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Liver and kidney toxicity induced by Afzal smokeless tobacco product in Oman

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ABSTRACT

Afzal, the common smokeless tobacco product (STP) in Oman, is believed to contain toxins that may impair the function of some organs such as liver and kidney. An aqueous extract from Afzal was added to drinking water to be administrated orally to Wistar albino rats ($n = 72$) young and adult from both genders weighing between 60–80 g and 150–240 g respectively for 8 weeks. Animals were divided into three groups: control (distilled water instead of Afzal extract), low-dose (3 mg nicotine/kg body weight/day) and high-dose (6 mg nicotine/kg body weight/day). The animals were euthanized and their blood, liver and kidney were collected for biochemical and histopathological investigations. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed for the liver function, while blood urea nitrogen (BUN) and creatinine (CRT) were assayed for the kidney function. The results showed a significant increase in the ALT, AST, BUN and CRT levels ($P < 0.05$) in both Afzal-treated groups (low and high doses) compared with the control. Histopathological findings revealed the initial but seem to be serious degenerative alterations of periportal fibrosis in liver and edematous and calcified changes in renal glomerulus among Afzal-treated groups. Additionally, the weight gain of the Afzal-treated groups was lower than the control group. Our findings show that the exposure of Wistar rats to the Afzal extract has the potentials of causing decreased weight gain and dose-dependent functional and structural damage to the biochemical and histological profiles of liver and kidney as well as serious biochemical effects.

1. Introduction

Afzal is a common illegally-used smokeless tobacco product (STP) which gains popularity among adults and school age-students. Like most STPs, this blend is composed mainly of tobacco, slaked lime (Calcium hydroxide) and some additives (IARC, 2007). Afzal users place it inside the mouth for a period of time and the formed tobacco mixture with the saliva most likely ends up into the systemic circulation. It is believed that STPs cause a number of cancers like oral and pancreatic (IARC, 2007). Moreover, they contain many toxic substances like heavy metals and nicotine which act as the major attractive factor in those products due to its addictiveness property. The chemical analysis of Afzal has shown that it is contaminated with some health hazards substances like heavy metals, high nicotine, nitrate, nitrite, other anions and tobacco-specific nitrosamines (TSNAs) (Al-Mukhaini et al., 2014, 2015, 2016).

Many studies highlighted toxic effects of some organs in response to the frequent use of STPs (IARC, 2007). Liver, kidney, heart, lungs, esophagus, pancreas, and testes are common vulnerable organs that showed STP-associated tissue damage (Boffetta et al., 2005; IARC, 2007; Adedayo et al., 2011). Toxins in STPs are metabolized mainly in the liver. Nicotine is a major tobacco alkaloid which causes liver inflammation due to the adverse actions of its secondary metabolites (Yanagita et al., 2012). Additionally, it has been found that smokeless tobacco diminishes the liver's ability to detoxify dangerous substances (Alwar et al., 2013) and cause kidney dysfunction (Okonkwo et al., 2013).

Afzal has been shown to contain cadmium and lead at elevated levels (Al-Mukhaini et al., 2014). These heavy metals have been found in high levels in serum of smokers resulting in glomerular dysfunction (Cooper, 2006). Nicotine constituent in Afzal was elevated, which is capable to accelerate nephropathies with an increased incidence of micro-albuminuria progressing to

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proteinuria, followed by type-1 diabetes mellitus induced renal failure (Cooper, 2006).

When the parenchymal cells of the inflamed liver are damaged or destroyed, specific enzymes leak out form the liver tissue to the blood stream. Those enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are considered as common clinical biomarkers for tissue damage in the liver (Ozer et al., 2008). Similarly, blood urea nitrogen (BUN) and creatinine (CRT) are end-product toxins in the blood which are excreted by the kidney and used as renal function biomarkers (Okonkwo et al., 2013).

Therefore, it is imperative to assess the liver and kidney functions and their histopathological changes when dealing with Afzal to elucidate the health hazards of this product.

2. Materials and methods

This study was approved by the Sultan Qaboos University Animal Ethics Committee (SQU/AEC/2013-14/6). Wistar albino rats ($n = 72$) young (4 weeks old) and adult (20 weeks old) of both genders weighing between 60–80 g and 150–240 g respectively were obtained from the small animal house at Sultan Qaboos University, Oman. The animals were kept in cages at room temperature ($23 \pm 2\,^{\circ}C$), relative humidity of about 60%, and 12 h dark–light cycles (light on at 6.00 AM) and given water and normal pellet diet ad libitum.

Animals were acclimatized for a week prior to the experiment and randomized according to their weight into the proper groups. They were divided into three groups of 24 rats each: high dose group (received 6 mg nicotine/kg b.w./day), low dose group (received 3 mg nicotine/kg b.w./day) and the control group (received distilled water mixed with their drinking water instead of Afzal extract). Each group was kept in separate cages.

Afzal extracts were prepared freshly every week by means of drying and grinding according to the modified method of Pramanik (2012). The prepared solution was extracted in distilled water.

The calculated amount (in grams) needed for each group was prepared according to group's weekly body weight measurements. Consideration of not exceeding the lethal dose of nicotine [LD50 = 10 mg nicotine/200 g rat body weight (b.w.)] (RTECS, 1986) was taken in mind. The low and high dose amounts were calculated according to the concentration of nicotine in Afzal 48.8 mg/g (Al-Mukhaini et al., 2015).

Extract was shaken for 1 h at 37 °C and then filtered twice using (Whatman no. 1) filter paper through Buckner funnel. The resulting concentrated extracts were diluted with the normal drinking tap water in the ratio of 1:30 ml of the concentrated extract to the tap water. While, the control groups received only the same volume as the extract of distilled water mixed with the drinking water.

The animals received the prepared aqueous Afzal extract mixed in drinking water for 8 weeks. The doses of Afzal were selected based on the lethal dose of nicotine (LD50) into low dose (3 mg nicotine/body weight/day) and high dose (6 mg nicotine/kg body weight/day) (Ukoha et al., 2012). Blood samples were collected in week 2 and on the day of sacrifice for nicotine/cotinine detection in order to verify Afzal consumption. At the day of sacrifice, the animals were anaesthetized by intramuscular injection of Ketamil/Xylazil-20. Targeted organs, liver and kidney were dissected out. Thirty-six males Wistar rats were used for histopathological study and 24 of both genders for serum collection (nicotine/cotinine detection) were used for biochemical tests. The rest of samples were kept for other analyses.

Animals were sacrificed after 8 weeks of treatment using Ketamil/Xylazil-20 injection. The animals were quickly dissected and blood was collected by cardiac puncture. Serum was separated by centrifugation at 3000 rpm for 20 min (CL3 OR-centrifuge, Thermo scientific, UK) and was stored at −80 °C till the analysis. Before biochemical test, the serum samples were subjected to verification analysis to prove Afzal consumption by the animals through nicotine/cotinine GC/MS analysis (Broadaway et al., 2008).

For nicotine/cotinine analysis, the serum samples of control and Afzal-treated groups were collected in week 2 from tail vein (1 ml) and again at sacrifice and were analyzed by GC/MS using the method of Broadaway et al. (2008). The extraction solvent was made using 1 ml of dichloromethane and 1 ml of 1:1 petroleum ether: di-ethyl ether. Another solution was made by combining a 2:1 ratio of solvent to serum and samples were shaken and allowed to settle at room temperature. The clear organic layer was collected, placed into a new test tube and evaporated under a nitrogen stream at room temperature. In order to suspend nicotine, 1 ml of methanol was added to the test tube. The mixture was then transferred to an amber vial for general screening run by GC–MS analysis (Perkin Elmer Clarus 600 GC System, USA) looking for the nicotine/cotinine peak. The same operation conditions were described in previous work (Al-Mukhaini et al., 2015).

After confirmation by GC/MS that animals' consumption of Afzal, the serum samples were used for the estimation of biochemical parameters; ALT and AST for liver function observation, and BUN and CRT for kidney function observation. Those parameters were assessed using assay kits obtained from Roche, Germany and by COBAS C 111 analyzer. Before running the samples, the standard calibration curves and quality control run were performed and the coefficient of variation percentage (CV%) was calculated for each analyte. All other reagents used were of analytical grade and were prepared using clean glass and distilled water.

All animals were sacrificed using cervical dislocation. Livers and kidneys were dissected out and fixed in Karnovsky, dehydrated and embedded in resin. Semi-thin sections (0.5 μm) and ultra-thin sections (70 nm) were cut using ultra-microtome (Reichert-Jung, Canada). Semi-thin sections were stained using toluidine blue and were examined using Olympus light microscope. The ultra-thin were collected on grids, stained with uranyl acetate and lead citrate and were examined under transmission electron microscope (JEOL JEM-1230, Japan).

Physical parameters of rats were observed and calculated as a percentage change (gain) of body weight. Data were expressed in mean ± SEM (standard error mean). The significance of the differences in mean between control and treated animals was determined using one-way ANOVA (Analysis of Variance) and followed by multiple comparison tests for observed means, LSD and Duncan post-test, using IBM-SPSS statistics – version 21 software. P values <0.05 were considered significant.

3. Results

3.1. Nicotine/cotinine analysis by GC/MS

All Afzal-treated serum samples showed the peaks for nicotine or its secondary metabolites. While, the control samples were free of those peaks (Fig. 1). The nicotine peak was obvious in the collected serum samples in both week 2 treatment and at the termination of study, which proves that Afzal has been ingested by the tested animals.

3.2. Biochemical parameters

The tested animals for the biochemical parameters were of both genders (adults and young) groups including their corresponding control groups. The coefficient of variation percentage (CV%) was calculated for the control values and repeated 21 times after plotting the standards curves for the four analytes (ALT, AST, urea (BUN)

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