RESEARCH ARTICLE

High-casein diet restores the redox balance in the liver and pancreatic health of mice when exposed to radiation during call mode from mobile phones

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Abstract

Introduction: The current lives of human beings cannot be imagined without cellular phones and electronic gadgets. Still, the probability of being exposed to their harmful radiation is unabated which demands an immediate intervention to shield humankind from being affected by using such devices. In our current investigation, we aimed to check the role of a high-casein diet as a protective measure against the biological effects of electromagnetic radiation (EMR) emerging from mobile phones on hepatic and pancreatic systems *Methods:* Swiss albino mice (n=24) were fed on a standard laboratory diet (ND; n=12) and an isocaloric high-casein diet (HCD; n=12). Six mice from each dietary group were exposed to mobile phone radiation for 3 hours/day within a 3 months span. Histopathological alterations were studied in hepatic and pancreatic tissues by using periodic acid-schiff (PAS)-hematoxylin and routine H&E staining methods. Biochemical evaluation of total serum protein, lipid profile and glucose, total glutathione, oxidized glutathione, and antioxidant enzymatic activities of hepatic tissues were performed. *Results:* Hyperglycemia was noticeable along with a reduced number and area of islets of Langerhans in radiation-exposed mice when compared to other groups. This was supported by the observation of depleted glycogen reserves in their liver. Additionally, distorted hepatic morphology with nuclear degeneration was suggestive of apoptotic progression. The total glutathione pool was reduced on radiation exposure, hint at redox imbalance. HCD was competent in preserving the normal pancreas and liver functioning in radiation-exposed mice.

Keywords: Casein, Electromagnetic radiation, Glutathione, Karyorrhexis, Redox imbalance, Triglyceride.

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Introduction

The rise in mobile phone usage in the modern world was inevitable since its inception but digitalization and the recent global pandemic have increased its usage exponentially. The resourcefulness and multitasking facilities of mobile phones have proven to be addictive over due course of time. However, it has now been converted into an indispensable commodity along with other electronic gadgets for adults who have to work from home and children who depend on online classes and exams. Therefore, the propensity to be exposed to its irradiated electromagnetic waves has heightened noticeably and culminated in its fatal effects at the system, cellular, and genetic levels.[1] With the introduction of 5G technologies, the issues of pre-existing electromagnetic radiation (EMR) exposure will exacerbate at environmental and personal levels. Safety limits of the exposure to non-ionizing radiation, stipulated by the responsible bodies, merely protect the industry that manufactures electronic gadgets, thereby neglecting environmental homeostasis and human health.[2]

Electrohypersensitivity (EHS) is now a recognized clinical condition in many countries of the European Union and Canada, and Sweden with the soaring electropollution.[3] The term EHS was primarily coined by William Rea in 1991 and designated a new clinical condition where patients reportedly complained of health issues when exposed to electromagnetic fields.[4] It has been estimated that 3-5% of the population in several countries is affected by EHS, so millions of people worldwide may be affected by EHS.[5]

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EMR from mobile phones can manifest adverse effects on cerebral blood flow[6] and the permeability of the blood-brain barrier (BBB)[7] in humans. Their repercussions are observed as the loss of attention and immediate memory, tinnitus, elevated levels of anxiety, emotivity, and impairment of other cognitive functions. Recent reports have claimed increased aggression among teenagers addicted to games on mobile phones.[8] *In-vivo* studies have shown that mobile radiation affects different regions of the brain, induces diabetes-like changes, and disrupts redox balance in hepatic

and reproductive systems thereby hampering physiological homeostasis.[9-12]

Radiation generated from mobile phones is known to have deleterious effects in the form of both thermal and non-thermal effects but only a handful of studies show that it enhances the signs of diabetes in humans. A randomized human study in Pakistan by Zafar *et al.* showed elevation of hyperglycemia in diabetic patients after calling with mobile phones for 15 minutes/day in 2 months.[13] In this light, a randomized study was conducted by our laboratory in Kolkata where 63% of residents of an area within the radius of 50 m from a radio-transmitting mobile tower showed moderate to severe hyperglycemia when compared to controls [unpublished data]. This led us to hypothesize whether EMR can induce toxic effects on pancreatic and hepatic systems and to find a possible remedy in the form of an inexpensive diet.

High-casein diet (HCD) is isocaloric in nature and fortified with casein, which comprises 70–80% of milk protein and is considered a complete protein source because all essential amino acids are available. It is hydrophobic in nature and gets digested slowly and causes the slow release of amino acids into the bloodstream. Previous research shows that casein has potent antioxidant activity *in-vitro;* however, studies that show its mechanism of action to perpetuate *in-vivo* antioxidant activity upon induction of stress by EMR exposure are scarce and inconclusive. [11, 14, 15]

Materials and Methods

Reagents

Sodium chloride, sulfosalicylic acid, tris-HCl and triethanolamine were purchased from Sisco Research Laboratories, India. Casein was obtained from LOBA Chemi, India. 2-vinylpyridine, 5.5'-dithiobis-2-nitrobenzoic acid (DTNB), GSH, GSSG, and β-NADPH were procured from Sigma Aldrich. Assay kits for the estimation of glucose, protein, total cholesterol, and triglycerides were bought from ARKRAY Healthcare Pvt. Ltd. Surat (India).

Selection of Animal and Care

Total 24 adult, male swiss albino mice (*Mus musculus*) weighing 20 $\pm$ 10 g were used for this study. They were kept under standard laboratory conditions (12 h:12h light/dark cycle, 25 ± 2˚C) with food and water *ad libitum*. This study was ethically approved by the Institutional Animal Ethics Committee (IAEC) of Presidency University [Approval No.: PU/IAEC/MS/21] and all animal experiments were performed according to the guidelines laid down by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Government of India.

Radiation Exposure

Animals moved freely when they were exposed to EMR in Plexiglas cages *via* a global system for mobile (GSM) communication handsets (Model: Micromax Bharat 2, Q402) marked with specific absorption rate (SAR) of 1.5 W/kg at 1g body weight. The handsets were connected to 4G mode of the Indian communication network (Jio, India), generally at a frequency range of 1.8–2.3 GHz, kept at 100% battery charge before radiation every day and they were set on call answered mode to irradiate the animals for 3 hours/day.

Experimental Design

Animals were divided randomLy into four groups containing six animals each and they were treated as mentioned in Table 1. Groups ND and HCD were maintained only on a normal diet (ND) and HCD, respectively and considered as control groups of the experiment. Animals in group ND + R3 were fed on ND and group HCD + R3 were fed on the HCD diet. Both the groups ND + R3 and HCD + R3 were exposed to mobile phone-generated EMR for a period of three months. The compositions of the two in-house diets are given in Table 2 formulated in our laboratory and as described by Bhattacharya *et al.*[11] The day following the cessation of the treatment period, animals were anesthetized prior to euthanization with ketamine hydrochloride intraperitoneally (IP). The blood was collected after cardiac puncture and the pancreas and liver tissues were collected carefully for histological analysis and biochemical assays.

Histoarchitectural Study

The liver and pancreas were carefully removed from the animals of all groups and fixed in buffered formal saline for subsequent hematoxylin-eosin (H&E) staining.[10] A portion of the liver was preserved in Bouin's fixative for periodic acid-schiff (PAS) hematoxylin staining.[16]

H&E Staining

The tissues were then dehydrated with graded alcohol, cleared in xylene before embedding in paraffin wax (melting point 56°C). Serial sections of approximately 4–5 µm thickness were cut in a rotary microtome (Medimeas, MRM1120) and then passed through xylene followed by rehydration with graded ethanol (100, 90, 70%) and water. The sections were stained with hematoxylin, followed by blueing under tap water, dehydrated with graded ethanol, countered-stained with eosin, cleared in xylene and mounted in Canada balsam. The slides were left to air dry before observation under the light microscope (Zeiss Axioscope A1, Thornwood, NY, USA).

PAS-hematoxylin Staining

PAS staining is generally used to detect glycogen and other polysaccharides. The liver sections were dewaxed, rehydrated and treated with 0.5% periodic acid for 5 minutes. After several episodes of washing, these sections were covered with schiff's cold solution for 15 minutes and washed under running tap water for 10 minutes. The sections were counter stained with hematoxylin for 1.5 minutes, followed by blueing under tap water and dehydrated using graded alcohol and clearing in xylene. The sections were mounted in Canada

balsam and viewed under a light microscope (Zeiss Axioscope A1, Thornwood, NY, USA).

Serum Glucose Estimation

Glucose levels in serum were estimated using the glucose oxidase and peroxidase (GOD-POD) method. The glucose oxidase enzyme converts glucose into gluconic acid and hydrogen peroxide. The latter is coupled with phenol and 4-aminoantipyrine (4-AAP) to form colored quinoneimine dye. The absorbance of colored dye was measured at 505 nm (UV-2600, Shimadzu, Europe) and is directly proportional to glucose concentration in the sample. In brief, 10 µL of serum sample and standard were added separately to the 1000 µL of Working Glucose Reagent (100 mM phosphate buffer, glucose oxidase, peroxidase, 4-AAP, 10 mM phenol) and then incubated for 10 minutes at 37°C leading to the formation of red adduct which is proportional to the glucose present in the samples.

Serum Protein Estimation

Total protein in serum was measured using the modified Biuret method. The cupric ions in the reagent react with the peptide bonds in protein, in an alkaline solution to form a colored chelate. The absorbance of this chelate was measured spectrophotometrically at 578 nm (UV-2600, Shimadzu, Europe). Briefly, 10 µL of the sample and standard were added separately to the 1000 µL of Biuret reagent (7 mM $CuSO_4$, 200 mM NaOH, 20 mM sodium-potassium tartrate) and mixed well. The absorbance of the final color after incubation at 37°C for 5 minutes was proportional to the total protein concentration in the sample.

Estimation of Enzymatic Antioxidants

Superoxide dismutase (SOD) in hepatic tissues was measured biochemically.[17] The tissue was homogenized in an ice-cold 0.1M potassium phosphate buffer (pH 7.2) at a 50 mg/mL tissue concentration. The homogenate was then centrifuged at 10,000 x*g* for 20 minutes at 4°C. The enzyme activity was assessed in a medium containing 0.05M potassium phosphate buffer with 0.5 mM ethylenediaminetetraacetic acid (EDTA). The auto-oxidation of hematoxylin with or without adding the supernatant of tissue homogenate was estimated at 560 nm in a UV-vis spectrophotometer (UV-2600, Shimadzu, Europe) and the percentage of inhibition was recorded. 50% inhibition of auto-oxidation is equivalent to the activity of 2 units of SOD and the percent change was used to determine the units of SOD in the samples.

Catalase (CAT) activity in the hepatic tissue was estimated biochemically.[18] Hepatic tissue was homogenized in an ice-cold 0.05 M Tris-HCl buffer (pH- 7.0), maintaining a tissue concentration of 50 mg/mL. The mixture was centrifuged at 10,000g x 20 minutes at 4°C and the supernatant was collected. 2.5 mL double distilled water and 0.5 mL 0.35 mM H_2O_2 solution were mixed well in a spectrophotometric cuvette and the absorbance was at 240 nm (UV-2600, Shimadzu, Europe). To this mixture, 40 µL of the supernatant was added, mixed well and six readings were taken at 30 second intervals. The activity of catalase was expressed in µM H_2O_2 consumed per mg tissue per minute.

Estimation of Total Reduced and Oxidized Glutathione

Total reduced glutathione (GSH) and oxidized glutathione (GSSG) was estimated by the method described by Rahaman *et al.*[19] The protocol is based on the thiol-mediated conversion of Ellman's reagent (DTNB) to 5-thio-2-nitrobenzoic acid (TNB). The formation of TNB is proportional to the GSH concentration in the sample monitored spectrophotometrically at 412 nm.

Lipid Profile Estimation

Total cholesterol (TC) was estimated using the cholesterol oxidase and peroxidase (CHOD-POD) method. The cholesterol esters were hydrolyzed to cholesterol by cholesterol esterase (CE) and the CHOD enzyme converted cholesterol into cholest-4-en-3-one and hydrogen peroxide which gave a colored product with phenol and 4-aminoantipyrine (4-AAP). Absorbance was measured at 505 nm (UV-2600, Shimadzu, Europe) and was directly proportional to the cholesterol concentration in the sample. Briefly, 10 µL of serum sample and 200 mg/dL standard were added separately to the 1000 µL of working cholesterol reagent (Good's buffer [pH 6.7], CE, CHOD, POD, 4-AAP) followed by mixing and incubation at 37˚C for 10 minutes.

High density lipoprotein (HDL) was estimated by mixing equal volumes of serum samples and 200 mg/dL of polyethylene glycol 6000. The mixture was kept at room temperature for 10 minutes and centrifuged at 2000 rpm×10 minutes to collect the clear supernatant. Subsequently, 10 µL of supernatant was then used in place of the serum sample and the previous protocol to estimate TC was followed against 50 mg/dL HDL standard.

Triglycerides (TG) were hydrolyzed by lipoprotein lipase (LPL) to produce glycerol and free fatty acids (FFA), the former was further phosphorylated to produce glycerol-3-phosphate which reacts with oxidase to form dihydroxyacetone phosphate and H_2O_2 that forms a colored product in the subsequent step. In short, 10 µL of the serum and 200 mg/dL of standard triglyceride were mixed with 1000 µL of working triglyceride reagent (50 mmol/L Pipes buffer, 5 mmol/L 4-chlorophenol, 5 mmol/L Mg^{++}, 1 mmol/L ATP, lipase, peroxidase, glycerol kinase, 0.4 mmol/L 4-AAP, glycerol-3-phosphate oxidase) and incubated at 37˚C for 10 minutes and the absorbance readings were recorded at 505 nm in a UV-Vis spectrophotometer (UV-2600, Shimadzu, Europe).

Statistical Analysis

All data were expressed in terms of mean ± SE. For statistical interpretation, each parameter's quantitative data from various groups was analyzed by one-way ANOVA followed by PostHoc and Tukey HSD analyses to determine the significance level. *P-values* less than 0.05 were considered significant.

Results

HCD Protects Against Hyperglycemic Changes Induced by Mobile Phone Radiation

Mobile phone radiation caused an increase in the serum glucose levels of animals fed on ND whereas HCD was able to counter such elevation of glucose levels in the blood ($p < 0.01$) but was significantly altered from the control levels ($p < 0.01$) (Figure 1A). The total protein levels in the blood remained unaltered in all the groups ($p > 0.05$) (Figure 1B).

HCD Prevents the Shrinkage of Islets of Langerhans of the Pancreas of Radiation-Exposed Groups

The normal sections of the pancreas usually depict a generous number of islets of Langerhans lying amidst pancreatic acini as seen in the control pancreas (Figure 2a). The average size of the islets of Langerhans is reduced in the radiation-exposed animals maintained on the ND (Figure 2b). Moreover, the number of functional islets of Langerhans is also less. The average size of the islets of Langerhans was compensated in the case of animals exposed to radiation and kept on HCD (Figure 2c).

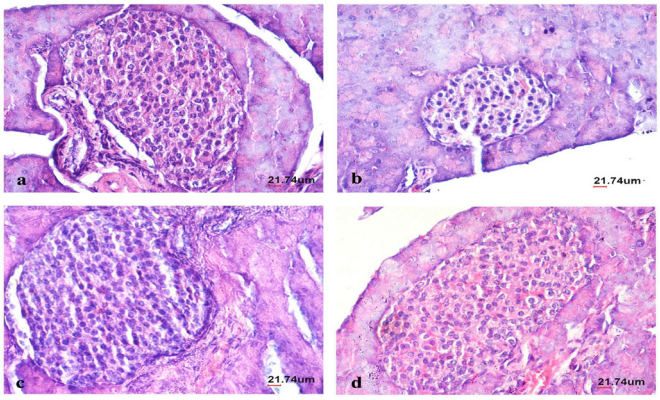

Figure 2: Pancreatic histoarchitecture as assessed by H & E staining. (a) corresponded to the ND control group. (b) The atrophy of the islets of Langerhans is represented in the pancreatic section of the ND + R3 group. (c) HCD prevented the atrophy of the islets in the radiation-exposed HCD + R3 group. (d) The feature of normal islets and pancreatic acini in the HCD control group

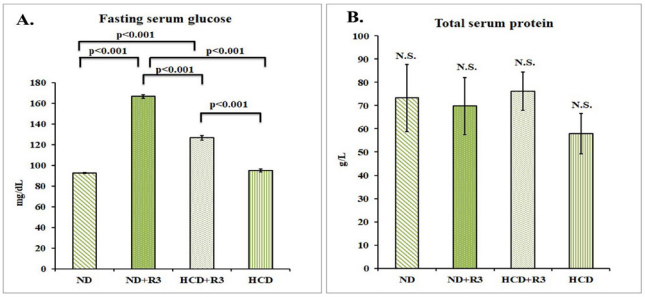

Figure 1: Graphical representation of fasting serum glucose (A.) and total serum protein levels (B.) in control and radiation-exposed groups of mice fed on ND and HCD.

HCD Prevents the Loss of Glycogen Storage Capacity in the Liver Due to Radiation Exposure

The control mice kept on ND showed regular morphology of the liver parenchyma with distinct hepatic lobules highlighted with regions of stored glycogen upon PAS-hematoxylin staining (Figures 3a and 4a). The hepatic sections of ND + R3 mice revealed a deficit in glycogen storage capacity as indicated by their pale-pinkish appearance (Figures 3b and 4b). The nuclear disintegration is quite apparent in these tissues with larger frequencies in the hepatocytes around the central canal with prominent karyorrhectic and karyolytic changes (Figure 4b). HCD, on the other hand, has protected against the depletion of glycogen storage in the liver of radiation-exposed mice (Figure 3c and 4 c). The histoarchitecture and characteristics of mice fed on HCD only were at par with that of the ND control group (Figures 3d and 4 d).

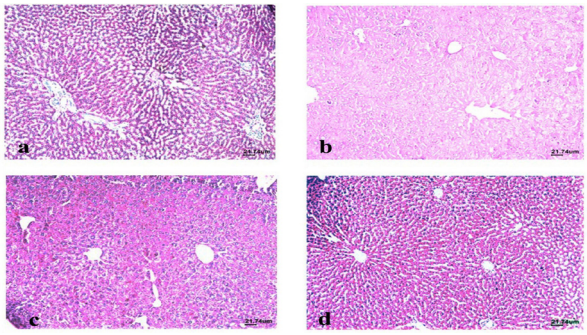

Figure 3: Photomicrographs highlighting the glycogen deposition when stained with PAS-hematoxylin. (a) Groups of mice fed on HCD (b) Pale appearance of the hepatocytes is indicative of the compromised glycogen storage capacity of mice in the ND + R3 group (c) with respect to that of HCD + R3 group. (d) had glycogen storage capacity and hepatocyte morphology comparable to the controls group fed on ND.

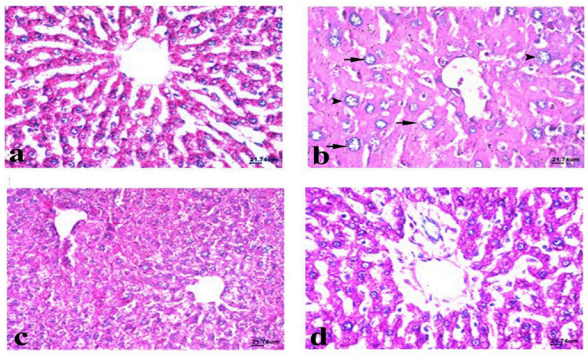

Figure 4: Photomicrograph of liver section of various groups stained with PAS-hematoxylin. On EMR exposure to ND fed mice, the hepatocytic cords were indistinct and the sinusoidal spaces were disorganized. (a) Features of the regular hepatic parenchyma, nucleus and distinct sinusoidal spaces were observed in both ND and HCD Signs of karyolysis (thin, black arrow) and karyorrhexis (black arrowhead) were prominent in ND + R3 group (b). (c) HCD helped to prevent the karyolytic and karyorrhectic alterations due to EMR exposure in HCD + R3 mice group. (d) groups.

Table 1: Design of experimental animal models

Groups	*Diet*	*Mobile Phone Generated EMR Exposure*
ND	Normal diet	-
ND + R3	Normal diet	+
HCD + R3	High-casein diet	+
HCD	High-casein diet	-

Table 2: Formulation of two sets of diets

%Mass	*Composition*	*Normal Diet (%)*	*High Protein Diet (%)*
Proteins	Wheat meal	4.6585	4.719
	Chick peas	10.4625	6.975
	Casein	5	20
	TOTAL	20.12105	31.694
Carbohydrates		53.7355	45.077
Lipids		8.0725	7.275
Vitamins and Minerals		7.22	7.20
Energy		367.37 kcal	370.38 kcal

Adapted from Bhattacharya et al., 2020.[10]

HCD Compensates for the Hepatic Redox Imbalance on Exposure to EMR from Mobile Phones

The complex redox status in the hepatic system of the mice exposed to radiation reveals elevated SOD activity (Figure 5A). In contrast, the CAT activity is downplayed in the animals of the ND + R3 group (Figure 5B). It is remarkable to note that the total GSH store has drained to a striking level in the radiation-exposed groups when compared to the controls (Figure 5C). In addition, GSSG levels spiked up in the liver of the mice belonging to the ND + R3 group (Figure 5D).

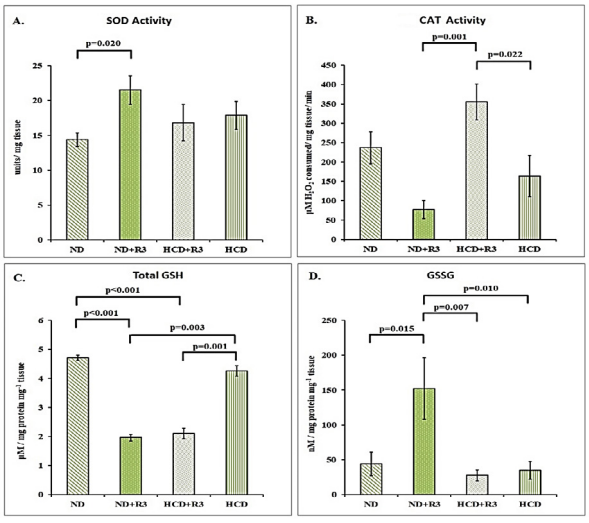

Figure 5: Evaluation of ROS-scavenging enzymes and glutathione levels.

Table 3: Estimation of lipid profile.

Parameters	*ND*	*ND + R3*	*HCD + R3*	*HCD*
TC (mg/dL)	74.84 ± 8.65	64.75± 10.79	44.85 ± 7.31	62.26 ± 14.77
HDL (mg/dL)	20.49 ± 4.44	13.285± 1.89	22.39 ± 3.35	31.13 ± 3.81
LDL (mg/dL)	34.62 ± 9.27	25.96 ± 5.46	18.82 ± 6.19	32.95 ± 9.19
Glycerol free TG (mg/dL)	108.26 ± 9.67[b]	161.54 ± 5.43[a]	41.67 ± 6.11[a,b]	67.64 ± 10.43[a, b]

Value bearing superscripts are significantly different by ANOVA analysis ($p < 0.05$). a with respect to Group ND; b with respect to Group ND + R3.

HCD Reduces the Hike in Free Triglyceride Levels in the Blood due to Cellular EMR Exposure

The levels of TC, HDL and LDL did not vary significantly among the groups of mice. However, the increase in the glycerol-free TG in the serum of the mice of the ND + R3 group was outstanding (Table 3).

Discussion

The drawbacks of using mobile phones has been established before. Still, they only became obvious, especially after the onset of the global pandemic in 2019.[5] During the pandemic, we distanced ourselves physically. Still, we were brought together virtually via the internet and phone conversations. Hence, mobile phones and other electronic gadgets have eased our difficulties in coping with the unprecedented situation by shifting our work and education to a virtual platform. This, in turn, brings humans close to electrical gadgets for a fairly long time; especially the cell phones that we place near our ears during on-call conversations, which gradually threaten human health.

The mice were irradiated in a freely moving condition since the duration of radiation exposure per day is long. Any restraint during this period could affect the results of the experiment by inducing more stress on the experimental animals. The condition of the radiation exposure was kept at par with their natural laboratory environment to mimic the real-life scenario while using mobile phones for calling purposes.

The plasma glucose level's elevation is noteworthy among mobile or cordless phone users.[3, 20] Similar observations were found in our previous unpublished study among human subjects and also in the current study. The protein levels in blood did not increase significantly, which indicates that physiological homeostasis is maintained in all the groups of the animals to keep the protein levels at normal. It might be possible that HCD provides a constant and elevated supply of amino acids which, on entering the cells, might aid in gluconeogenesis and restore pancreatic functions to compensate for the loss of glucose metabolism.

Karyorrhexis is a phenomenon where the nucleic acids are destroyed and fragmented, whereas karyolysis is mediated

by nucleases of the dying cells' lysosomes to dissolute the nuclear material.[21] In this study, we found karyorrhectic and karyolytic alterations in hepatocytes which supports our previous finding of DNA damage and apoptosis progression in hepatic tissue and other studies dealing with a chemical stressor like chloroacetonitrile.[11,22] Drastic induction of death of hepatocytes validates the incompetence to fulfill the glycogen stores in the liver, successfully restored when mice were maintained on HCD on simultaneous exposure to radiation.

The GSH reserve once depleted was not restored by mitigation with antioxidants such as vitamins E and C as documented by Vaziri *et al.* in 2000 when a GSH synthase inhibitor induced the oxidative stress in a hypertensive model of rats.[23] This validates the result obtained in this study i.e., the depletion of GSH storage in hepatic tissue on exposure to radiation in both the groups fed on different diets. This implies the deleterious effect of oxidative stress induced by the radiation. Surprisingly, the GSSG level was elevated only in the radiation-exposed group maintained on ND with respect to that fed on HCD. This proves that HCD had an antioxidant effect on the hepatic system, even when the mice were exposed to radiation and it might be manifested by the free-radical scavenging and metal-chelating casein-derived peptides, which is reflected by the alterations in the levels of antioxidant enzymes, SOD and CAT activities, as they helped to reduce oxidation of the GSH.[14] The contrasting activities of SOD and CAT in the liver of the radiation-exposed mice seemed to be confounding. However, this phenomenon can be well understood by the results from the study conducted by Turk *et al.*, where a hike in SOD and diminution of CAT activities were due to the feedback mechanism to compensate for the antioxidant systems in Type-2 diabetes patients.[24] Although lipid profiles are known to be high in diabetic patients with insulin resistance, except for HDL levels, we have encountered a significant elevation of TG levels only in the blood of the radiation-exposed group administered with ND which was consistent with reports of Schaefer *et al.* in 2002.[25, 26]

Limitations of the Study

The limitation of this study is that a diabetic model of mice was not included to compare the alterations among the groups since we had to abide by the restrictions on number of animal use imposed by IAEC. We could not check the insulin level because the infinitesimal amount of blood acquired from the mice did not allow us to conduct more experiments.

Conclusion

Our findings suggest an inclination towards the progression of Type-2 diabetes mellitus among radiation-exposed mice which may be facilitated by being exposed to radiation from mobile phones. Hence, there is a need for extensive future research in this aspect to prevent such perturbations from affecting our daily lives and the environment. The HCD, through our study, is highlighted as one such precautionary measure to be taken meanwhile to protect from any uninvited physiological disorders acquired on exposure to radiation from mobile phones when they are being used.

Conflict of Interest

The authors declare that there is no conflict of interest.

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During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.