double beam Shimadzu spectrophotometer model UV-2450 against a reagent blank. The results were expressed in IU/L. For the *in vivo* experiments the protein concentration was approximately maintained at 40 µg µL^{-1} of microsomal suspension according to requirement.

Assay of Alkaline Phosphatase: Alkaline phosphatase was measured by reaction with p-nitrophenyl phosphate (pNPP substrate) (Bessey *et al.*, 1946). After incubation of the liver microsomal extract with pNPP substrate for 30 min at room temperature the reaction was finally stopped by 1(N) NaOH. The absorbance was measured at 405 nm using a double beam Shimadzu spectrophotometer model UV-2450 against a reagent blank. The results were expressed in IU/L. For the *in vivo* experiments the protein concentration was approximately maintained at 40 µg µL^{-1} of microsomal suspension according to requirement.

Assay of Malondialdehyde (MDA)-Thiobarbituric Acid (TBA) method: The whole liver or kidney tissue protein extract were at first precipitated with 20% trichloroacetic acid solution and then was incubated for half-an-hour at -20°C followed by centrifugation for 10 min at 13,000 rpm in cold. Here the pellet was collected and to it again 10% trichloroacetic acid solution was added and recentrifuged in cold. To this was added 500 µL of 6.7% Thiobarbituric Acid (TBA) solution, vortexed and the mixture incubated at 100°C for 10 min. After cooling, absorbance was recorded at 532 nm in a Shimadzu spectrophotometer; model UV-2450 (Esterbauer and Cheeseman, 1990). The results were expressed as n moles of per milligram protein using a molar extinction coefficient of 33,000. For the *in vivo* experiments, 5 mg concentration of whole tissue extract protein of the liver or kidney were taken and directly subjected to MDA estimation as described above.

Measurement of Conjugated Dienes: To the whole liver or kidney tissue protein extract 1 mL of n-hexane was added, vortexed and centrifuged at 2000 g for 2 min. The upper n-hexane layer was withdrawn and used for measuring conjugated dienes at absorption maxima of 234 nm. The absorbance was recorded in a double beam spectrophotometer (Shimadzu spectrophotometer, model UV-2450) and quantified by using $\sum 235 = 25$ mM^{-1} cm^{-1} (Aikens and Dix, 1991). The results were expressed as pico moles of per milligram protein. For the *in vivo* experiments, 5 mg concentration of whole tissue extract protein of the liver or kidney were taken and directly subjected to estimation of conjugated dienes, as described above.

Histology and morphometric analysis for assessing cellular damage of liver tissue: The liver and kidney were fixed in 10% formalin and embedded in paraffin. Sections (5 µm) were cut from each type of organ, more or less from similar positions. The paraffin embedded tissue sections (5 µm) were deparaffinized using xylene and ethanol (absolute, 95, 90, 80 and 70% diluted in water). The slides were washed with Phosphate Buffered Saline (PBS) and permeabilised with permeabilisation solution (0.1 M citrate, 0.1% TritonX-100). The deparaffinized sections were stained with haematoxylin and eosin. Digital images were captured with Olympus CAMEDIA digital camera, Model C-7070 wide zoom (magnification, 10x) (Merck Source, 2002).

Statistical analysis: All values were expressed as Mean±Standard Deviation (SD) of three different sets of experiments, each set consisting of three controls and fifteen experimental animals. Data were analysed using origin 61 data analysis and graphing software. All statements were significant based on a probability $p \leq 0.05$.

RESULTS

Under the condition of feeding carbonated soft drink to guinea pigs as described in experiments below, damages to protein, lipid occur as presented below (I). Protein damages (IA) were studied by oxyblot assay (in liver) and by assaying carbonyl, bityrosine, tryptophan, thiol (in both liver and kidney). These damages were recovered by supplementation of vitamin C along with carbonated soft drink. In case of damage to lipid (IB), it has been found that both malondialdehyde and conjugated diene formation are increased with carbonated soft drink feeding, which can be reduced with vitamin C supplementation.

Cellular, tissue and organ damage have been studied as presented below (II) by IIA-tunel assay (in liver and kidney), IIB-FACS assay (in blood), IIC-enzyme assay and IID-histopathology and morphometric analysis (in liver). Under the experimental condition damages at the cellular level occur in liver, kidney and blood as have been found here which are recovered by vitamin C supplementation.

While studying damages by histopathological and morphological analysis (in liver and kidney), it has been found that no significant changes occur in the organs under carbonated soft drink fed condition. However, liver enzymes are significantly elevated, showing damages to be limited to enzymatic level only but not to organ damage as a whole. The elevated enzymatic level could be brought back to near normal by vitamin C supplementation to animals:

I: Following results contain oxidative damage in animal tissue proteins and lipids by carbonated soft drink and prevention by vitamin C
IA: Protein oxidation in guinea pig tissues due to consumption of carbonated soft drink as manifested by oxyblot, carbonyl, bityrosine, tryptophan and thiol assay

Oxyblot assay showing protein damage and subsequent prevention by vitamin C: One group of guinea pigs was fed with carbonated soft drink for a period of 0, 7, 15, 30, 45 and 60 days along with vitamin C (2 mg day^{-1}) in water to protect animals from scurvy. The other group was fed same quantity of soft drink along with supplementation of vitamin C (20 mg day^{-1}).

The guinea pigs of both groups were sacrificed on completion of the treatment period, i.e., 0, 7, 15, 30, 45 and 60th day and kidney tissues were collected to carry out oxyblot of the tissue proteins.

Figure 1 shows the oxyblot profile of kidney tissue proteins of animals treated with carbonated soft drink against those treated under vitamin C supplementation. The results clearly show a time-dependent increase in the oxidative damage of the proteins (Fig. 1, Lanes 2, 4, 6 and 8) compared to the zero day control (Fig. 1, Lane 1) as well as significant protection of such damage by vitamin C (Fig. 1, Lanes 3, 5, 7 and 9).
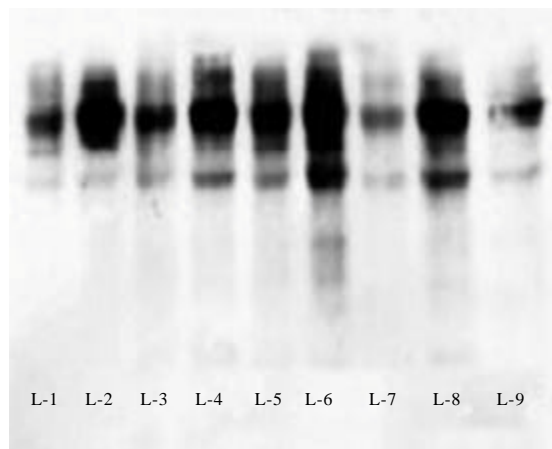
Fig. 1: Oxyblot of kidney proteins from guinea pigs orally administered carbonated soft drink with and without vitamin C supplementation. One set of guinea pigs were exposed to carbonated soft drink against water as control (as described under Materials and Methods, Banerjee *et al.*, 2013) before being sacrificed on 0, 15, 30, 45 and 60th days, respectively. Another set were exposed to carbonated soft drink along with 20 mg vitamin C (as described under Materials and Methods) before being sacrificed after 15th, 30th, 45th and 60th days, respectively. L-1: Water as the drink; L-2: Carbonated soft drink for 15 days, L-3: Carbonated soft drink along with vitamin C for 15 days, L-4: Carbonated soft drink for 30 days, L-5: Carbonated soft drink along with vitamin C for 30 days, L-6: Carbonated soft drink for 45 days, L-7: Carbonated soft drink along with vitamin C for 45 days, L-8: Carbonated soft drink for 60 days, L-9: Carbonated soft drink along with vitamin C for 60 days. The figure represents best of three different sets of experiments, each set consisting of control and experimental

Carbonyl and bityrosine formation as well as tryptophan and thiol loss in guinea pig tissues due to consumption of carbonated soft drink and its prevention by vitamin C: Assay of carbonyl (Fig. 2 and 3) and bityrosine (Fig. 4 and 5) formation as well as trypophan (Fig. 6 and 7) and thiol (Fig. 8 and 9) loss in liver and kidney tissue proteins were also done to ascertain the degree of oxidative damage induced by the consumption of carbonated soft drink. Possible prevention by vitamin C as an antioxidant during such treatment was also explored in these experiments (Fig. 2-9). The same group of animals as described above was used for such experiments. The results clearly vindicate the oxidative damage observed in the oxyblot assay as well as the potential of vitamin C as a potent antioxidant in the prevention of such carbonated soft drink-induced oxidative damage *in vivo*.

IB) Lipid peroxidation in guinea pig tissues due to consumption of carbonated soft drink as manifested by malondialdehyde and conjugated diene formation and its prevention by vitamin C: In the present experiments oral administration of carbonated soft drink to guinea pigs in amounts, consonant with general human consumption up to 60 days (Fig. 10-13) brought about significant increase in lipid peroxidation as measured by both thiobarbituric acid (TBA) estimation (Fig. 10 and 11) as well as conjugated diene formation (Fig. 12 and 13), in liver and kidney tissues of the treated animals. However, supplementation of vitamin C along with the soft drink ameliorated such trend to a significant extent during experimental period:
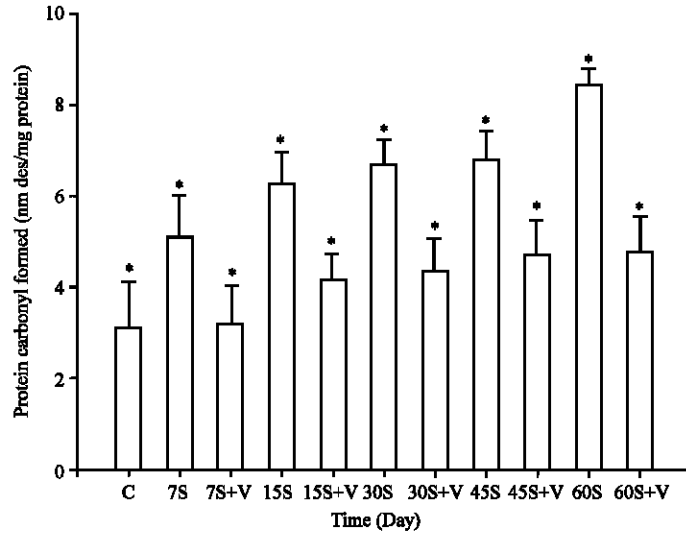
Fig. 2: Time-dependent carbonyl formation in liver tissue proteins of guinea pigs treated with carbonated soft drink and protection by vitamin C *in vivo*. Protein carbonyl levels in liver tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding protein carbonyl levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of carbonyl groups are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *represent $p < 0.001$
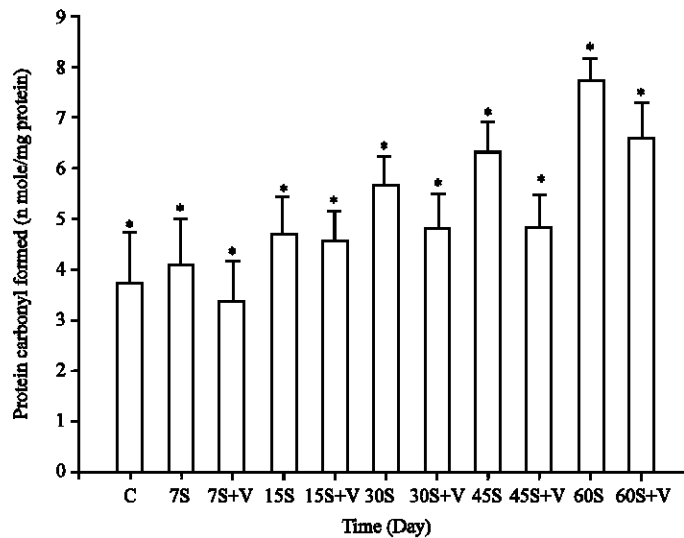

Fig. 3: Time-dependent carbonyl formation in kidney tissue proteins of guinea pigs treated with carbonated soft drink and protection by vitamin C *in vivo*. Protein carbonyl levels in kidney tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding protein carbonyl levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and assay are described under 'Materials and Methods'. Soft drink is represented by 'S' and vitamin C as 'V'. *represents $p < 0.001$
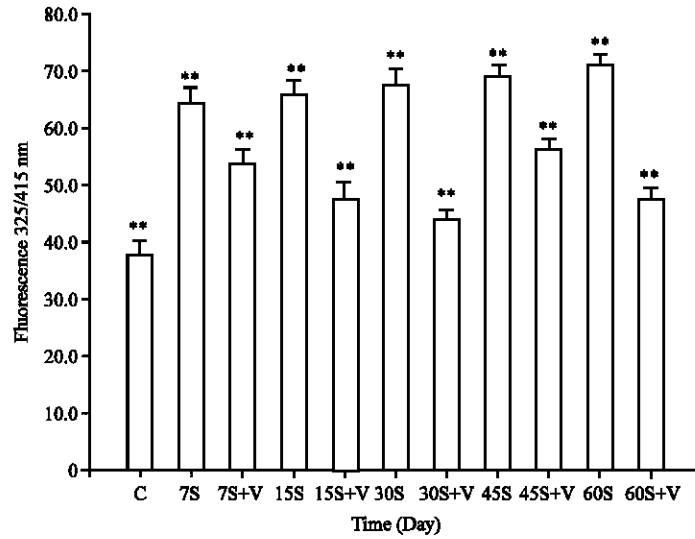
Fig. 4: Time-dependent bityrosine formation in liver tissue proteins of guinea pigs orally treated with carbonated soft drink and protection by vitamin C *in vivo*. Protein bityrosine levels in liver tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding protein bityrosine levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of bityrosine groups are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. **represents $p < 0.001$
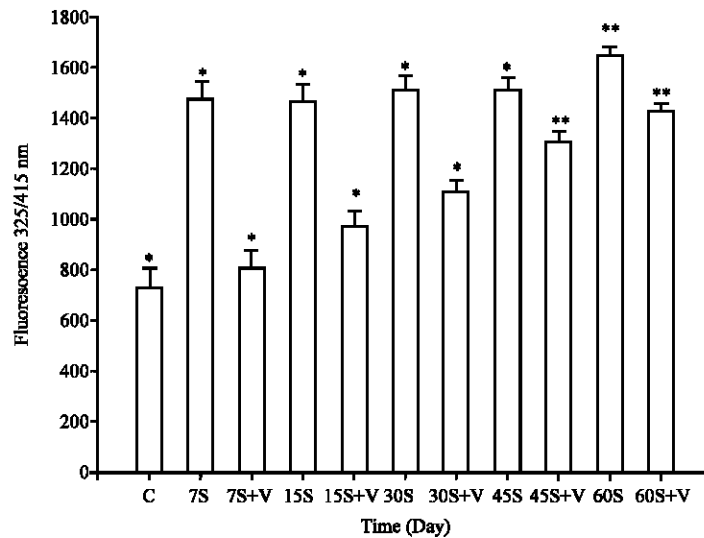

Fig. 5: Time-dependent bityrosine formation in kidney tissue proteins of guinea pigs treated with carbonated soft drink and protection by vitamin C *in vivo*. Protein bityrosine levels in kidney tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding protein b ityrosine levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of bityrosine groups are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *represents $p < 0.001$ and **represents $p < 0.01$
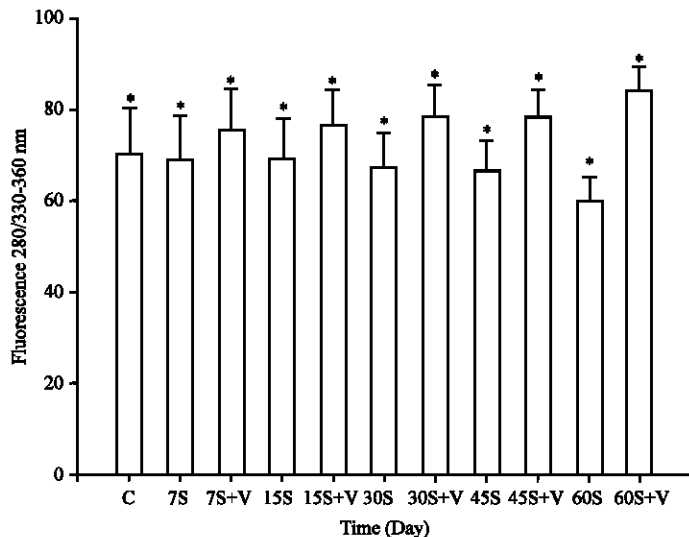
Fig. 6: Time-dependent loss of tryptophan residues in liver tissue proteins of guinea pigs treated with carbonated soft drink and protection by vitamin C *in vivo*. Protein tryptophan levels in liver tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding protein tryptophan levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of tryptophan groups are described under 'Materials and Methods'. Soft drink is represented by 'S' and vitamin C as 'V'. *represents $p < 0.001$
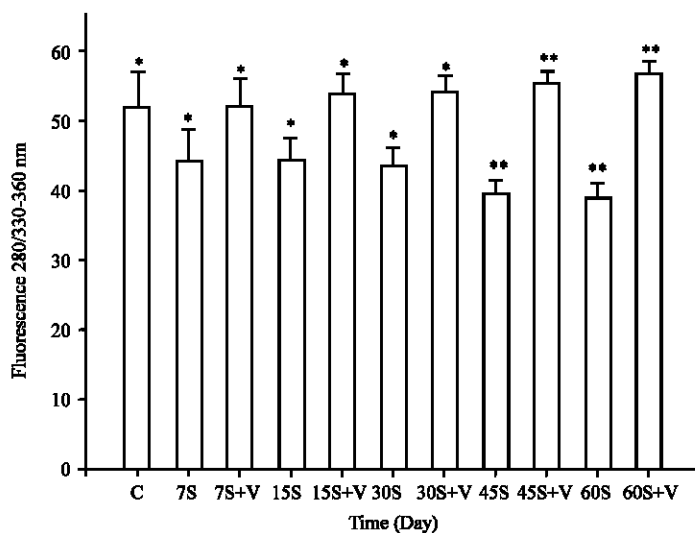

Fig. 7: Time-dependent loss of tryptophan residues in kidney tissue proteins of guinea pigs treated with carbonated soft drink and protection by vitamin C *in vivo*. Protein tryptophan levels in kidney tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding protein tryptophan levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of tryptophan groups are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *represents $p < 0.001$ and **represents $p < 0.01$
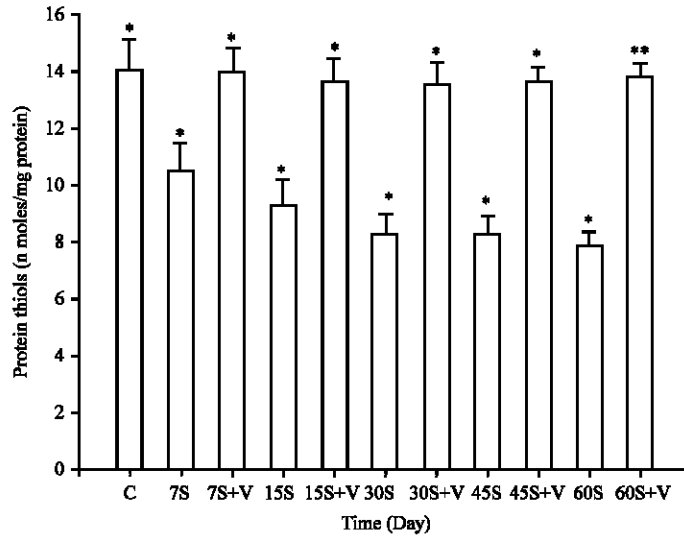
Fig. 8: Time-dependent loss of thiol in liver tissue proteins of guinea pigs treated with carbonated soft drink and protection by vitamin C *in vivo*. Protein thiol levels in liver tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding protein thiol levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of thiol groups are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *rpresents p<0.001 and **represents p<0.01
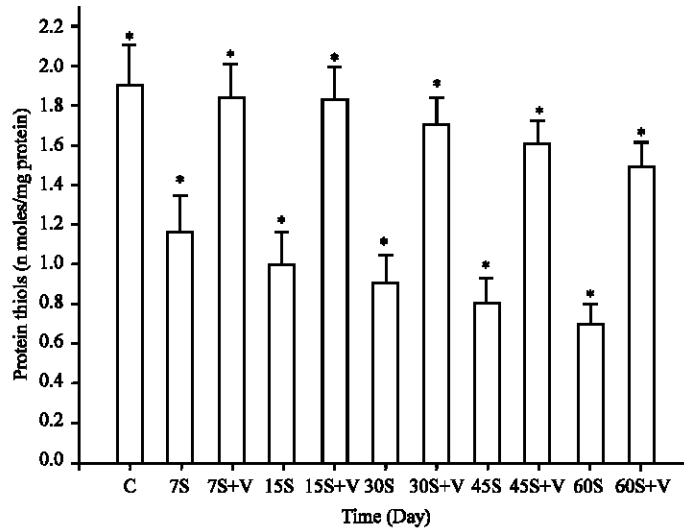

Fig. 9: Time-dependent loss of thiol in kidney tissue proteins of guinea pigs treated with carbonated soft drink and protection by vitamin C *in vivo*. Protein thiol levels in kidney tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding protein thiol levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of thiol groups are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *rpresents p<0.001
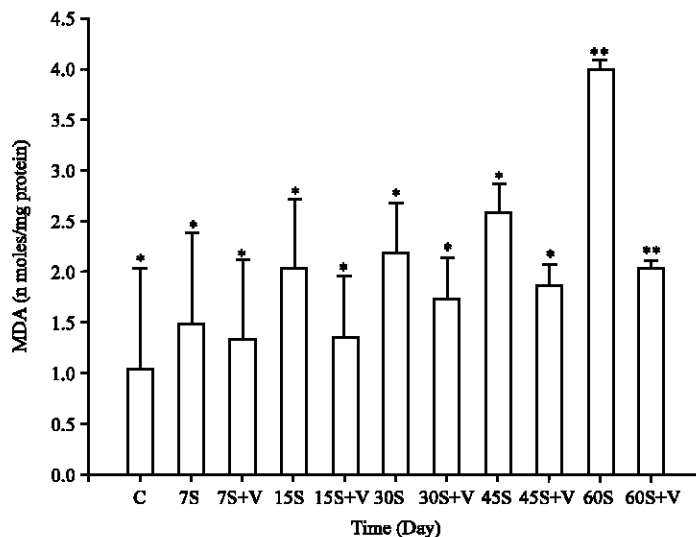
Fig. 10: Time-dependent formation of MDA in whole liver tissue extract induced by oral administration of carbonated soft drink to guinea pigs and protection by vitamin C as estimated by the Thiobarbituric Acid (TBA) method. Lipid MDA levels in liver tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7S, 15S, 30S, 45S and 60S, respectively) and corresponding lipid MDA levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of MDA levels are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *rpresents $p < 0.001$ and **represents $p < 0.01$
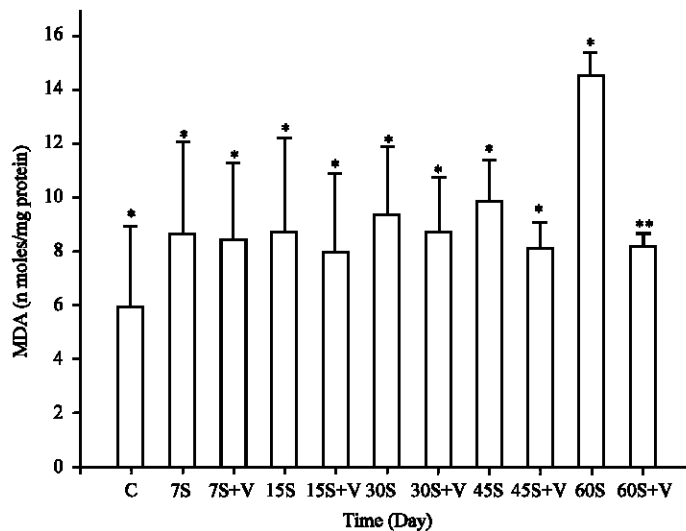

Fig. 11: Time-dependent formation of MDA in whole kidney tissue extract induced by oral administration of carbonated soft drink to guinea pigs and protection by ascorbic acid as estimated by the Thiobarbituric Acid (TBA) method. Lipid MDA levels in kidney tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding lipid MDA levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of MDA levels groups are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *represents $p < 0.001$ and **represents $p < 0.01$
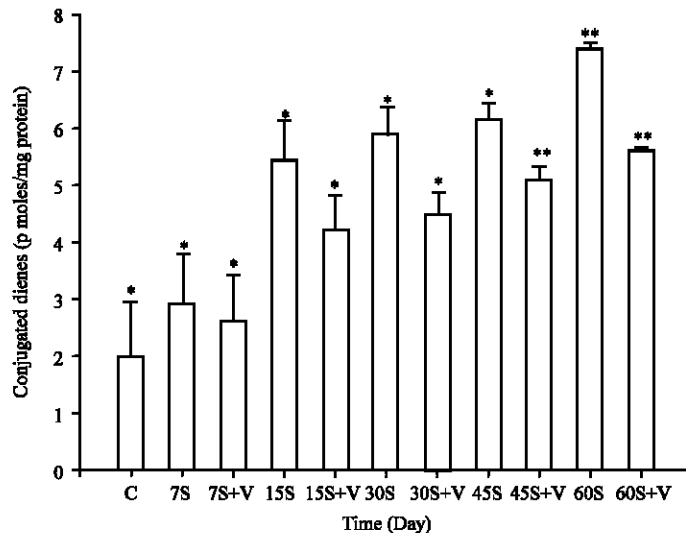
Fig. 12: Time-dependent formation of conjugated dienes in whole liver tissue extract induced by administration of carbonated soft drink to guinea pigs and protection by ascorbic acid. Conjugated diene levels in liver tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45 and 60S, respectively) and corresponding conjugated dienes levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of conjugated dienes levels are described under 'Materials and Methods'. Soft drink is represented by 'S' and vitamin C as 'V'. *represents $p<0.001$ and **represents $p<0.01$
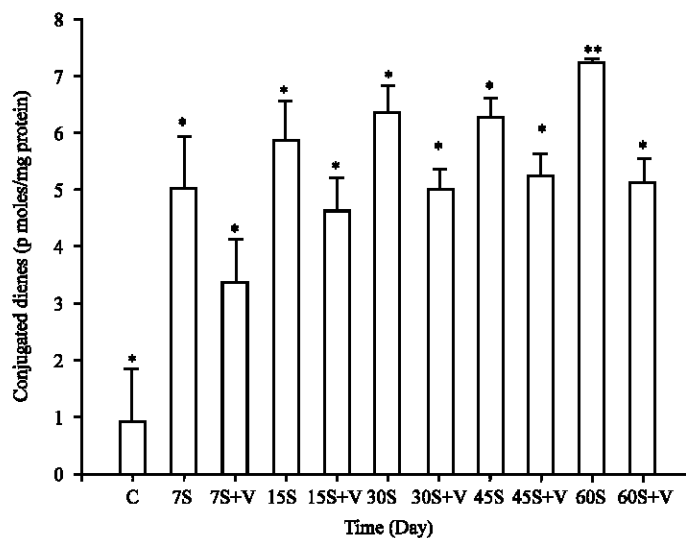

Fig. 13: Time-dependent formation of conjugated dienes in whole kidney tissue extract induced by oral administration of carbonated soft drink to guinea pigs and protection by ascorbic acid. Conjugated dienes level in kidney tissue proteins of guinea pigs orally administered with carbonated soft drink for 7, 15, 30, 45 and 60 days (represented by 7, 15, 30, 45S and 60S, respectively) and corresponding conjugated dienes levels in animals supplemented with vitamin C (20 mg day^{-1}) along with carbonated soft drink administration for 7, 15, 30, 45 and 60 days (represented by 7S+V, 15S+V, 30S+V, 45S+V and 60S+V, respectively). Detailed conditions of incubation and estimation of conjugated dienes levels are described under 'Materials and Methods'. Soft drink is represented by 'S' and vitamin C as 'V'. *rpresents $p<0.001$ and **represents $p<0.01$

| (a) | (b) | (c) | (d) |
| Control-TUNEL (T) | Control-DAPI (D) | 7 days-TUNEL (T) | 7 days-DAPI (D) |

| (e) | (f) | (g) | (h) |
| 15 days-TUNEL (T) | 15 days-DAPI (D) | 30 days-TUNEL (T) | 30 days-DAPI (D) |

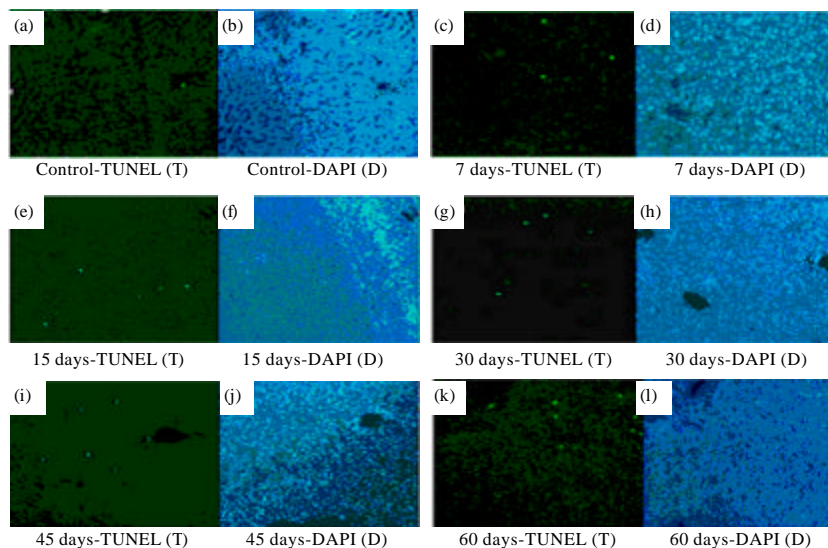| (i) | (j) | (k) | (l) |
| 45 days-TUNEL (T) | 45 days-DAPI (D) | 60 days-TUNEL (T) | 60 days-DAPI (D) |

Fig. 14(a-l): Detection of DNA strand breaks as cell death indicator by TUNEL assay in liver cells of guinea pigs orally administered carbonated soft drink along with vitamin C in clinical dose i.e., 20 mg day^{-1} per animal (10x magnification). The liver sections of the treated animals were stained with fluorescein labeled dUTP. In above figure, uniform cross section, depicts the DNA strand breaks in apoptotic cells as green fluorescent spots in the control and experimental liver tissue sections of the guinea pigs fed on water as control group along with vitamin C in therapeutic dose i.e., 20 mg day^{-1} while others were given carbonated soft drink supplemented with vitamin C in the same therapeutic dose in a time frame of 7, 15, 30, 45 and 60 days, respectively. Liver sections corresponding to fluorescein labeled dUTP stain were counterstained with DAPI to identify the cell nuclei. Here TUNEL stained samples are represented by 'T' and DAPI stained by 'D'

II: Studies of possible cellular, tissue and organ damage in liver, kidney and blood by carbonated soft drink *in vivo* and their prevention by vitamin C

IIA) Determination of carbonated soft drink induced cellular apoptosis in liver and kidney tissues in guinea pig *in vivo* and its prevention by vitamin C by TUNEL assay: Tissue sections from sacrificed animals fed with carbonated soft drink showed discernible apoptosis of liver and kidney cells as determined by TUNEL assay (Banerjee *et al.*, 2013) while the same cells from animals fed with carbonated soft drink supplemented with vitamin C showed significantly low levels of cell death (Fig.14 and 15) reconfirming that the observed increase in cell death in the liver and kidney of the carbonated soft drink treated animals was apparently due to oxidative damage induced by the consumption of such drinks.

IIB) Determination of carbonated soft drink induced cellular apoptosis in blood cells in guinea pig *in vivo* as measured by fluorescence-activated cell sorting (FACS) of annexin V and propidium iodide labeled whole blood cells and prevention by vitamin C: Whole blood cells of guinea pigs orally administered carbonated soft drink for the given experimental periods were subjected to flow cytometric studies in order to assess any possible apoptosis of such cells. Results, with reference to the experimental work Banerjee *et al.* (2013) show that blood cells from treated guinea pigs undergo a time-dependent increase in cellular apoptosis, which was significantly prevented by supplementation of vitamin C in such treated animals (Fig. 16).

Table 1: Percentages distribution of normal, early apoptotic, late apoptotic and necrotic cells

Days	Upper left quadrant (Q$_1$) Necrosis cell population (annexin V-FITC-/PI+)	Upper right quadrant (Q$_2$) Late apoptosis cell population (annexin V-FITC+/PI+)	Lower left quadrant (Q$_3$) Control cell population (annexin V-FITC-/PI-)	Lower right quadrant (Q$_4$) Early apoptosis cell population (annexin V-FITC+/PI-)
0	0.0000000	0.0000000	99.23 ±3.02	0.77±0.040
7	0.08±0.002	0.04±0.001	99.70±3.12	0.18±0.020
15	0.0000000	0.0000000	99.70±3.20	0.30±0.020
30	0.01±0.001	0.01±0.001	99.75±3.32	0.23±0.010
45	0.14±0.020	0.02±0.001	99.82±3.71	0.02±0.001
60	0.02± 0.001	0.01± 0.001	98.39±3.61	1.58±0.150

(a) Control-TUNEL (T) (b) Control-DAPI (D) (c) 7 days-TUNEL (T) (d) 7 days-DAPI (D)

(e) 15 days-TUNEL (T) (f) 15 days-DAPI (D) (g) 30 days-TUNEL (T) (h) 30 days-DAPI (D)

(i) 45 days-TUNEL (T) (j) 45 days-DAPI (D) (k) 60 days-TUNEL (T) (l) 60 days-DAPI (D)
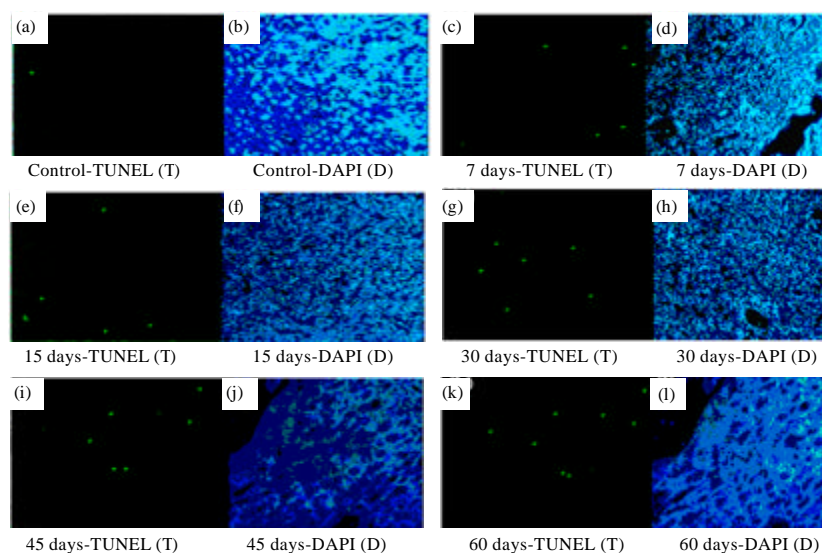
Fig. 15(a-l): Detection of DNA strand breaks as cell death indicator by TUNEL assay in kidney cells of guinea pigs exposed to carbonated soft drink along with vitamin C in clinical dose i.e., 20 mg day^{-1} per animal (10x magnification). The kidney sections of the treated animals were stained with fluorescein labeled dUTP. In the above figure, uniform cross section, depicts the DNA strand breaks in apoptotic cells as green fluorescent spots in the control and experimental kidney tissue sections of the guinea pigs fed on water as control group along with vitamin C in therapeutic dose i.e., 20 mg day^{-1} while others were given carbonated soft drink supplemented with vitamin C in the same therapeutic dose in a time frame of 7, 15, 30, 45 and 60 days, respectively. Kidney sections corresponding to fluorescein labeled dUTP stain were counterstained with DAPI to identify the cell nuclei. Here TUNEL stained samples are represented by 'T' and DAPI stained by 'D'

The annexin V-FITC/PI double staining assay was employed to classify blood cells in early apoptosis or late apoptosis stages, respectively.

Table 1 and Fig. 16 depict the apoptotic profiles of blood cells isolated from the guinea pigs administered carbonated soft drink and those treated with carbonated soft drink along with vitamin C supplementation, respectively for 0, 7, 15, 30, 45 and 60th day. Results, with reference to the experimental work by Banerjee *et al.* (2013) clearly show a time-dependent increase in early as well as late apoptotis in treated animals against those administered carbonated soft drink supplemented with vitamin C as in this work. Indicating that such degenerative change in the blood cells induced by consumption of carbonated soft drink is directly related to oxidative damage and can be prevented by vitamin C.
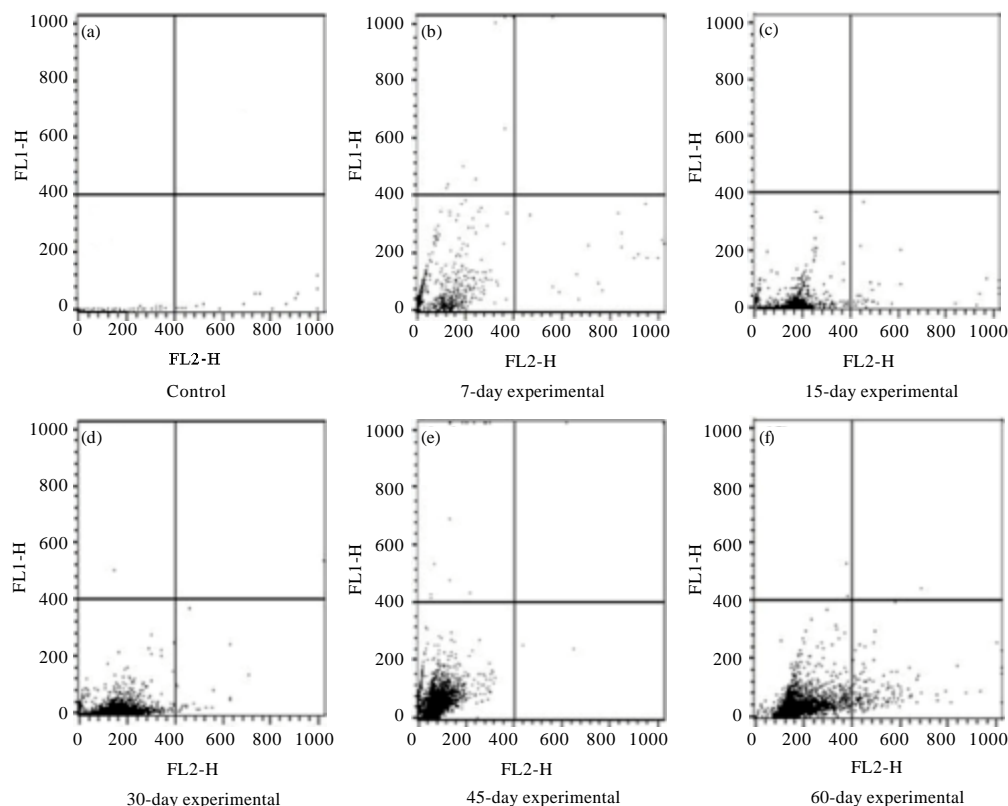
Fig. 16(a-f): Determination of carbonated soft drink induced cellular apoptosis in guinea pig *in vivo* as measured by Fluorescence-activated Cell Sorting (FACS) of annexin V and propidium iodide labelled whole blood cells and prevention of the same by vitamin C. Annexin V-FITC/PI stained blood cells was exposed to carbonated soft drink along with vitamin C for a time period of 0, 7, 15, 30, 45 and 60th day. Cells were then processed for annexin V-FITC/PI staining according to the protocols discussed under 'Material and Methods' and analyzed with flow cytometry. Here the picture marked as control are the blood cells of the guinea pig which has been given water as drink along with vitamin C in clinical dose and others are given soft drink as drink along with vitamin C in clinical dose for a time frame of 7, 15, 30, 45 and 60th day, respectively. The annexin V-FITC-/PI-population (Q3-lower left quadrant) was regarded as control cells, whereas annexin V-FITC+/PI-cells (Q4-lower right quadrant) were taken as a measure of early apoptosis, annexin V-FITC+/PI+ (Q2-upper right quadrant) as late apoptosis and annexin V-FITC-/PI+ (Q1-upper left quadrant) as necrosis. Results are representative of three independent experiments

IIC) Determination of carbonated soft drink induced liver enzyme damage in guinea pig *in vivo* and its prevention by vitamin C: Studies have been done to examine whether consumption of carbonated soft drink results in any adverse effects in the liver functioning of treated animals *in vivo* through activity assays of liver function indicator enzymes.

Indeed in consonance with the degenerative changes observed in liver cells of treated animals, marker enzymes for liver function tests show marked increase due to the feeding of carbonated soft drink *in vivo* while simultaneous feeding of vitamin C along with such drink decreases such activities during the exposure period as shown in following figures (Fig. 17-19).

IID) Histology and morphometric analysis for assessing cellular damage of liver tissue induced by carbonated soft drink: In the present study we have also attempted to examine
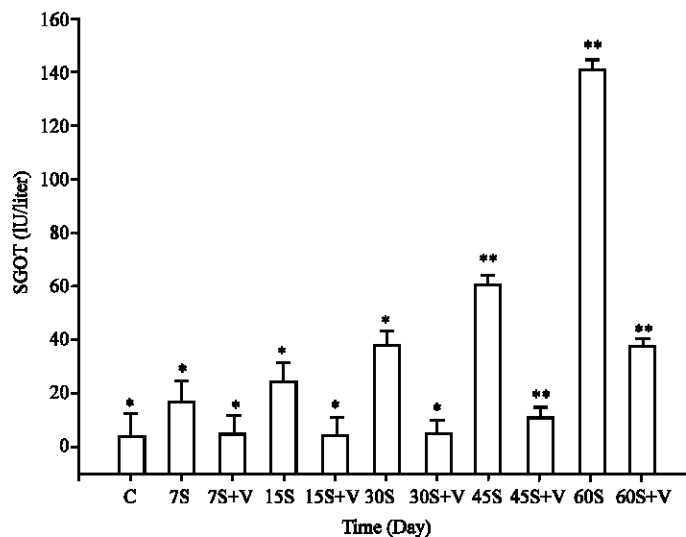
Fig. 17: Time dependent assay of serum glutamic oxaloacetic transaminase activity and effect of vitamin C treatment in guinea pigs fed with carbonated soft drink. Time-dependent liver tissue SGOT activities of guinea pigs orally administered carbonated soft drink and corresponding effect of vitamin C supplementation with such drink. Conditions of incubation and estimation of SGOT are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *represents $p<0.001$ and **represents $p<0.01$
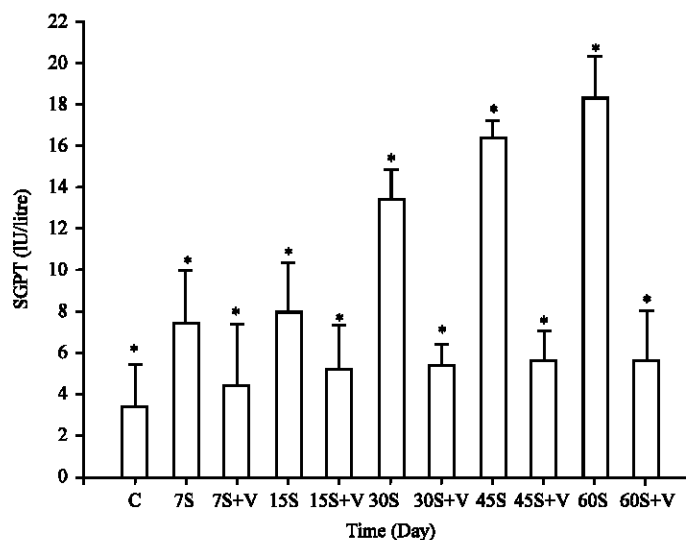

Fig. 18: Time dependent assay of serum glutamic pyruvic transaminase activity and effect of vitamin C treatment in guinea pigs fed with carbonated soft drink. Time-dependent liver tissue SGPT activities of guinea pigs orally administered carbonated soft drink and corresponding effect of vitamin C supplementation with such drink. Conditions of incubation and estimation of SGPT are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *represents $p<0.001$

whether consumption of carbonated soft drink results in any cellular damage of liver tissue of treated animals *in vivo* through histology and morphometric analysis.

Tissue sections from sacrificed animals fed with carbonated soft drink showed little to no histology and morphometric change as determined by histology and morphometric analysis
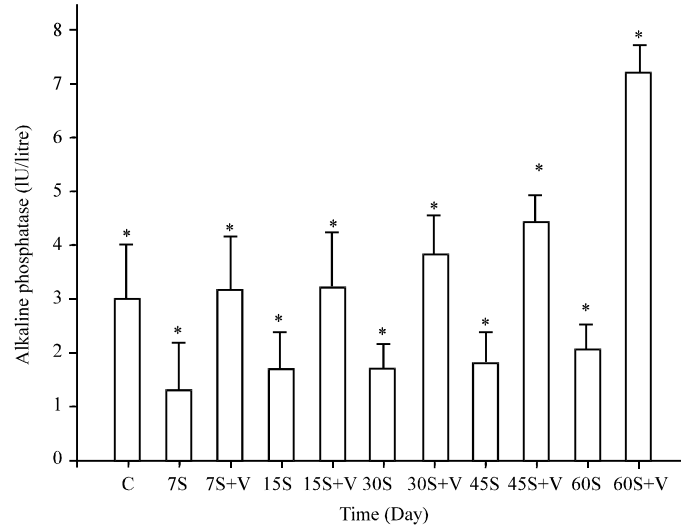
Fig. 19: Time dependent assay of alkaline phosphatase activity and effect of vitamin C treatment in guinea pigs fed with carbonated soft drink. Time-dependent liver tissue alkaline phosphatase activities of guinea pigs orally administered carbonated soft drink and corresponding effect of vitamin C supplementation with such drink. Conditions of incubation and estimation of alkaline phosphatase are described under 'Materials and Methods'. Here soft drink is represented by 'S' and vitamin C as 'V'. *represents $p < 0.001$
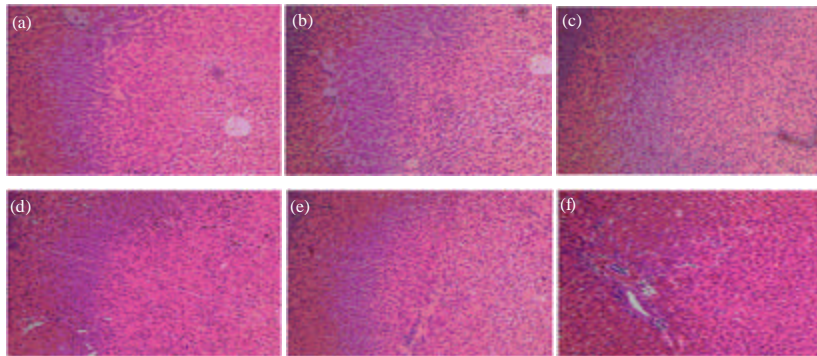

Fig. 20(a-f): Histology and morphometric analysis for assessing cellular damage of liver tissue induced by carbonated soft drink (10x). The set of guinea pigs were exposed to water or carbonated soft drink (as described under Materials and Methods) before being sacrificed on 0, 15, 30, 45 and 60th days, respectively of water/soft drink exposure, (a) Control picture represents guinea pigs given water as the drink, (b) 7 Days picture represents guinea pigs given carbonated soft drink as the drink for 7 days, (c) 15 Days picture represents guinea pigs given carbonated soft drink as the drink for 15 days, (d) 30 Days picture represents guinea pigs given carbonated soft drink as the drink for 30 days, (e) 45 Days picture represents guinea pigs given carbonated soft drink as the drink for 45 days and (f) 60 Days picture represents guinea pigs given carbonated soft drink as the drink for 60 days. Here a subclinical dose of vitamin C i.e., 2 mg was given to all guinea pigs to prevent scurvy. The figure represents best of three different sets of experiments, each set consisting of control and experimental

(Fig. 20) while again the same cells from animals fed with carbonated soft drink supplemented with vitamin C showed almost no change (Fig. 21) indicating the fact that though carbonated soft drink have a significant damage effect upto enzymatic level in organ like
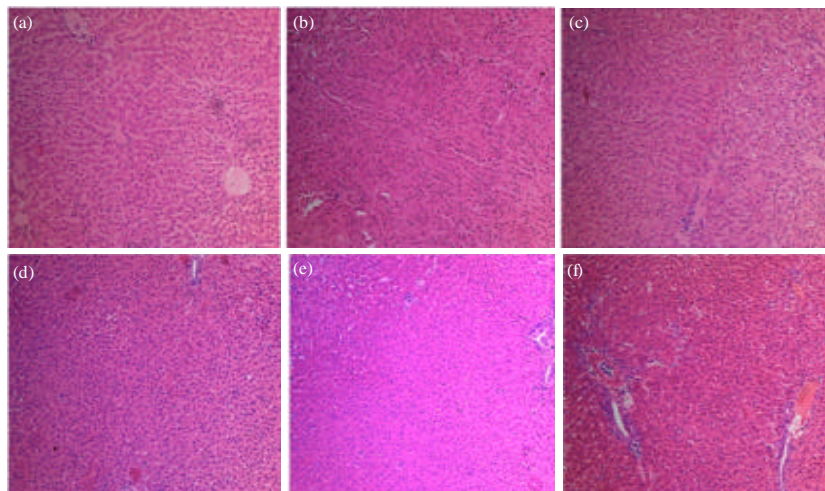
Fig. 21(a-f): Histology and morphometric analysis for assessing cellular damage of liver tissue induced by carbonated soft drink (10x). The set of guinea pigs were exposed to carbonated soft drink along with 20 mg vitamin C (as described under Materials and Methods) before being sacrificed after 0, 15, 30, 45 and 60th days, respectively, (a) Control picture represents guinea pigs given only 20 mg vitamin C dissolved in water as the drink, (b) 7Days picture represents guinea pigs given carbonated soft drink as the drink along with 20 mg vitamin C for 7 days, (c) 15 Days picture represents guinea pigs given carbonated soft drink as the drink along with 20 mg vitamin C for 15 days, (d) 30 Days picture represents guinea pigs given carbonated soft drink as the drink along with 20 mg vitamin C for 30 days, (e) 45 Days picture represents guinea pigs given carbonated soft drink as the drink along with 20 mg vitamin C for 45 days, (f) 60 Days picture represents guinea pigs given carbonated soft drink as the drink along with 20mg vitamin C for 60 days. The figure represents best of three different sets of experiments, each set consisting of control and experimental

liver in treated animals it had apparently almost no adverse effect on the organ as a whole. This establish the fact that carbonated soft drink has a degenerative effect but to a certain degree, which is almost restricted upto cellular to enzymatic level in organs like liver and kidney.

DISCUSSION

Studies published from this laboratory earlier (Banerjee *et al.*, 2013) have shown that carbonated soft drink contain oxidants those are capable of oxidative damage of liver, kidney and serum protein in guinea pigs which cannot synthesise vitamin C, when administrated with such drink in measured doses.

Oxyblot assay as described earlier (Banerjee *et al.*, 2013) showed both liver tissue protein oxidation and protein degradation in guinea pigs when fed with carbonated soft drink which contains excess of sugar. That excess sugar consumption increases production of destructive free radicals and lower level of key antioxidants have been reported by Mohanty *et al.* (2000). Type II diabetes resulting from high sugar consumption is also associated with an increase in free radical generation, leading to damage of fats, proteins and DNA (Tappy and Le, 2010). High fructose feeding in rats showed increased level of oxidative stress marked and increased reactive oxygen species in circulatory leukocytes (Al-Awwadi *et al.*, 2005).

Oxidative damages as observed here in guinea pigs fed on carbonated soft drink can be protected by supplementation of vitamin C. Similarly carbonyl, bityrosine formation and loss of tryptophan and thiol residues under carbonated soft drink consumption are prevented by vitamin

C as have been observed here. Increased in lipid peroxide and conjugated dienes formation under such condition is ameliorated to a significant extend by administration of vitamin C as have been reported here. Carbonated soft drink consumption on lipid peroxide activity in serum and liver of animals has also been reported (Awhin, 2012).

Guinea pigs on carbonated soft drink have been shown to undergo a time dependent cellular apoptosis. The increase is though small but was noted in early apoptosis period between 30-60 days as reported from this laboratory (Banerjee *et al.*, 2013). Certain polysaccharides have shown induced apoptosis of lymphoma cell line (Hattori *et al.*, 2004). Vitamin C supplementation to carbonated soft drink fed guinea pigs as observed in the studies reported here have shown marked decrease in cellular apoptosis.

Carbonated soft drink consumption brings about changes in the activities of alanine amino transferase and aspertate amino transferase in serum and kidney in rats (Jeroh *et al.*, 2012) and also on alkaline phosphatase activity in serum and liver of animal (Awhin, 2012).

Studies have been done and reported here on the liver function tests of guinea pigs fed on carbonated soft drink and then supplemented with vitamin C. Marker enzymes like SGOT, SGPT and alkaline phosphatase have shown increase in activities when guinea pigs were fed on carbonated soft drink, which could be prevented by supplementations with vitamin C.

The above results indicate that the overall efficacy of vitamin C plays important role as an antioxidant for prevention of oxidative damage as well as cellular and organ damage induced by consumption of carbonated soft drink. This establishes a strong causal relationship between oxidative damage induced by carbonated soft drink and its consequence cytotoxic effects if not protected by sufficient antioxidant.

CONCLUSION

Carbonated soft drink consumption to guinea pigs causes oxidative damages to liver, kidney and serum proteins with apoptosis of the tissues which can be prevented by supplementation of vitamin C along with soft drink.

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