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Evaluation of Smokeless Tobaccos-induced Hepatotoxicity and Nephrotoxicity in Swiss Albino Mice

Grimchi T. Sangma, Manash P. Borah, Ibanrikynti Turnia and Surya B. Prasad*

Cell and Tumor Biology Laboratory, Department of Zoology, School of Life Sciences, North-Eastern Hill University, Umshing-Mawkynroh, Shillong – 793022, Meghalaya, India; grimchisangma@gmail.com, manashpborah30@gmail.com, turnia77@gmail.com, sbnehu@gmail.com

Abstract

The massive global increase in tobacco use has become a public health problem worldwide. Gutka or gutkha and zarda are some of the widespread smokeless tobacco products commonly consumed in India. The present study aims at analyzing the histopathology and biochemical changes in the liver and kidney of Swiss albino mice following the treatment with some smokeless tobaccos. The animals in different groups were administered with low and high doses of aqueous solutions/filtrates of a gutkha and a blend of combined pan masala and S-FOUR zarda daily by oral gavage for a month. After treatment, the liver and kidneys were collected for histopathological examination through light and transmission electron microscopy and reduced glutathione determination. At the same time, blood samples were collected for measuring the serum marker enzymes/biochemicals for liver and kidney functions. Histological and ultrastructural observations of the liver and kidney showed marked deleterious alterations in the structural architecture, suggesting the development of cytopathological tendency of the tissues after treatment with smokeless tobaccos. Serum biomarker enzymes for liver and kidney functions showed a considerable increase in all the parameters, thus, indicating adverse effects on their functional capabilities which substantiate the structural variations and development of hepatotoxicity and nephrotoxicity after treatment. The treated animals showed a significant decrease in GSH levels in these tissues which may cause decreased antioxidant and protective ability and may aid in developing the toxicity in the hosts. These toxicities could be attributable to various chemical constituents present which are needed to be analyzed and made known on the commercially available smokeless tobacco products/packets. It is suggested that addictive use of smokeless tobaccos could be a risk factor for many diseases and in developing countries like India, recognition of harmful consequences of their recurrent usage should be widely known to the public.

Keywords: Glutathione, Hepatotoxicity, Nephrotoxicity, Smokeless Tobacco

1. Introduction

Tobacco is an agricultural product processed and prepared from the leaves of the tobacco plant of the genus *Nicotiana* in the family *Solanaceae*. The majority of tobacco products available in the market are made mainly from two species of the tobacco plant, *Nicotiana tabacum and Nicotiana rustica* which are cultivated extensively in many countries around the world[1]. India is the second-most populous country and the third-largest producer and consumer of tobacco in the world[1]. There is an enormous global increase in tobacco use and the tobacco epidemic is one of the biggest public health threats the world has ever faced. In 2008, the World Health Organization (WHO) named tobacco as the world's single greatest preventable cause of death and estimated that by 2030, more than 80%

*Author for correspondence

of tobacco deaths will be in developing countries[2]. Tobacco products can be divided into smoked tobaccos (combustible tobaccos) and smokeless tobaccos. Smoked tobaccos require burning prior to consumption and are available in the forms of beedis, cigarettes, cigars, pipes, kreteks or by using devices like hooka, hookli, chhutta, dhumti or chillum[3,4]. Smokeless tobacco is tobacco that is not burned and the tobacco products are used by means other than smoking, such as chewing, sniffing or placing between the teeth and gum. It is also known as chewing tobacco, oral tobacco, spit or spitting tobacco. Smokeless tobacco is used in a wide variety of forms in many countries of the world and as of 2017, the WHO states that smokeless tobacco use is a significant part of the overall world tobacco problem[4].

The growing popularity in the use of smokeless tobacco appears to be increasing because of mistaken perception of safety, indoor smoking bans, the ability to hide use, increased social acceptance and reported "positive" physiological effects such as relaxation, increased concentration, heightened alertness and diminished hunger[5]. The commonly used smokeless tobacco products in India include gutka or gutkha, khaini, zarda, etc.

Gutkha is a cheap betel-quid substitute containing tobacco in powdered form, along with areca nut, slaked lime, catechu and perfumery substances[6,7]. It is popularly used in the Indian sub-continent and in other regions of South Asia[6]. Unlike paan, gutkha is relatively non-perishable and packaged in small single-use packets that are easy to carry and use anytime, anywhere. Gutkha usually refers to the product with tobacco and pan masala is the same product without tobacco[8]. Gutkha is held in the mouth and chewed. Saliva is generally spat out, but it is also sometimes swallowed.

Among adults in India, khaini is the most commonly used type of smokeless tobacco. The main ingredients of khaini (or khoinee in Bangladesh) are sun-dried tobacco leaves, slaked lime (calcium hydroxide) and sometimes areca nut. Lime is often added to the tobacco just before use. This mixture is prepared by the user keeping the ingredients on the left palm and rubbing it with the right thumb. The prepared pinch is kept in the buccalsulcus[6]. People may use khaini from 3 to 30 times a day and a regular khaini user may carry a double-ended metal container, one side of which is filled with sun-dried tobacco and the other slightly moistened slaked lime.

Ever since the Meghalaya Government imposed a ban on the sales of gutkha in 2014, only a few brands are sold in the market secretly and used mainly by labourers, drivers, auto-workers and young adults. Our preliminary survey showed that Shikhar gutkha is mostly consumed in different parts of Meghalaya. Also, if this gutkha was not available, people use a blend of Shikhar pan masala and S-FOUR zarda. The said zarda compensates for the lack of tobacco in the gutkha, producing a gutkha-like taste. Hence, Shikhar gutkha and a blend of Shikhar pan masala and S-FOUR zarda were selected for determining toxicity in animal model based on the average minimum and maximum packets commonly consumed by a person per day. Although some toxic effects of smokeless tobaccos have been studied, there is no record of such studies in Meghalaya in spite of a number of people, both male and female, consuming different types of smokeless tobaccos. The present studies were undertaken to examine the possible toxic effects of the selected smokeless tobaccos and related glutathione changes in the liver and kidney of mice under laboratory conditions.

2. Material and Methods

2.1 Chemicals

Reduced glutathione and 5,5'-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Chemical Company St. Louis, MO, U.S.A. Hematoxylin (Delafield's) and eosin (2% w/v) were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Copper sulfate, propylene oxide, araldite Cy212, Dodecenyl Succinic Anhydride (DDSA), tridimethylamino methyl phenol, dibutylphdyhalate, osmium tetroxide, cacodylate buffer, lead citrate, uranyl acetate and other chemicals used in the experiments were of analytical grade and purchased from SRL Pvt. Ltd., Mumbai, India.

2.2 Animal Maintenance

Inbred Swiss albino mice colony is being maintained under conventional laboratory conditions at room temperature of 22 ± 2°C. The animals were kept in polypropylene cages on dust-free rice husk as the bedding materials,

with free access to food pellets (Amrut Laboratory, New Delhi) and drinking water *ad libitum*. Female mice in the age group of about 10–12 weeks weighing about 28–30 g were used for the experiments. The maintenance, use of the animals and the experimental protocol of the present study have been approved by the North-Eastern Hill University, Shillong vide Ph.D. registration no. 2457 of 08-12-2014 and Institutional ethics committee (animal models), North-Eastern Hill University, Shillong, India dated 15-12-2015.

2.3 Preparation of Aqueous Extract of Smokeless Tobaccos

Smokeless tobaccos Shikhar gutkha, Shikhar pan masala and S-FOUR zarda were purchased from local vendors in Shillong. Each available small packet of Shikhar gutkha contains 2 g of the product. Twenty-five packets (50 g) of this product was further ground to fine pieces and dissolved in 500 ml distilled water and stirred overnight. Each available large packet of Shikhar pan masala contains 12 g and related S-FOUR zarda packet contains 2 g of the product. Four packets each of the pan masala (48 g) and zarda (8 g) were ground together into fine powdered form and added to 500 ml distilled water and stirred overnight. Both the smokeless tobacco extracts were then filtered using Whatman filter paper 1001–110 (Grade qualitative filter paper, pore size 11 μm) and approximately 100 ml of the filtrates were obtained. These filtrates were used for the treatment of the mice through oral gavage as described below. To know the concentration of the filtrate, 10 ml of the filtrate was slowly evaporated at a low temperature of 30°C to dryness and the residue was collected which showed approximately 24–26 mg per ml of the filtrate.

2.4 Treatment Schedule

The doses for treatment were calculated on the basis of the human daily consumption of 5 and 20 packets of Shikhar Gutkha per day (taking the body weight 60 Kg). On comparing with human consumption, the low dose that is to be administered per average 30 g of a mouse was found to be 0.25 mg (0.01 ml of filtrate) and that of high dose was 1 mg (0.04 ml of filtrate). Similarly, for the treatment of mice with Shikhar pan masala and S-FOUR zarda combined, low and high doses were calculated equivalent to human consumption of 1 packet each of large Shikhar pan masala and S-FOUR zarda per day for low dose and 2 packets per day each of the same for the high dose, amounting to 0.31 mg (0.01 ml of filtrate) and 0.62 mg (0.02 ml of filtrate) respectively, per 30 g mouse.

A total of 20 mice were randomly divided into five groups consisting of 4 in each group. Group 1 mice were fed with basal diet throughout the experiment and served as control. Group 2 mice were administered with an aqueous solution of a low dose of Shikhar gutkha by oral gavage daily. Group 3 mice were administered with aqueous solution of high dose of Shikhar gutkha by oral gavage daily. Group 4 mice were administered with an aqueous solution of low dose of Shikhar pan masala and S-FOUR zarda by oral gavage daily. Group 5 mice were administered with aqueous solution of high dose of Shikhar pan masala and S-FOUR zarda by oral gavage daily. The mice in different groups were treated for 30 days and fed with food pellets and drinking water *ad libitum*.

2.5 Histopathological Studies - Light Microscopy

At the end of the treatment, the liver and kidney of mice in different groups were collected and processed for histopathological studies. Tissues were fixed in Bouin's fixative and processed for dehydration in progressively increasing concentrations of ethanol (30, 50, 70, 90 and 100%), cleared in xylene, infiltrated by liquid paraffin and cooled to form a solid paraffin block containing tissues. Paraffin tissue sections (5–7 μm in thickness) were cut with a microtome (Sipcon) and mounted on clean glass slides, smeared with a drop of Mayer's albumin. The sections were deparaffinized by mild heating the slide on the burner flame and placing it in the xylene. The tissue sections were hydrated by passing through a decreasing concentration of alcohol baths and water. The sections were stained in hematoxylin for 3–5 minutes, washed in running tap water for 3–5 min until sections turn "blue" and differentiated in 1% acid alcohol (1% HCl in 70% alcohol) for 5 minutes. The sections were washed in running tap water until the sections are again blue by dipping in an alkaline solution (e.g. ammonia water) followed by tap water wash. Further, sections were stained in 1% Eosin Y for 10 minutes, washed in tap water for 1–5 min and dehydrated in increasing concentration of alcohols, cleared in xylene and mounted

in DPX mounting media. The sections were thoroughly examined under the microscope and photographed. The stock solutions of hematoxylin and eosin were diluted 1:1 with distilled water before staining.

2.6 Ultrastructural Studies - Transmission Electron Microscopy

The liver and kidney collected from animals in different treatment groups were fixed in 3% glutaraldehyde (in 0.1 M cacodylate buffer) for 2 hours at 4°C. Fixed tissues were washed and rinsed in 0.1 M cacodylate buffer, cut into small pieces and post-fixed in 1% osmium tetroxide (OsO_4) for 15 min at 4°C. Tissues were then processed for dehydration with an ascending grade of acetone (30 - 50 - 70 - 80 - 90 - 95%, twice for 15 minutes each) at room temperature and kept in dry acetone (prepared by adding $CuSO_4$ crystals in excess to absolute acetone and filtered). The tissue samples were treated with propylene oxide twice for 1 h at room temperature followed by different ratios of propylene oxide and embedding medium (3 : 1 for overnight; 1 : 1 for 1 hour; 1 : 3 for 1 hour and in pure embedding medium twice for 2 hours at 50°C). The embedding medium was prepared by mixing 1 ml Araldite Cy212, 10 ml Dodecenyl Succinic Anhydride (DDSA), 0.4 ml tri-dimethylamino methyl phenol and 1 ml dibutylphdyhalate. Ultrathin sections (60–70 nm) were cut in an ultramicrotome (Ultratome-RMC, MTX, USA). Tissue sections collected on copper grids were stained with lead citrate (5%) and uranyl acetate (5%, 1 : 1 v/v). Viewing was done in an electron microscope (Joel TEM C XII) at a voltage of 80 KV and photomicrographs were taken.

2.7 Analysis of Liver and Renal Functions

The blood sample was collected from retro-orbital venous sinus bleeding and serum was separated. Analysis for liver function test and renal function tests were done using Clinical Chemistry Analyzer (SYNERGY BIO-1904C) at North East Diagnostic Centre, Shillong, Meghalaya, to measure the serum levels of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) for liver function and urea, cholesterol and creatinine for renal function respectively.

2.8 Reduced Glutathione (GSH) Determination

Reduced glutathione (GSH) content of liver and kidney from the mice under different experimental conditions was determined as total sulfhydryl contents using the method of Sedlak and Lindsay (1968)[9]. Briefly, 5% of tissue homogenates were made in 0.02 mol/L EDTA, pH 4.7 by sonication (Soniprep 150). To the homogenate or pure reduced form of glutathione (100 µl), 1 ml of tris-EDTA buffer (0.2 mol/L, pH 8.2) and 0.9 ml of EDTA solution (0.02 mol/L, pH 4.7) was added which was followed by addition of 20 µl of Ellman's reagent (0.01 M DTNB in methanol). After 30 minutes of incubation at room temperature, the reaction mixtures were centrifuged at 3000xg for 20 min and the absorbance was read against a reagent blank at 412 nm in a Carey-100 spectrophotometer.

2.9 Statistical Analysis

The results were expressed as mean ± S.D. The data were analyzed by one way ANOVA using the OriginPro version and statistical significance between control and different treatment groups was drawn as $*p \leq 0.05$ level.

3. Results

Diagnostic evaluation of liver and kidney is largely based on a thorough examination of sections stained with hematoxylin and eosin (H and E). Hematoxylin has a deep blue-purple colour and stains nucleic acids by a complex, incompletely understood reaction. Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Histological observations of the liver of control mice showed the normal arrangement of hepatic cells, sinusoid spaces and Central Vein (CV) and Portal Vein (PV). However, in gutkha treated mice, irregularity in the central vein structure and congestion with hemorrhages in the portal veins was observed in the liver [Figure 1]. In the Pan Masala Zarda (PMZ) treated mice, the liver histopathology revealed vacuolization, hemorrhage and shrinkage of central vein and portal vein [Figure 2].

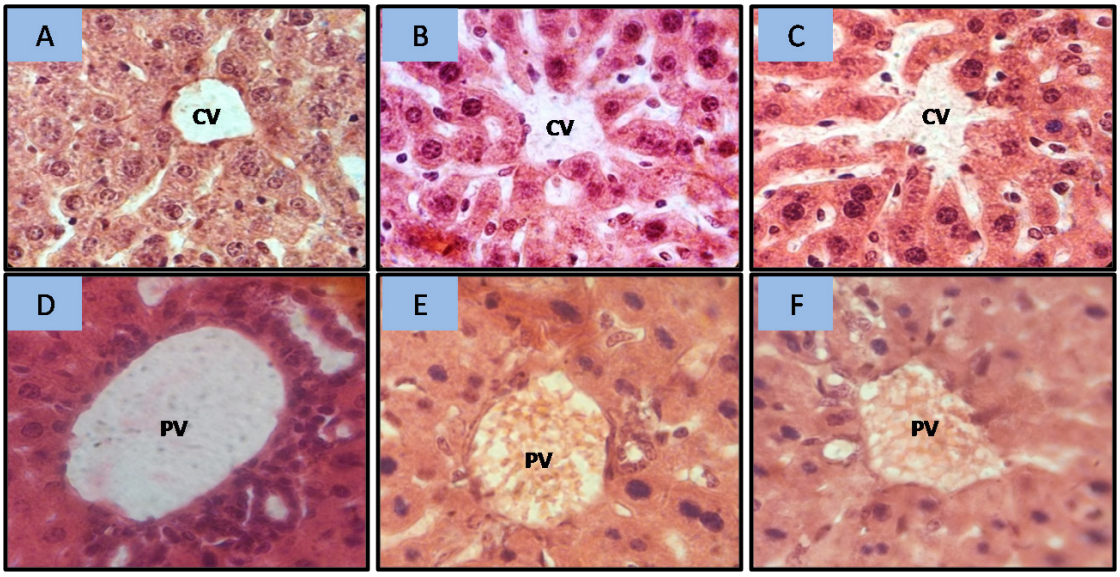


Figure 1. Light micrographs of the liver sections from mice under different experimental conditions. Control (A and D) showing normal Central Vein (CV) and Portal Vein (PV). Photographs B and E are for a low dose of gutkha treatment showing structural features of CV and PV respectively, while C and F are for the same features after a high dose of gutkha treatment. Irregularity in central vein structure (B and C) and congestion in portal vein structure and hemorrhage (E and F) were observed after both the doses of treatments. (Magnification: x40).

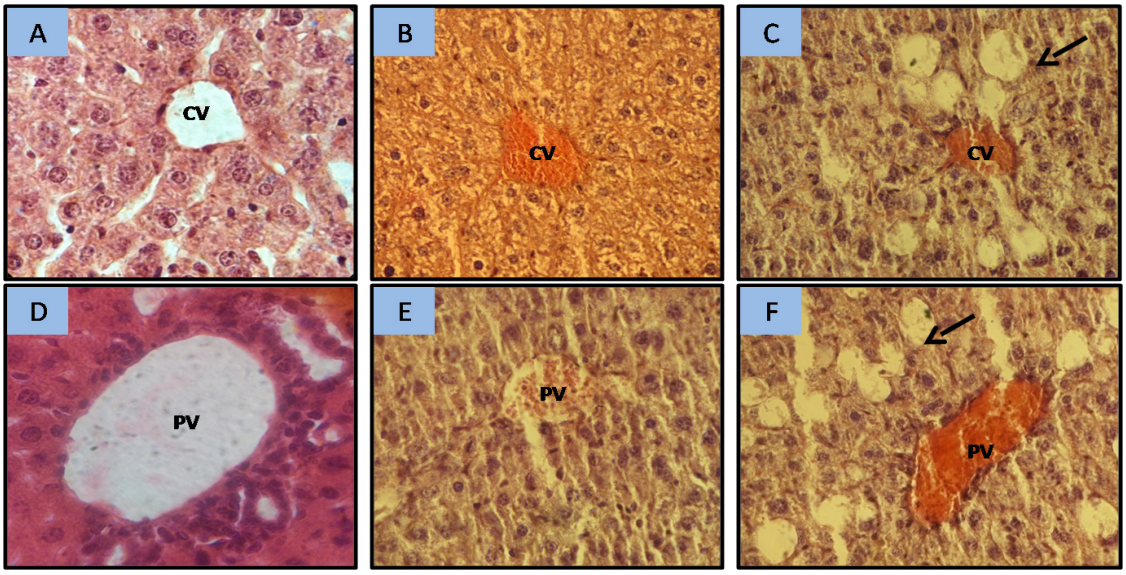


Figure 2. Light micrographs of histological sections of the liver from mice under different experimental conditions. Control (A and D) showing normal Central Vein (CV) and Portal Vein (PV). Photographs B and E are for a low dose of Pan Masala Zarda (PMZ) treatment showing structural features of CV and PV respectively, while C and F are for the same features after a high dose of PMZ treatment. Irregularity in central vein structure (B and C) and congestion and hemorrhage in the portal vein (E and F) were observed after both the doses of treatments. Tissue vacuolization was prominently noted after a high dose of PMZ treatment (C, F). (Magnification: x40).

Histology of kidney from control mice showed the normal renal architecture of the outer cortex and inner medulla. Cortex showed the normal structure of renal glomeruli surrounded by a double-walled Bowman's capsule. The kidneys of both gutkha and Pan Masala Zarda treated groups showed disruption in the glomerular and tubular structures [Figure 3].

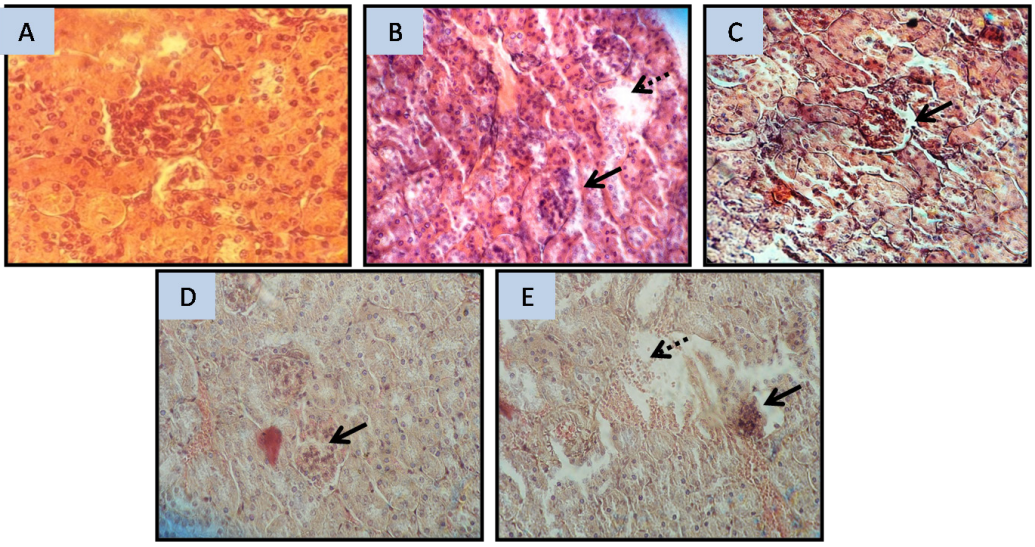


Figure 3. Light micrographs of the histopathology of a kidney from mice under different experimental conditions. A. Control, kidney section showing normal architectural features. B and C are the section photographs of low and high doses of gutkha treatment, whereas D and E are the kidney sections after low and high doses of Pan Masala Zarda (PMZ) treatment. Treatment with both the doses developed conspicuous structural abnormalities in the kidney such as glomerular disruption (regular arrows) and tubular degeneration (dotted arrows). (Magnificationx40).

Ultrastructural features of the liver of control animals showed the normal architecture, with a smooth and circular nucleus, normal mitochondrial cristae and smooth endoplasmic reticulum. Irregularity in the nuclear membrane structure developing more inter-membranous gaps and mitochondrial deformities was noted after a low dose of gutkha treatment [Figure 4 B]. At a high dose of gutkha treatment pronounced dilation and irregularity in the rough endoplasmic reticulum and mitochondrial deformities [Figure 4 D] were observed. Both the doses of PMZ treatment of mice caused the development of irregularity in the nuclear morphology and dilation in the rough endoplasmic reticulum and mitochondrial deformities in the liver [Figure 5].

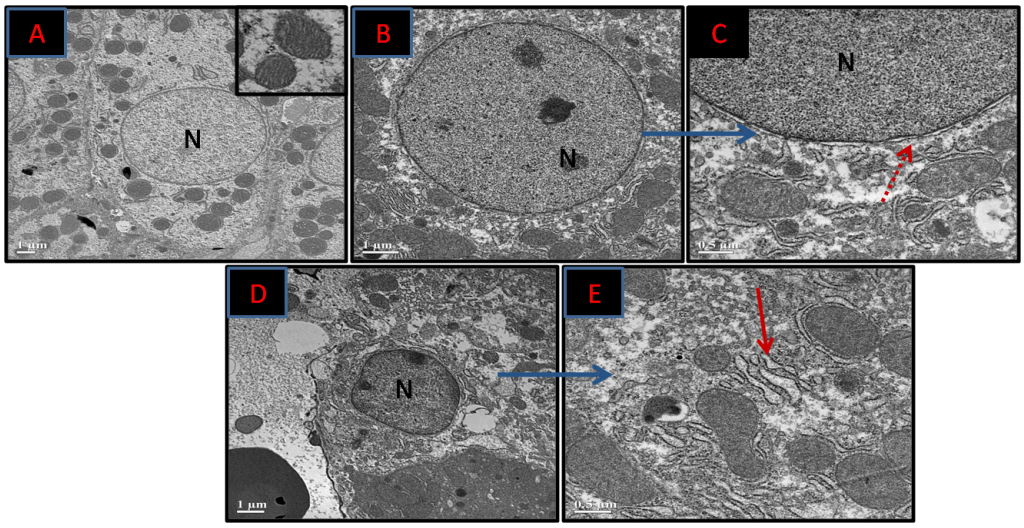


Figure 4. Transmission electron micrographs of liver from the control (A) and gutkha-treated mice. Micrographs of the liver after low (B) and high (D) dose of gutkha treatment. Micrographs C and E are the enlarged views of B and D respectively. Irregularity in the nuclear membrane structure developing more intermembranous gaps and mitochondrial deformities was noted after a low dose of gutkha treatment (C, dotted red arrow). At a high dose of gutkha treatment pronounced dilation and irregularity in the rough endoplasmic reticulum and mitochondrial deformities (E, regular red arrow) were also observed. N = nucleus.

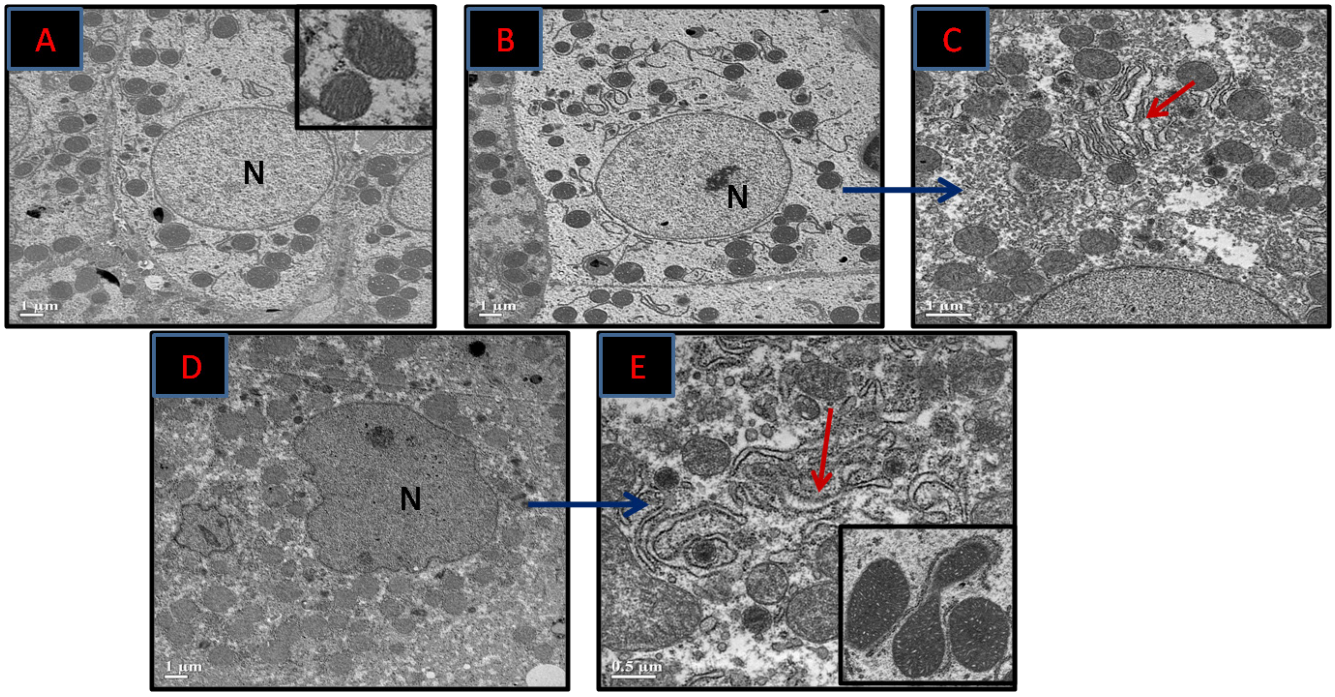


Figure 5. Transmission electron micrograph of liver from control (A) and Pan Masala Zarda (PMZ) treated mice. Micrographs of the liver after low (B) and high (D) dose of PMZ treatment. Micrographs C and E are the enlarged view of B and D respectively. Irregularity in the nuclear morphology and dilation in the rough endoplasmic reticulum (regular arrows) and mitochondrial deformities were observed after both the doses of PMZ treatment (C, E). Inset in micrograph 'A' shows normal mitochondrial features whereas inset in 'E' shows mitochondria with dilated cristae. N = nucleus.

Abnormality and degeneration in the smooth endoplasmic reticular membrane and disrupted mitochondria were noted in the kidney after both the doses of gutkha treatments [Figure 6]. In the kidney of PMZ-treated animals, distortion in the basement membrane and appearance of cytoplasmic vacuoles were observed [Figure 7].

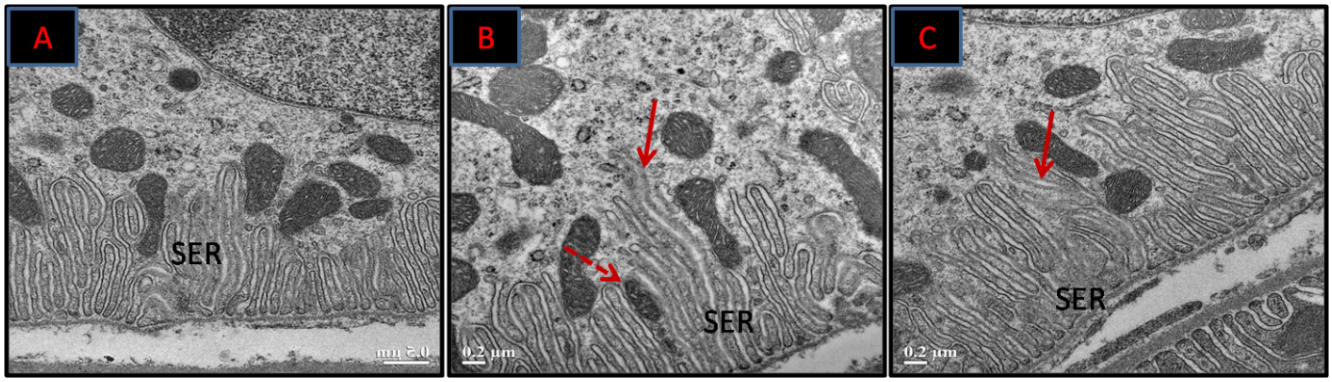


Figure 6. Transmission electron micrograph of the kidney from the control (A) and gutkha-treated (B-low dose and C-high dose) mice. Abnormality and degeneration in Smooth Endoplasmic Reticular (SER) membrane/structure (regular arrows) and disrupted mitochondria (dotted arrow) were noticeable after treatment with both the doses of gutkha.

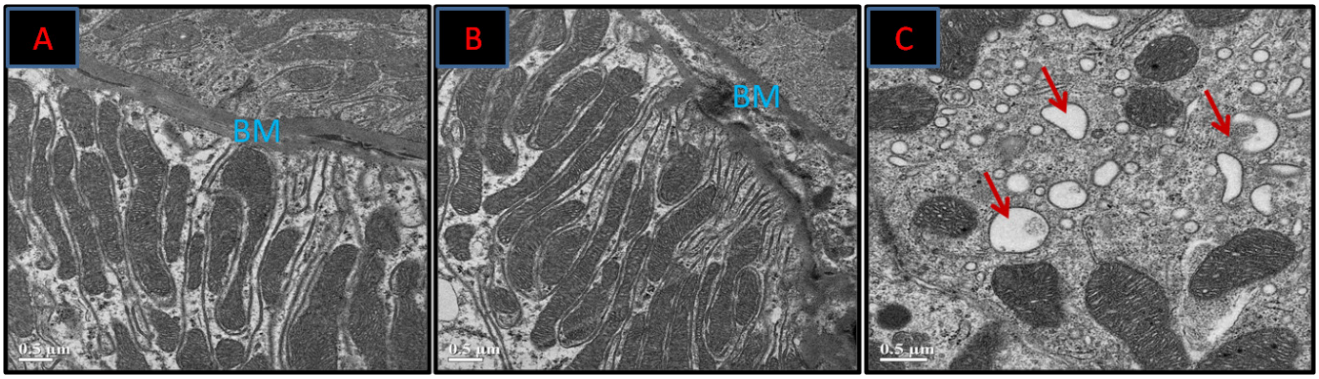


Figure 7. Transmission electron micrographs of the kidney from the control (A) and Pan Masala Zarda (PMZ)-treated (B-low dose and C-high dose) mice. Normal basement membrane (BM) organization was observed in control (A), whereas distorted basement membranes were formed after a low dose of PMZ treatment. At the high dose of treatment (C), the appearance of a number of cytoplasmic vacuoles (regular arrows) was also noted.

Biochemical analysis for liver functions showed a significant increase in all the parameters in the serum i.e. ALT, AST and ALP in both gutkha and PMZ-treated groups [Figure 8].

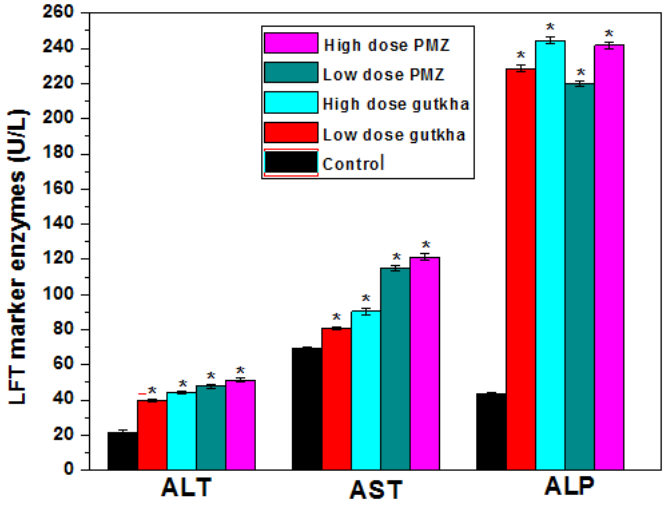


Figure 8. Changes in the serum marker enzymes for Liver Function Tests (LFT) in gutkha and Pan Masala Zarda (PMZ) treated mice. In both the treated groups significant increase in LFT marker enzymes was observed. ALT = Alanine Transaminase; AST = Aspartate Transaminase; ALP = Alkaline Phosphatase (ALP). Results are expressed as mean, ± SD., n = 4, *p≤0.05 as compared to the corresponding control.

In the kidney function analysis, a significant increase was observed in serum cholesterol, urea and creatinine as compared to that in control [Figures 9, 10].

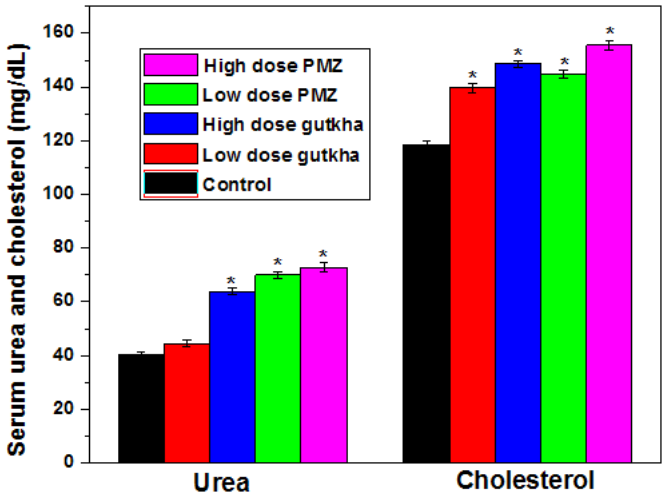


Figure 9. Changes in the serum cholesterol and urea concentration in gutkha and Pan Masala Zarda (PMZ) treated mice. In both the treated groups significant increase in serum urea and cholesterol was observed. Results were expressed as mean, ± SD., n = 4, *p≤0.05 as compared to the corresponding control.

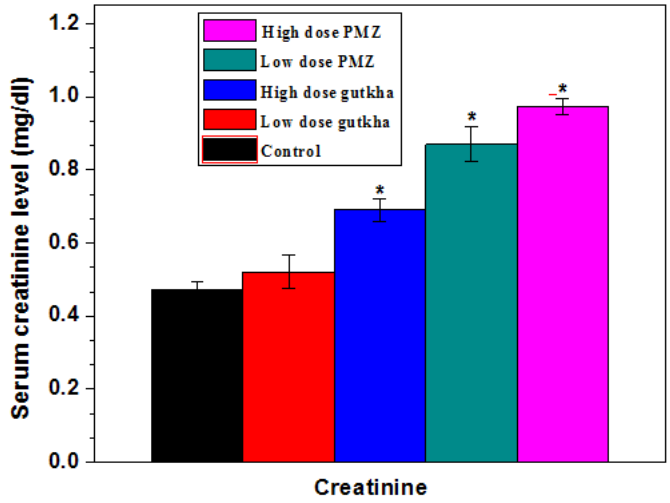


Figure 10. Changes in the serum creatinine levels in gutkha treated and Pan Masala Zarda (PMZ) treated mice. In both the treated groups significant increase in serum creatinine was observed. Results were expressed as mean ± SD., n = 4.

Administration of both low and high doses of aqueous extracts of Shikhar gutkha and PMZ to mice significantly decreased the liver GSH levels whereas in the case of kidney only the high dose of gutkha but both the doses of PMZ caused a significant reduction in GSH levels [Table 1].

Table 1. Changes in the total GSH content (μ moles/g wet wt.) in the tissues of mice under different treatment conditions

Treatment groups	Liver	Kidney
Group 1 (Control)	12.92 ± 0.272	9.68 ± 0.056
Group 2 (low dose gutkha)	11.86 ± 0.083*	8.42 ± 0.021
Group 3(high dose gutkha)	11.28 ± 0.007*	8.13 ± 0.006*
Group 4(low dose PMZ)	11.61 ± 0.022*	8.02 ± 0.005*
Group 5(high dose PMZ)	11.27 ± 0.017*	7.90 ± 0.056*

The results are expressed as mean ± S.D., n = 4. The significance of the changes between control and treated groups was tested by ANOVA, $p \leq 0.05$ (*as compared to control). PMZ = Pan Masala Zarda

4. Discussion

Different forms of tobacco, including smokeless tobaccos, are addicting and nicotine is the substance in tobacco that causes addiction. Once a person is addicted to tobacco use, it is quite difficult for him/her to leave its use. Many of the risks to health and life caused by tobacco consumption develop over a long period and take decades to become fully evident. Liver and kidney are important organs of metabolism, detoxification, storage and excretion of xenobiotics and their metabolites and are especially vulnerable to damages[10]. Evaluations of histopathological changes have been commonly used for detecting organ-specific effects related to chemical exposure and toxicity[11,12]. The histopathological observations of liver and kidney from a month-long treatment of Swiss albino mice with different combinations of smokeless tobaccos revealed alterations in the normal architecture of the liver and kidney and also their functions. The treatment caused irregularity in the central vein structure and shrinkage of portal veins, irregular arrangement of sinusoidal spaces and occurrence of vacuoles in the liver [Figures 1, 2], thus, highlighting the deleterious effect of the smokeless tobaccos on the histological organization of liver. The ultrastructural features of the liver showed the most striking changes in the nuclear membrane, mitochondrial cristae and smooth endoplasmic reticulum. Whereas the liver cells from control mice showed a smooth and round nuclear membrane, the treated mice liver cells developed irregular nuclear membrane with regular dilations at a few places around the nucleus. At the same time, the mitochondrial cristae were disrupted and the membranes of the smooth endoplasmic reticulum were dilated after treatment [Figures 4, 5]. These abnormalities in the nucleus and mitochondria may adversely affect the normal transport and energy metabolism in the cells and may assist to develop hepatotoxicity and alter the liver functions. This view could be supported by the findings of biochemical analysis of liver functions which showed that the treatments severely affected liver functions as observed by a significant increase in serum biomarker enzymes [Figure 8]. Liver enzymes are normally found within the cells of the liver. When the liver is injured or damaged, the liver enzymes such as ALT, AST and ALP are released into the blood[13]. ALT is more specific to the liver than AST and its activity is usually greater than AST activity at the earlier stages of the hepatocellular disease. AST tends to be released more than ALT in chronic liver disease such as cirrhosis[14]. ALP is a useful diagnostic marker of cholestatic hepatobiliary lesions and its activity is increased in liver diseases[15]. The findings from the study show that the serum marker enzymes for liver function (ALT, AST and ALP) were elevated consequent to treatment with different combinations of smokeless tobaccos, with higher elevation seen in Shikhar Pan Masala Zarda treatment. Such elevations in the markers for liver function have also been observed in other investigations on various types of smokeless tobaccos on different animal models[16-18]. Varying patterns of degenerations in the glomerulus and proximal convoluted tubules observed in the kidneys under different treatment conditions [Figure 3] may lead to the long retention of metabolic wastes. Stevens and Lowe[19] have also suggested that improper/non-clearance and persistence of metabolic wastes in the kidney may lead to its loss of the sensitive homeostatic mechanisms. Ultrastructural features of kidney showing the degeneration in the smooth endoplasmic reticular membrane, disrupted mitochondria, distortion in the basement membrane and appearance of cytoplasmic

vacuoles after both the treatments [Figures 6, 7] may also support the development of nephrotoxicity. In the studies related to the toxic effects of the organophosphorus pesticide, dimethoate on some organs of mice showed blood vessels congestion, hemorrhage, vasodilation and hypertrophy in the liver whereas kidney showed some changes including glomerular degeneration, hemorrhage, tubular widened lumen, glomerular shrinkage and compressed blood vessel[20]. The toxicity of oral administration of other smokeless tobacco extract in rats has been reported to cause a dose-dependent reduction in body weight, tissue pathological changes in the digestive system (mainly the liver and stomach), the urinary system (kidney) and the respiratory system (lungs)[21]. To corroborate the nephrotoxicity in terms of functional parameters of the kidney, serum creatinine, urea and cholesterol levels were determined. Creatinine is commonly measured as an index of glomerular function[22]. High levels of serum creatinine can indicate kidney damage/failure[23]. Besides creatinine, urea is a major nitrogenous end product of protein and amino acid catabolism, produced by the liver and distributed through intracellular and extracellular fluid[24]. It is filtered out by the glomeruli in the kidney. The measure of the concentration of urea in serum is useful in the differential diagnosis of acute renal failure where blood urea nitrogen-creatinine ratio is increased[25]. We observed an increase in the level of serum urea and creatinine after both the smokeless tobacco treatments [Figures 9, 10]. This increase in serum creatinine and urea may suggest the development of nephrotoxicity, and also supports the findings of histopathological abnormalities in kidney indicating possible toxicities in the host. The observed increase in serum cholesterol level in smokeless tobacco-treated animals [Figure 9] may also have adverse effects in the hosts. This observation has also been seen in a study conducted on smokeless tobacco consumers[26,27] but no such study in animal models has been established. Some studies conducted on Swedish snuff (smokeless tobacco) however, have found no changes in the levels of cholesterol between snuff users and non-tobacco users[28,29]. The toxicity of smokeless tobacco gutkha on the reproductive system of adult male mice, showing decreased levels of circulating testosterone, body weight and liver weight have also been reported[30].

In order to further explore the possible mechanism of the development of toxicities after gutkha and PMZ treatment of mice, reduced glutathione was determined in the kidney and liver collected from mice under the same treatment conditions used for histopathology. Reduced glutathione (GSH, γ-l-glutamyl-l-cysteinylglycine) is the most abundant antioxidant in the cells and plays an important role in a multitude of cellular processes including the protection of various organs against oxidant-induced injury and inflammation[31]. The consequence of exposure of mice to the smokeless tobaccos in present studies resulted in a significant decrease in the GSH levels in the liver and kidney. Such depletion of GSH levels may decrease the antioxidant potential and protective ability of the cells and may lead to increased burden of oxidative stress and extensive damages in these tissues, thereby, assisting the development of hepatotoxicity and nephrotoxicity in the hosts. The depletion of physiological antioxidants like reduced glutathione (GSH) in liver and kidney has also been reported as a possible mechanism of cadmium-induced oxidative toxicity in chicken[32]. Avti et al.[33] reported the effects of a short-term, high-dose and long-term, low-dose exposures to the smokeless tobacco extract on the antioxidant defense status and histopathological changes in liver, lung and kidney of Wistar rats. The tobacco products contain a variety of harmful chemicals that could be responsible for developing harmful effects in the body. The toxicities and oxidative stress-inducing actions of smokeless tobacco extract determined by colony formation assays using Chinese Hamster Ovary (CHO) cells also indicated that all preparations of smokeless tobacco extract significantly decreased GSH levels and increased malondialdehyde (MDA) generation[34]. In fact, the balance between the amount of free radicals generated and antioxidants such as GSH to scavenge them to protect the body against their deleterious effects could be an important factor in the development of the majority of organ toxicities.

In conclusion, the findings from the present study suggest that smokeless tobaccos, even when consumed at a low dose, are able to cause considerable damage to the structural and functional aspects of the liver and kidney. These effects of smokeless tobacco products could be attributable to various harmful chemical constituents including nicotine present in gutkha and PMZ. Therefore, it is necessary to determine their chemical constituents and it must be mandatory to mention different chemical constituents on the commercially available smokeless tobaccos. Further, it is proposed that consumption of tobaccos in any form is harmful and their consumption should not be encouraged. We need to make more efforts for strengthening smokeless tobacco control policies and

also to increase public awareness on the harmful effects of smokeless tobacco use and implement effective cessation programmes.

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