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Original Article

Effect of paper industry leachate on various serological indices and serum proteins of wistar rats

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Abstract
Pulp and paper industry consumes more resources as compared to any other industry around the globe. The toxicity of waste water (leachate) resulting from paper processing has become a serious issue now a day. So, current research was aimed to assess various toxic aspects of pulp and paper industry waste water. Study design involved the division of Wistar ratsof about 245±5g in three groups (n=5) *viz,* Control group (4ml/ kg normal saline), Group 1 (4ml/ kg leachate) and Group 2 (4ml/ kg 1:10 diluted leachate). All the animals received the treatment through intraperitoneal injection. After 24h of the injection, all the animals were sacrificed and blood was collected, sera were separated and processed for further analysis. Serological analysis revealed that leachate induction leads to significant positive variations in level of serum aspartate aminotransferases (AST) ($P<0.0001$), cholesterol ($P<0.0001$) and High density lipoproteins (HDL) ($P<0.0001$) while a significant negative change in triglycerides ($P=0.0002$) and creatinine ($P=0.0370$) level in both experimental groups. Alanine aminotransferases (ALT) level showed a significant increment in Group 1 and a decrement in Group 2 compared to control group ($P<0.0001$). SDS page analysis revealed an overall decreased expression of various proteins in both experimental groups. The protein fractions of 317, 212, 178, 134, 89, 74 and 68 KDa were found in Group 1 compared to control (331, 215, 180, 139, 112, 91, 75 and 69 KDa). However, protein fraction of 202, 171, 136 and 91 KDa were present in Group 2 compared to control. These findings confirmed that paper industry leachate is extremely noxious and induce alterations in various serological parameters and also interfere with the expression of proteins.
Key Words: Leachate, proteins, pulp and paper industry, toxicity

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INTRODUCTION

According to a careful estimate, the wastewater discharge volume for every ton of manufactured pulp ranges between 30 and 180 m^3(Gullichsen, 1991; Puhakka, 1990) while 20-70 m^3 for paper and paperboard (Miner and Unwin, 1991). This discharged wastewater from paper and pulp industry have a characteristic foul smell and is usually dark brown in color, often contains a significant amount of biological oxygen demand (BOD), high organic material, chemical oxygen demand (COD), as well as of high pH (Pokhrel and Viraraghavan, 2004). The characteristic dark brownish color of discharged water is because of lignin and its derivatives produced as a result of alkaline extraction during bleaching of paper. This dark coloration has been reported to be injurious not only to aquatic life but it also inhibit the penetration of significant amount of light to support photosynthesis hence disturbs the natural ecosystem (Dilek and Bese, 2001; Merilainen and Oikari, 2008; Rintala and Puhakka, 1994; Singh *et al.*, 2002).

It is of extreme concern that even 1 mg/L of phenolic contaminations resulting from paper processing in drinking water is enough to make it unfit for drinking because of characteristic foul taste and smell. Leachate often contain sizeable amount of a vast array of chlorinated compounds but they are seldom in a concentration which can induce acute toxicity. For instance, for a 70 kg adult human being, the minimum lethal doses through oral route of administration of the two very common compounds of bleaching effluents pentachlorophenol and tri-chloromethane are 1.2 and 14.8g respectively (Dreisbach, 1980; Schroeder, 1965).So, the intimate risk to human health is via bioaccumulation of these organo-chlorines or because of very long-term exposure

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to contaminated water or affected food resources.The aquatic life in water, polluted with the discharge of pulp and paper mill were often reported to be containing highly deleterious compounds including tetrachlorocatechols, tri, and tetrachloroguai-acols, acetovanillone (a by-product of lignin) as well as tri, tetra, and pentachlorophenols (Paasivirta *et al.*, 1980; Tana, 1988). Among these, acetovanillone results in some noxious chlorinated acetones upon chlorination and chemical degradation. 1, 1, 3-trichloroacetone, a bleachery effluent which is most toxic compound of all, have been measured up to 2.4 mg/ liter (McKague *et al.*, 1981). Presence of these noxious chemical substances in paper industry leachate is of great concern. So, the present study was aimed to assess various serological indices as well as changes in various serum proteins.

MATERIALS AND METHODS

Animals

The current study was carried out on healthy Wistar rats of about 250g. All the animals were kept in cages made up of stainless steel under standard lab conditions of light and temperature (22°C ± 1°C). Animals were supplied with food (standard Rat pellets) and fresh drinking water available *ad libitum*. For acclimatization, all rats involved in the study were housed under standard laboratory condition for 15 days before the commencement of the experiment. Protocols involving animals and their care were concurrent with international laws and policies.

Study Design and Animal grouping

Wistar rats were randomly assigned into three groups (n=5) namely Control Group, Group 1 and Group 2. Both the experimental groups were treated with leachate. Group 1 was injected with 4ml/ kg leachate and Group 2 was given 4 ml/ kg of 1:10 diluted leachate intra-peritoneally. After 24 hours of the leachate injection, animals were dissected and blood was collected without any coagulant and processed for further analysis.

Serum Separation and Quantitative Assessment

Blood samples were allowed to stand for 30 minutes at room temperature followed by centrifugation at 2000g for 20 minutes. The obtained serum samples were processed for quantitative *in vitro* determination of various serological parameters *viz,* serum aspartate aminotransferases (AST), alanine aminotransferases (ALT), cholesterol, high density lipoproteins (HDL), triglycerides and creatinine. These assessments were done by using protocols given with ready to use kits.

SDS-PAGE for evaluation of serum proteins variations

For preparation of polyacrylamide gel, Laemmli method was adopted (Laemmli, 1970). 8%, 10% and 12% gels were used to resolve low molecular weight proteins. But only 8% gel was found satisfactory and processed further. In short, BioRad gel plates were taken, assembled and placed in the electrophoresis tank according to manufacturer's instruction manual; 1× running buffer was filled in the electrophoresis chamber up to the marked level. Comb was carefully removed to keep the integrity of wells in its original shapes. 5µl "protein marker" (Fermentas PageRuler TM unstained protein ladder # SM0661) was loaded in the first well while 15µl of samples were loaded in the following wells. The electrophoresis was performed according to the method described elsewhere (Abbasi *et al.*, 2015). Lastly, the gel was scanned and saved for further analysis.

Estimation of Relative Protein Fractions

Gel analyzer version 2010a was used for the densitometry analysis for electrophoretically resolved "protein fractions".

Ethical Statement

The experimental protocol was approved by ethical committee of Department of Zoology, University of the Punjab, Lahore, Pakistan.

Statistical Analysis

All the reported values are means ± SEM. Values were statistically calculated using Prism Graph pad 5 software (San Diego, CA). One way ANOVA was applied to determine statistical significance with $P < 0.05$ considered significant.

RESULTS

Aminotransferases

Serum level of aminotransferases viz, Alanine aminotransferases (ALT) and aspartate aminotransferases (AST) showed a statistically significant change in both the experimental groups against the control. ALT level was found to be

showed a significant positive increment of 42.06% in Group 1 while a significant negative change of about 13.56 % in Group 2 (P < 0.0001). In contrast to it, AST level showed an overall significant positive change of 104.21 and 28.79 % in Group 1 and Group 2 respectively when compared to control (P<0.0001) (Fig. 1).

Triglycerides and Cholesterol

Serum level of triglycerides as well as cholesterol of both the experimental groups showed statistically significant alterations when compared to control. Triglycerides showed a significant negative change of 29.28% and 20.72% in Group 1 and Group 2 respectively (P=0.0002). While in contrast to triglycerides, hypercholesterolemia was observed in both the experimental groups (P<0.0001). Group 1 and Group 2 showed a significant positive change of 35.90% and 47.64% respectively (Fig. 2).

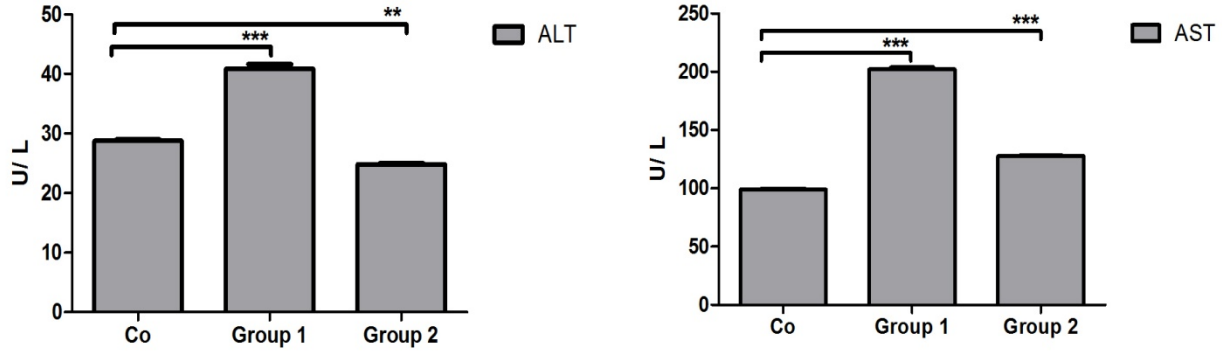

Figure 1: Serum level of aminotransferases (ALT & AST). Alanine aminotransferases (ALT) and aspartate aminotransferases (AST) showed a statistically significant increase in Group 1 as compared to the control group, while Group 2 did not show any significant change. The values are expressed as mean ± S.E.M of three animal series.

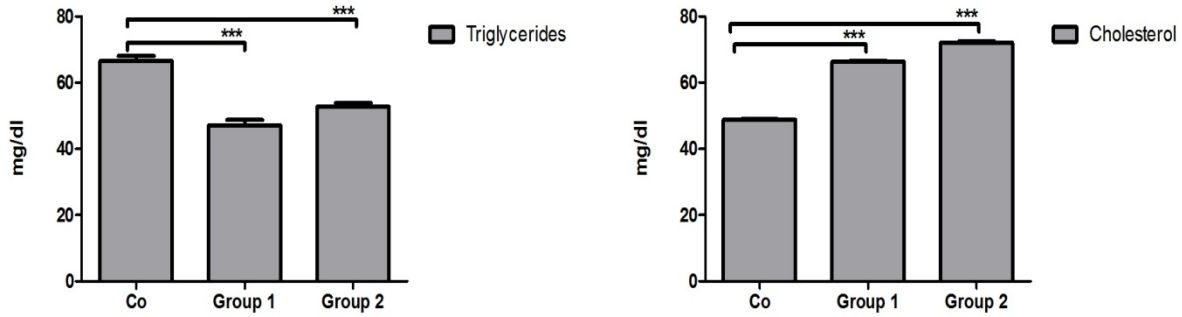

Figure 2: Level of triglycerides and cholesterol in sera. The level of triglycerides and cholesterol showed an opposite trend. Triglycerides were found to be decreased significantly in both studied experimental groups while on the other hand, cholesterol level was found to be enhanced in both the experimental groups compared to the control. The values are expressed as mean ± S.E.M of three animal series.

High Density Lipoprotein (HDL)

Serum HDL level showed a significant positive increase in both the experimental groups when compared to control (P<0.0001). Group 1 showed a significant increment of about 23.16% while Group2 showed a positive change of 37.07% against control group (Figure 3).

Creatinine

Creatinine level in the sera of both the experimental groups showed statistically significant changes against the control group (P=0.0370). Group 1 showed a significant negative change of 25% while Group 2 showed a decrement of 12.50% when compared to control (Figure 4).

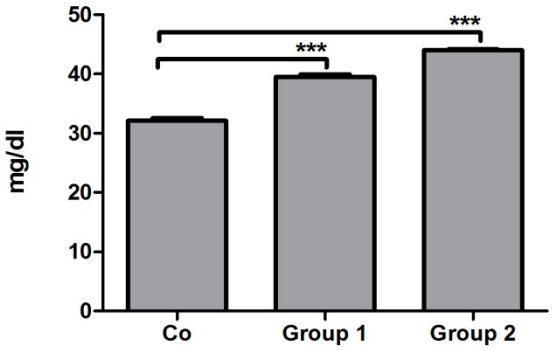

Figure 3: Level of high density lipoprotein (HDL) in sera. The level of HDL was found to be increased significantly in both studied experimental groups as compared to the control. The values are expressed as mean ± S.E.M of three animal series.

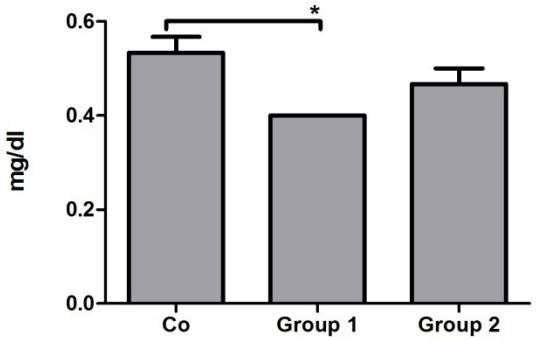

Figure 4: Level of creatinine in the sera. The level of creatinine was found to be decreased significantly in Group 1 as compared to the control while Group 2 did not show any significant change. The values are expressed as mean ± S.E.M of three animal series.

SDS Page results

The comparative analysis of the serum proteins profile of both the experimental groups and control group against protein ladder of 10-220 KDa revealed relative protein fractions from 68 KDa to 331 KDa. The protein fractions of 317, 212, 178, 134, 89, 74 and 68 KDa were found 24h after administration of 4ml/ kg leachate (Group 1) compared to control (331, 215, 180, 139, 112, 91, 75 and 69 KDa). However, protein fraction of 202, 171, 136 and 91 KDa were present after the administration of 4ml/ kg of 1:10 diluted leachate in Group 2 compared to control (Figure 5).

DISCUSSION

Induction of various chemicals, drugs, stress and pathological conditions leads to the failure of the parenchyma cells of hepatic lobules to carry out their normal functions which results in disturbed or impaired intermediary metabolism. The cellular damage and tissue necrosis alters the permeability of cell membranes because of which various enzymes leach out into serum (Center, 2007), most of them can be located in the cytosol of hepatocytes, and their raised level indicates the type and extent of damage imposed (Flier *et al.*, 1993). ALT and AST belongs to transaminase family of enzymes. ALT is produced in large quantities in liver and in small quantities in other body tissues including heart, muscles and kidney. While AST is found in various body tissue including heart, muscles, kidney, brain and lungs. ALT and AST are good biomarker for the assessment of liver function (Cadiot *et al.*, 1989). In the following study both amino-transferases (ALT, AST) showed a statistically significant increment in their serum level in Group 1 which is a clear indication of the cellular insult. The results of Group 1 are concordant with the current study and supported by previous studies (Garg *et al.*, 2009; Rahman *et al.*, 2000). Hepatic toxicants sometimes lead to severe liver injury resembling to viral hepatitis induced liver damage and the ultimate result of which is the increased level of these amino-transferases up to five times compared to the normal values (Lee, 2003). Group 2 showed a similar trend as of Group 1 regarding the AST while Group 2 showed a significant negative change in the serum level of ALT in contrast to Group 1. These findings are in accordance with a previous study in which similar findings have been reported in rat liver enzymes due to consumption of chemical effluents (Oloyede and Sunmonu, 2008).

In the present study, leachate administration leads to a statistically significant hypotriglyceridemia in both the experimental groups compared to control. This decrease may be due to increase in the removal rate of triglycerides (TG) from the blood or due to a decreased TG secretion by the liver. More specifically, perhaps leachate induction has induced an antilipolytic effect in case of TG.

Results of current study are concurrent with the result of previous study in which a significant negative change was noted in masoprocol-treated rats (Scribner *et al.*, 2000).

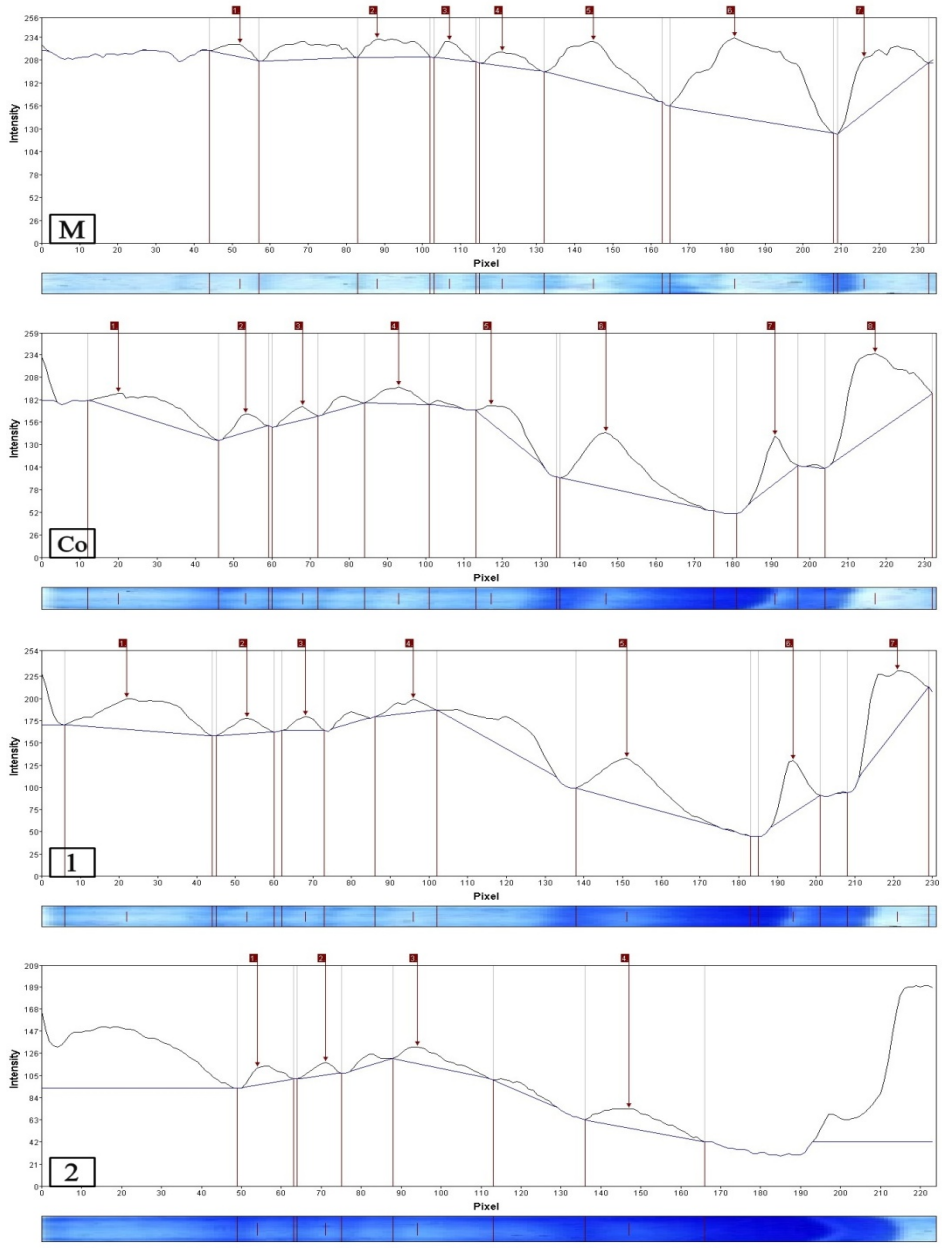

Figure 5: 1-D SDS-PAGE and Densitometric comparison of electrophoretically resolved serum proteins of leachate treated rat after 12h with concentrated (1) and 1:10 diluted (2) leachate with control (Co) and protein ladder (M).

Leachate induction introduced a significant hypercholesterolemia in both the experimental groups. Liver is the major organ involved in cholesterol homeostasis. Liver do so by three primary functions: cholesterol biosynthesis, uptake cholesterol from diet and metabolic conversion of cholesterol to various bile acids (Chiang, 1998; Goldstein and Brown, 1990). Genes responsible in the expression of those enzymes which are involved in homeostasis of cholesterol are regulated through sterols by feedback mechanism. Whenever cholesterol level inside the cell is increased it led to the decreased uptake as well

as its biosynthesis by liver (Ashby and Edwards, 1989; Li *et al.*, 1990; Liscum *et al.*, 1983; Ness *et al.*, 1994). The results of the present study are in accordance with a study in which induction of lead nitrate leads to hypercholesterolemic conditions in rats (Kojima *et al.*, 2004).

Lipids are not only an important part of cell membrane; several hormones are also lipid in nature as well and are required for several cellular activities. Lipids are transport in and out of the cell by several low and high density lipoproteins. HDL carries cholesterol to the liver, so, hepatic dysfunction could be a cause of an enhanced serum cholesterol (Gupta *et al.*, 2008). A significant high level of serum HDL has been noted in both the experimental groups after the induction of leachate. An increment in lipoproteins level is perhaps due to formation of cholesterol and phospholipids from excess fatty acids. These elevated parameters along with triglycerides are converted and released in to the blood stream in the form of lipoproteins. The concentration of cholesterol, HDL, LDL and VLDL found to be increased with the administration of the lethal dose of TAA. The hyperlipidemia noted in this study is might be due to increase in cholesterol biosynthesis activity or in NADPH which is an important requirement of fatty acid as well as of cholesterol biosynthesis (Chi, 1982; Kedar and Chakrabarti, 1982; Sharma *et al.*, 2003; Siddiqua *et al.*, 2010).

Serum creatinine level is an important biomarker to assess chronic kidney disease as well as widely used in evaluation of acute kidney injury (AKI) (Bolton *et al.*, 2002; Mehta *et al.*, 2007). Accurate estimation and very early detection are some widely known limitations in the assessment of renal dysfunction.Tubular creatinine secretion and some other non-renal factors like gastrointestinal elimination, hepatic function and muscle mass also influence the serum creatinine levels. A significant decrease was noted in Group 1 after the induction of leachate which is a clear indication of recovery from renal damage (Nankivell, 2011).

Liver, one of the largest glands of the body, absorbs the nutrients from small intestine and stored them as glycogen. In addition to it, liver is also involved in the biosynthesis of various plasma proteins and in the detoxification and neutralization of various exogenous toxins. Serum protein profile of control and both the experimental groups carried out by SDS page gel electrophoresis against protein marker (ladder) ranging between 10-220 KDa. This comparative analysis revealed relative protein fractions from 68 KDa to 331 KDa. There was found a general decrease in the level of various proteins expression in both the experimental groups. The protein fractions of 317, 212, 178, 134, 89, 74 and 68 KDa were found 24h after administration of leachate (Group 1) compared to control (331, 215, 180, 139, 112, 91, 75 and 69 KDa). However, protein fraction of 202, 171, 136 and 91 KDa were present in Group 2 compared to control (FARRUKH, 2015). This decreased level of various protein fractions is perhaps because of an altered function of liver induced by leachate administration which is also evident from a disturbed level of aminotransferases.

CONCLUSION

Taken together these results it could be concluded that leachate is a potentially toxic industrial effluent which leads to severe alterations in various serological parameters as well as certain serum proteins. So, proper waste treatment is needed prior to disposal of leachate.

Competing interests:

The authors declare that they have no competing interests.

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