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Experimental assessment of arsenic toxicity in garole sheep in India

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ABSTRACT

Arsenic, a dangerous bio-accumulative poison, is a grave threat affecting a large number of people as well as animals throughout the World, particularly in Bangladesh and West Bengal, India. It is also a matter of concern as continuously entering into food chain through biotic and abiotic products. The present study was conducted to evaluate the experimental effect of arsenic toxicosis on Garole sheep of West Bengal. One group was subjected to oral arsenic exposure @ 6.6 mg Kg^{-1} over 133 days when rests considered as negative control. Periodical arsenic estimation in wool, urine and feces along with hemato-biochemical alteration were checked thoroughly. It was evident from the study that long term arsenic exposure exerted a significant ($p < 0.01$) alteration compared to normal animal which were further supported by clinical abnormalities. Exposed animals showed histological changes throughout major internal organs like coagulative necrosis of liver, tubular nephritis of kidney and acanthosis of skin etc. The bio-accumulative and excretion pattern of arsenic inside body were also well understood by the arsenic estimation study of wool, urine and feces which may be helpful for discussion regarding arsenic entry into food chain via animals.

1. Introduction

Arsenic (As) is an element present ubiquitously in the earth's crust. Arsenic in drinking water (having maximum permissible limit of 0.05 μg ml^{-1}) has been recognized as a major public health concern in several regions of the world affecting not only the human population but also the livestock and agricultural products and thus entering into food chain [1]. Approximately 60 million people are at risk of arsenic exposure of Asia alone, of which 0.2 million people are exposed to arsenic endemic region in West Bengal, India. Half of the exposed people of this area exhibited the arsenic toxicosis showing skin lesion; remaining other half people are at risk due to consumption of water containing 10–12 times of minimum permissible limit of arsenic [2].

It is observed that most of the animals mainly ruminants in arsenic prone area do not show any specific clinical symptoms but from their feces and milk significant amount of arsenic is eliminated which further contaminate the pasture land and enter into human food chain [2]. Biswas et al. [3] reported that arsenic treated goats exhibited signs of toxicity from 3 week post-exposure, consisting of dullness and depression with slightly reddish coloured urine, oliguria and weakness, rough body coat with erected hairs and profound muscular weakness. Increased respiratory and heart rate were also observed after long term arsenic administration in goats. Arsenic also causes hepatotoxicity and liver damage in small ruminant like goat [4] and sheep [5]. Experimental Arsenic toxicity in sheep also affects alimentary system, adrenal system and

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respiratory system and ultimately causes death with a lethal dose of 33 mg kg^{-1} body weight in the form of Sodium Arsenite [6].

Among the small ruminant husbandry in India, Garole sheep rearing plays a crucial role in socio-economic condition of rural people of West Bengal. It carries mutated Boroola gene, characteristic of high fecundity, which implies a special economic importance to this animal [7]. The Sundarban area of South 24 Parganas and a small part of North 24 Parganas is in a deltaic zone. A previous study conducted by the School of Environmental Studies from Jadavpur University demonstrated that the extent of arsenic contamination in the ground water of South 24 Parganas was substantial. Garole sheep which live in this area are therefore chronically exposed to arsenic, and there is always a likelihood of arsenicosis in this breed which may affect consumers of affected sheep meat. Due to the paucity of information on arsenic toxicity in garole sheep, this study has been undertaken to further understanding of arsenicosis in sheep based on clinical evaluation, biochemical mining, hematological changes and histological findings. To the authors' knowledge, this is the first such study in garole sheep.

2. Materials and methods

2.1. Animals

Eight apparently healthy male Garole sheep (8–9 months of age, weighing between 10 and 13 Kg) purchased from arsenic-free villages of Kakdwip (as declared by Public Health Engineering Department, Govt. of West Bengal) were used in this experiment. They were caged individually in custom-made stainless steel metabolic cages and reared in arsenic free condition at small animal unit of Department of Pharmacology and Toxicology, West Bengal University of Animal and Fishery Sciences, Nadia, West Bengal. The animals were kept stall feeding with the supply of paddy straw and concentrates and ad lib. water.

Before starting the experiment, the animals were dewormed once with a mixture of levamisole and oxyclozanide (Fluzan, Jeps pharmaceuticals) at the dose rate of 7.5 mg kg^{-1} body weight. The animals were acclimatized in experimental environment for one month. Institution Animal Ethics Committee approved experimental protocol before starting the experiment. The animal experimentation was duly approved by the IAEC before.

2.2. Design of experiment

After conventional toning up all eight Garole sheep were randomly grouped into two groups. Four animals of group1 were kept as healthy negative control. As there is no such reference for experimental chronic dosage patterns in garole sheep so the rest four sheep (group2) were fed with sodium arsenite powder orally mixed with water @ 6.6 mg kg^{-1} b.wt. daily which is 1/5th of the lethal dose in merino sheep [5] for 133 days. Wool (from loin region), urine (by catheterization to avoid contamination) and feces (collected from metabolic cage) sample were collected every seven days interval in the early morning to find out residue of arsenic. Blood was collected aseptically by vacutainer tube from jugular vein at every 14 days interval for periodical biochemical and hematobiological analysis simultaneously.

2.3. Reagent

Diagnostic kits to assess Serum Glutamic Oxaloacetic Transaminase (SGOT) and Serum glutamic pyruvic transaminase (SGPT), Blood Urea Nitrogen (BUN) and Creatinine activity were obtained from Cogent, India. Other chemicals of analytical grade were purchased from Rankem Pvt. Ltd., E-Merck (India), and Sigma Aldrich (USA).

2.4. Estimation of total arsenic

Total arsenic was quantified by wet ashing procedure in hot plate using tri-acid mixture of nitric acid, perchloric acid and sulphuric acid (10:4:1) following the method of Dutta et al. [2]. Briefly, the digested samples were diluted with deionized Millipore water, passed through Whatman filter paper No. 4 (Rankem, India) and made the volume to 10 ml. Concentrated hydrochloric acid (5 ml) was added to it and shaken well. Then 1 ml of potassium iodide (5% w/v) and ascorbic acid (5% w/v) mixture was added and the aliquot was incubated for 45 min for transformation of arsenate to arsenite [8]. The final volume was made up to 25 ml with Millipore water and arsenic concentration read in Atomic Absorption Spectrometer (AAS) equipped with vapor generation accessories (model No. VGA77). The operating parameters were: lamp, arsenic hollow cathode lamp; wavelength, 193.7 nm; slit width, 0.5 nm; lamp current, 10.0 mA; vapor type, air/acetylene; air flow, 10.00 $Lmin^{-1}$; inert gas for hydride generation, Argon. Reducing agent (Aqueous solution of 0.6% sodium borohydride was prepared in 0.5% w/v sodium hydroxide) and 40% HCl were prepared freshly before use. The working standards were 2.5, 5, 10, 15 and 20 μgL^{-1} and prepared by same procedure as test sample.

2.5. Biochemical parameters

Serum Glutamic Oxaloacetic Transaminase (SGOT) and Serum glutamic pyruvic transaminase (SGPT) activity was measured by Reitman and Frankel [9] method using commercially available kit (Cogent, India) and following manufacturer's instructions. The activity was expressed as IU L^{-1}. BUN was measured from plasma samples by the DAM method and Plasma creatinine was estimated from plasma samples following Jaffes reaction by standard procedure depicted in manufacturer's instruction kit (Cogent, India) and both the quantities were expressed in $mgdl^{-1}$.

2.6. Hematological parameters

From blood sample hemoglobin level was determined at 14 days interval by indirect acid haematin method as described by Coffin [10] and expressed as gm/dl. Total erythrocyte count was done following standard method of Wintrobe as described by Schalm et al. [11].

2.7. Histopathology

Samples of liver, kidney, skin and intestine were collected from all the sheep in 10% buffered formal saline for histopathological examination as these organs are rich in oxidative system and more susceptible to arsenic toxicity [12].

2.8. Statistical analysis

Each of the three parameters i.e. arsenic concentration in faeces, urine and wool was analyzed by two -way ANOVA using the following linear model;

$$y_i = \mu + d_j + ex_k + dex_{j(k)l} + e_{ijklm}$$

where, y_i is the as conc. in faces/urine/wool, μ is the overall mean, d_j is effect due to days of experiment; ex_k is effect due to exposure of

arsenic, $dex_{j(k)l}$ is effect due to interaction between day of experiment and arsenic exposure, e_{ijklm} is the residual error. SYSTAT 12 statistical software was used for the analysis.

3. Results

3.1. Clinical manifestation of sheep after daily arsenic ingestion

The symptoms exhibited by the arsenic exposed group were complex in nature. It was noted that no clinical manifestation was observed in first four weeks of arsenic administration. But after six weeks animals were suffering from general weakness, lethargy, dull & depression. The body weight was taken after seven weeks of daily oral administration of arsenic and it was noticed that all arsenic ingested animals had lost their body weight about 3–4 kg. Depilation of hair was profound in two sheep of arsenic exposed group after 110 days at oral surface and neck region. No alteration of visible mucous membrane was observed up to four weeks but later became congested first and then pale after 12 weeks onwards.

On clinical examination, body temperature was found within normal range but there was a variation in temperature (103.1° ± 0.6). Increased heart rate, respiration rate and decreased rumen motility were observed in exposed animals in comparison to the negative control animals. A superficial examination by pulling of skin showed that elevation remained even more than 1 min indicating dehydration as well as reduced tonicity of skin. Unformed characteristic natures of feces deviating from usual pellet form in some sheep were started from seven weeks onwards.

Gross post mortem findings showed distended abomasum and rumen along with splenomegaly.

3.2. Concentration of arsenic in different substrates from sheep

3.2.1. Feces

The average arsenic concentration in feces of exposed group following daily oral administration of sodium arsenite was significantly ($p < 0.01$) higher than that of non exposed group. The least square means were 14.656 ± 0.441 $mgKg^{-1}$ and 0.281 ± 0.402 $mgKg^{-1}$, respectively (Table 1). It was observed that arsenic excretion through feces in exposed group was maximum on day 7 and then there was gradual and significant ($p < 0.01$) reduction (Fig. 1A) which suggested alteration of body metabolism and increasing accumulation of arsenic in different internal organs.

3.2.2. Urine

It was observed that the excreted arsenic concentration in urine showed maximum elevation on day 7 and then declined gradually and significantly ($p < 0.01$), followed the same pattern as Arsenic concentration in feces (Fig. 1A). The average arsenic concentration in urine of exposed group was significantly ($p < 0.01$) higher than that of non exposed group. The least square means – were 5.636 ± 0.097 mgL^{-1} and 0.056 ± 0.008 mgL^{-1}, respectively (Table 1).

3.2.3. Wool

It was observed that arsenic deposition in wool of exposed animals increased gradually and significantly throughout the period of experiment – (Fig. 1A). The average arsenic concentration in wool of exposed group was significantly ($p < 0.01$) higher than that of non exposed group. The least square means were 4.772 ± 0.044 $mgKg^{-1}$ and 0.531 ± 0.040 $mgKg^{-1}$, respectively (Table 1).

3.2.4. Hemoglobin (Hb)

The least square mean levels of hemoglobin (gdl^{-1}) were 9.648 ± 0.028 and 7.904 ± 0.031 in arsenic exposed and non exposed group, respectively which differed significantly ($p < 0.01$) (Table 2). Mean values of hemoglobin (Hb) of arsenic exposed and normal sheep were 9.62 ± 0.1 and 9.63 ± 0.08 at 0 day. After 133 days of oral administration the value reduced to 5.875 ± 0.23 in arsenic exposed group. Value remained almost constant in non exposed group (Fig. 2-A1).

3.2.5. Total erythrocytic count (TEC)

The TEC levels ($10^{12}L^{-1}$) on 0 day were 8.93 ± 0.03 and 8.82 ± 0.09 in arsenic exposed and non exposed group, respectively. After 133 days of arsenic exposure the value dropped (6.63 ± 0.04) in all arsenic exposed animals compared to healthy control group (Fig. 2-A2) indicated a sign of anemia. Least square means of TEC (10^{12}/L) of the arsenic exposed group (7.773 ± 0.024) and non exposed group (8.825 ± 0.022) indicated a significant fall ($p < 0.01$) of TEC in exposed group (Table 2).

3.3. Biochemical profiles

3.3.1. Blood Urea Nitrogen (BUN) and plasma creatinine

It was observed that the value of creatinine level ($mgdl^{-1}$) elevated gradually with the insult of induced arsenicosis in arsenic exposed animal (Fig. 2-C2). Serum Creatinine level ($mgdl^{-1}$) of arsenic exposed and normal animals were 1.55 ± 0.12 and 1.57 ± 0.103, respectively at 0 day whereas 4.18 ± 0.04 and 1.74 ± 0.04, respectively at the end of experiment. Least square means of serum creatinine level of arsenic exposed and non exposed animal also varied significantly ($p < 0.01$) (Table 2).

The Blood Urea Nitrogen (BUN) level ($mgdl^{-1}$) change also followed the same pattern like Creatinine. Blood urea nitrogen level of arsenic exposed animal were 11.62 ± 0.302 and 30.05 ± 0.714 at 0 day and 133th day, respectively where healthy control animal showed almost similar pattern (Fig. 2-C1). In control animal, level of BUN ($mgdl^{-1}$) was found significantly ($P < 0.01$) low in comparison to exposed animals (Table 2).

3.3.2. Serum Glutamic Oxaloacetic Transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT)

The mean levels (IU/L) of SGOT and SGPT in arsenic exposed group increased significantly ($P < 0.01$) than non exposed group (Table 2). At the end of the experiment SGOT and SGPT level (IU/L) of arsenic induced animals were 275.7 ± 5.494 and 54.3 ± 0.406, respectively (Fig. 2-B1 and B2) which was much higher than the negative control group as well as the corresponding values on day 0 (45.23 ± 2.653 and 22.19 ± 1.179).

Table 1
Arsenic (As) concentration in feces, urine and wool of Garole sheep (least square mean value ± SE).

Arsenic concentration ($mgKg^{-1}$ or mgL^{-1})	Exposed group	Non –exposed group
Faeces ($mgKg^{-1}$)	14.656 ± 0.441[a]	0.281 ± 0.402[b]
Urine (mgL^{-1})	5.636 ± 0.097[a]	0.056 ± 0.008[b]
Wool ($mgKg^{-1}$)	4.772 ± 0.044[a]	0.531 ± 0.040[b]

Different superscript indicates that means within the row differ significantly ($p < 0.01$).

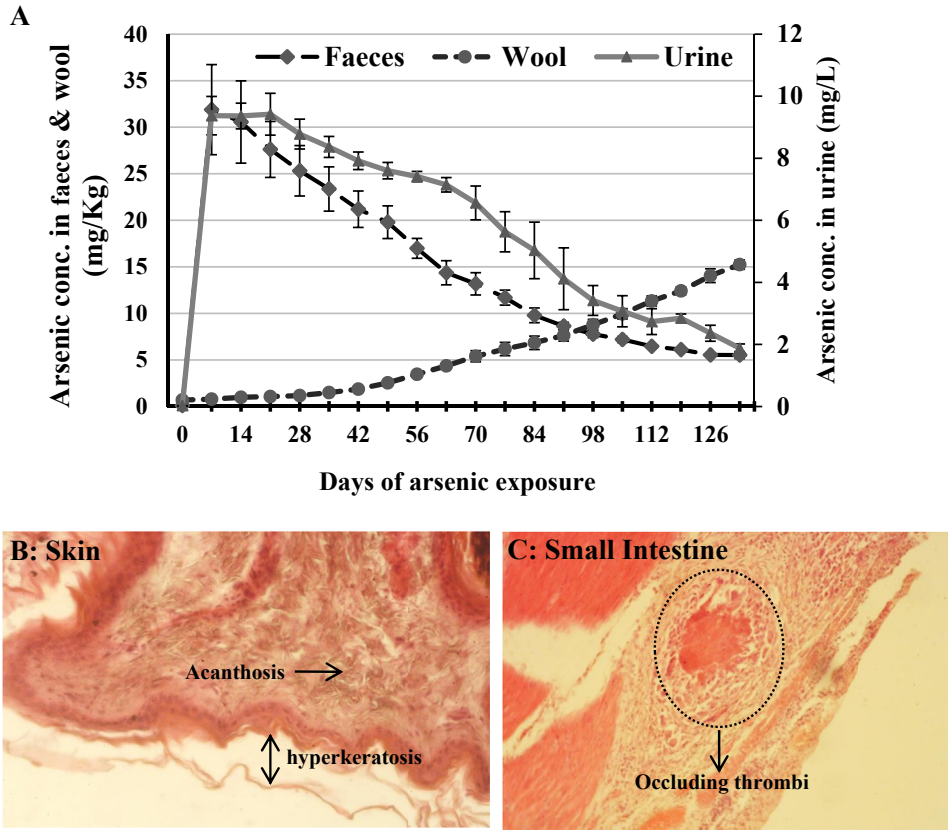

Fig. 1. Changes in the excretion and accumulation pattern of arsenic in body and subsequent changes of body surface skin as well as inner intestinal tract. **(A)** Arsenic concentration in faeces, wool ($mgKg^{-1}$) and urine (mgL^{-1}) of sheep after single oral administration of sodium arsenite @ 6.6 $mgKg^{-1}$ for 133 days. **(B)** Hyperkeratosis, acanthosis of arsenic affected skin (H and E stain, 45×). **(C)** Throbi adhered with wall of vessel and few empty space for blood channelization; also thickening of muscularis layer of intestine (H and E stain, 10×).

Table 2
Comparison of hematological and biochemical parameters between arsenic exposed and non-exposed garole sheep (least square mean ± SE).

Parameters (unit)	Unexposed control group	Arsenic exposed group
Haemoglobin (Hb)% (gdl^{-1})	9.648 ± 0.028*	7.904 ± 0.031**
Total erythrocytic count (10^{12} L^{-1})	8.825 ± 0.022*	7.773 ± 0.024**
Blood urea nitrogen ($mgdl^{-1}$)	13.297 ± 0.34*	22.085 ± 0.377**
Serum creatinine ($mgdl^{-1}$)	1.581 ± 0.066*	3.297 ± 0.073**
Serum glutamic oxaloacetic transaminase (IU L^{-1})	49.542 ± 0.735*	162.342 ± 0.815**
Serum glutamic pyruvic transaminase (IU L^{-1})	23.77 ± 0.502*	39.316 ± 0.556**

Different superscript indicates that means within the row differ significantly ($p < 0.01$).

3.4. Histopathological findings

Vacuolation and coagulative necrosis of hepatocytes was seen in the centrilobular, midzonal and periportal areas of the hepatic lobules with infiltration of degenerative neutrophils and lymphocytes. At focal areas there was presence of RBCs prolification of bile duct. Toxic necrosis of kidney was observed with degenerative changes in tubules and inflammatory changes (tubular nephritis) with evidence of cast and massive hemorrhage with cellular infiltration. Shrinkage of glomeruli in the focal areas was also evidenced. Hyperkeratosis, parakeratosis and acanthosis were the main findings when cutaneous sections were examined. Congestion, atrophy of the secretory gland architecture of the small intestinal mucosa due to proliferation of connective tissue and mononuclear cellular infiltration were observed. Thickness of muscularis mucosa was also evidenced. Congestion and atrophy of secretory gland along with thrombi formation and massive mononuclear cellular infiltration was observed in the large intestine.

4. Discussion

It is evident from the results that just after induction of toxicity by orally feeding of arsenic, garole sheep exhibited no clinical symptoms. Moreover the animals tried to remove toxic arsenic from their body through feces and urine (Fig. 1A) which confirmed the findings of De [13], Mandal [14], Rana et al. [15], Ghosh et al. [16], and Biswas [17]. After absorption, inorganic arsenic is accumulated in liver, spleen, kidney, lung and gastrointestinal tract and during metabolism most of the inorganic arsenic are metabolized to dimethylarsenic acid and monomethylarsenic acid, which then rapidly cleared from the tissue through urine [18] which is considered as a good indicator for current exposure [19] and may be excreted in the faeces without absorption [4] also.

In contrast to gradual decrease in excretion of arsenic through urine and feces there was a gradual increase of arsenic accumulation in wool of arsenic exposed sheep (Fig. 1A). The observation in relation to arsenic content in wool of sheep was closely similar with

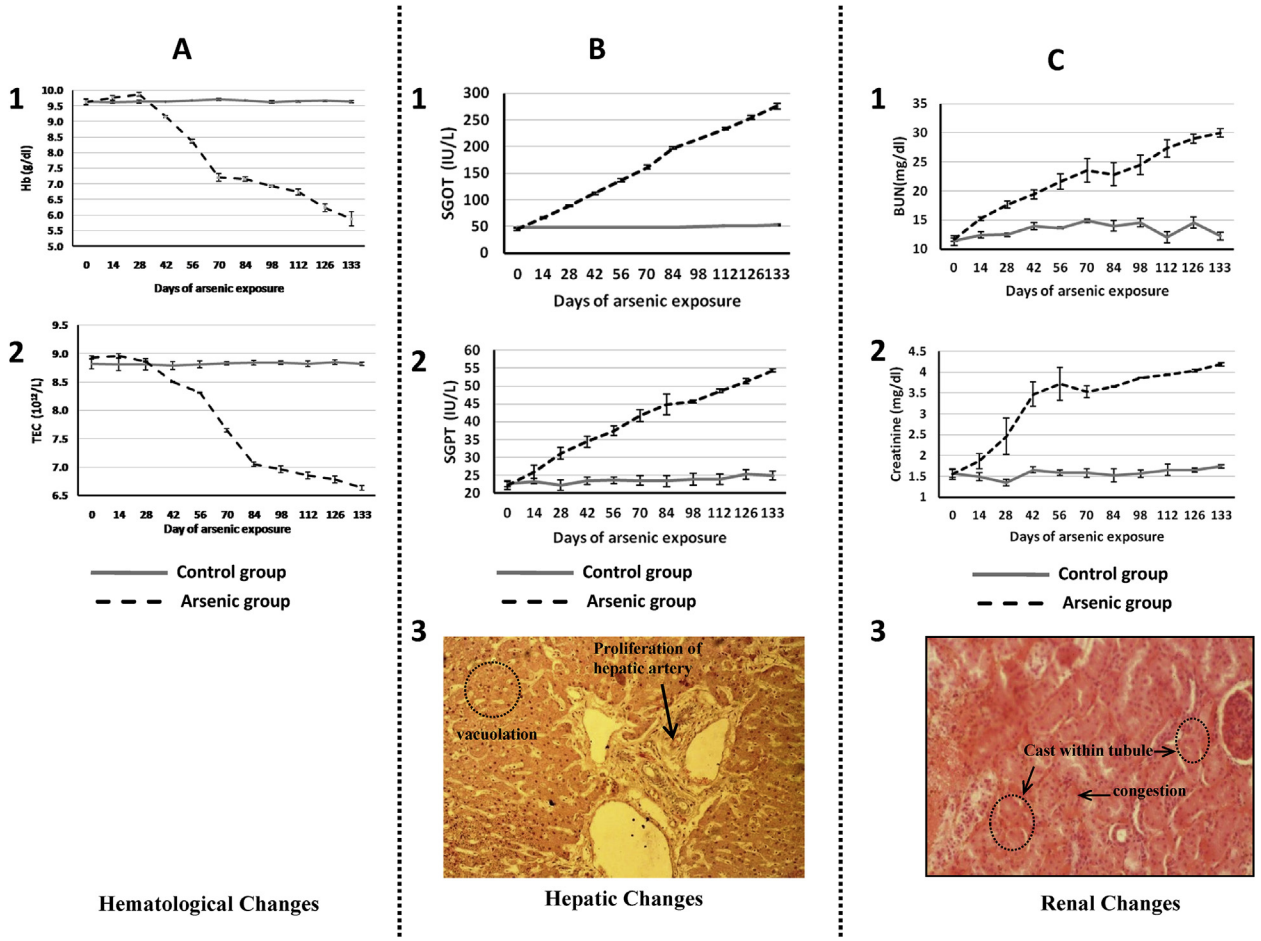

Fig. 2. Changes of different hematological (**A**) and biochemical parameters due to damage in hepatic (**B**) and renal system (**C**). **(A)** Hematological parameters include Hemoglobin concentration (g dl^{-1}) **(1)** and Total Erythrocytic Concentration (10^{12} L^{-1}) **(2)** in healthy control and arsenic exposed group of sheep after single oral administration of sodium arsenite @ 6.6 $mgKg^{-1}$for 133 days. **(B)** Liver of affected sheep showing vacuolation of hepatocytes and proliferation of hepatic artery (H and E stain, 10×) **(3)** which were supported by changes in liver specific enzymes, **(1)** SGOT (IU L^{-1}) and **(2)** SGPT (IU L^{-1}). Similarly, **(C)** Renal damage is attributed by congestion and hyaline cast within lumen of proximal tubule indicating toxic necrosis (H and E stain, 20×) **(3)** supported by changes in the specific renal markers change i.e. BUN level ($mgdl^{-1}$) **(1)** and Creatinine level ($mgdl^{-1}$) **(2)**.

the findings of Riviere et al. [20], who estimated the arsenic content in hair of cattle within the range of 0.80–3.4 mg kg^{-1}. The findings were supported by Radostis et al. [21], who reported that arsenic content in hair of cattle was as much as 5–10 mg kg^{-1} whereas, in animals not exposed to arsenic should contain less than 0.5 mg kg^{-1}.

The key enzyme of biomethylation of arsenic is present in liver [22] and kidneys are the major route of arsenic excretion but arsenic as a toxicant is linked to hepatic damage [3,23–25] and damage in the kidney including capillaries, tubules, and glomeruli [26] leading to kidney dysfunction [27] after chronic exposure. Exposure to As is known to cause severe toxic effects in almost all the major target organs like liver, brain, endocrine and cardiovascular system besides inhibiting DNA repair capability [28,29].

In the present investigation arsenic was excreted readily through feces and urine just after arsenic exposure to sheep when the body system was clinically healthy. But gradually hepatic enzyme like SGOT & SGPT level and renal function marker BUN and Creatinine level increased day by day due to toxic effect of arsenic which resulted in a simultaneous decrease of arsenic excretion through faeces and urine and increase of arsenic accumulation in wool. Increased level of Creatinine indicated the sign of renal failure [17]. The increased level of Creatinine was also recorded by Faries [30] in arsenic toxicated cattle. The action of arsenic on renal capillaries, tubules and glomeruli may cause severe renal damage [30]. The rise of urea might be due to the failure of kidney to remove metabolic products [15]. Faries [30] also recorded the increased level of BUN in inorganic arsenic toxicosis in a beef herd. The level of both SGOT and SGPT, two liver specific enzymes increased significantly as the experiment advances suggesting the possibility of alteration in the cell metabolism in liver as a result of toxic effect of arsenic and leaking out into the blood from the damaged tissues. The increased value of SGOT and SGPT was supported by the observation of Biswas et al. [3], in experimentally produced chronic toxicity in goats. Guha Mazumder et al. [32] reported the elevated level of SGPT in human patients of arsenic poisoning. Santra et al. [23] reported the hepatic damage caused by experimentally produced chronic arsenic toxicity in mice and there was elevated level of SGOT and SGPT. Histopathologically the damage was strongly supported by the lesion on kidney (Fig. 2-C3) and liver (Fig. 2-B3).

The biomethylation or metabolism process of arsenic become easily saturated and lead to the excess inorganic arsenic being deposited in the skin, hair and nails, where it tightly binds to keratin [33] and because of the high sulphydryl content of keratin, the highest concentration is found in hair and nails. Deposition in hair starts within two weeks of exposure, and arsenic stays fixed at this site for years [31]. The arsenic content in nails and hair has been used as a biomarker for arsenic exposure, including both current

and past exposure, while urinary arsenic is a good indicator for current exposure [19].

Clinically it was also supported by adverse findings of exposed animals only after 6 weeks onwards. The findings as recorded in the list of symptoms were corroborated with the reports of Anderson [34], Biswas [17], Howard and Smith [35], Kesavarzi et al. [5] and Radostis et al. [21]. Increased respiratory rate might be due to the toxic effect of arsenic and sign of compensatory mechanism against the adverse condition [36]. Increased heart rate was indicative of exaggerated heart for an attempt to maintain cardiac output and as a result of toxicosis. It has been reported that arsenic can lead to atherosclerotic diseases, peripheral vascular disease [37] and ischemic heart disease in arseniasis-hyperendemic villages of Taiwan [38]. Dehydration might be attributed due to various factors like toxicosis, anorexia, unformed faeces. Visible mucous membrane was pale which indicate anemia. Unformed faeces was possibly due to decreased metabolic activity as arsenic combines with sulphydryl group to inhibit the activities of enzymes [39] that was simulated with the reports of Petrusevski et al. [40]. Pathological changes in intestine (Fig. 1C) accounts an essential proof of arsenic toxicity on gastrointestinal system. Toxic effect of Arsenic on skin was also supported by the histopathological lesion (Fig. 1B).

Gradually decreasing of Hb percentage and TEC in animals of arsenic exposed group was indication of anemia. The findings were corroborated with the report of Kesavarzi et al. [5], Biswas [17], and Klaassen [31]. Biswas et al. [41] also reported the decreased level of Hb in experimentally produced chronic As toxicity in goats. Low Hb percentage in ruminants suffering from chronic arsenicosis was also reported by De [13], Mandal [14] and Ghosh et al. [16].

Besides, the anemia was due to suppression of the activity of metabolism and bone marrow as a residue of toxicant. Exposure of arsenic has been known to influence the activity of several enzymes of haem biosynthesis (ferrochelation, ALA synthesis). All eight steps of haem synthesis are catalyzed by enzymes which require functional sulphydryl group for optimal activity. Arsenic has affinity with the functional −SH group. The mechanism might have been ascribed for lowering level of Hb in arsenicosis [3,5,and38]. The decreased level of TEC in affected animals was also possibly due to hypoproteinemia or nutritional deficiency as a result of continuous inappetence to anorexia by the arsenic exposure.

5. Conclusion

From the present study it may be concluded that in long term exposure of arsenic on garole sheep, firstly the body tries to eliminate the arsenic as much as possible by faeces and urine but later due to toxic action of arsenic on different body system particularly vascular, hepatic, renal and gastrointestinal system, arsenic excretion diminishes through faeces and urine and simultaneously accumulated inside body resulting increasing concentration in wool. Different types of marked and significant damages by arsenic are reflected biochemically, hematologically and histopathologically in garole sheep. This arsenic accumulation tendency in the body of garole sheep is a threatening point to that particular livestock and human being also from the point of food chain in the Sundarban region of West Bengal.

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