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Identification of potential biomarkers of hepatotoxicity by plasma proteome analysis of arsenic-exposed carp *Labeo rohita*

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HIGHLIGHTS

- Plasma proteome of exposed carp showed increased abundance of ApoA1, A2ML, Wap65, TF.
- All these proteins indicate liver damage in arsenic toxicity.
- These proteins could serve as biomarkers of hepatotoxicity and chronic liver damage.

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ABSTRACT

Arsenic (As) is a toxic environmental contaminant and potential human carcinogen. Chronic intake of arsenic-contaminated water and food leads to arsenicosis, a major public health problem in many parts of the world. Early detection of arsenic toxicity would greatly benefit patients; however, the detection of arsenicosis needs to be done early before onset of severe symptoms in which case the tools used for detection have to be both sensitive and reliable. In this context, the present study investigated plasma proteome changes in arsenic-exposed *Labeo rohita*, with the aim of identifying biomarkers for arsenicosis. Changes in the plasma proteome were investigated using gel-based proteomics technology. Using quantitative image analysis of the 2D proteome profiles, 14 unique spots were identified by MALDI-TOF/TOF MS and/or LC–MS/MS which included Apolipoprotein-A1 (Apo-A1) (6 spots), α-2 macroglobulin-like protein (A2ML) (2 spots), transferrin (TF) (3 spots) and warm-temperature acclimation related 65 kDa protein (Wap65). The proteome data are available via ProteomeXchange with identifier PXD003404. Highly abundant protein spots identified in plasma from arsenic-exposed fish i.e. Apo-A1 (>10-fold), A2ML (7-fold) and Wap65 (>2-fold) indicate liver damage. It is proposed that a combination of these proteins could serve as useful biomarkers of hepatotoxicity and chronic liver disease due to arsenic exposure.

1. Introduction

Arsenic is a major toxic environmental contaminant and a potential human carcinogen. Chronic arsenic toxicity (arsenicosis), due to intake of arsenic-contaminated drinking water and food, is a major environmental health hazard throughout the world [1]. However, severity of this problem in India and Bangladesh is unprecedented and arsenic concentrations found to the level of concern (upto 3–4 ppm) [2,3]. Arsenic concentration in some of the mining areas in USA has also been reported to be up to 10 mg/L (10 ppm) which indicate an alarming situation (US EPA's cutoff for arsenic in drinking water is 10 ppb and WHO recommended value for India and Bangladesh is 50 ppb) [4]. Numerous epidemiological studies demonstrate that exposure to this hazardous metalloid is associated with an increased incidence of lung, bladder and skin cancer, as well as diabetes and cardiovascular disease in humans [5]. Nevertheless, the early detection of arsenic toxicity will be beneficial in preventing these adverse effects arising from arsenic

Abbreviations: As, arsenic; Apo-A1, apolipoprotein-A1; A2ML, α-2 macroglobulin-like protein; Hpx, hemopexin; IPA, ingenuity pathway analysis; TF, transferrin; Wap65, warm-temperature acclimation associated 65 kDa protein; leg1A, liver enriched gene protein 1A; Hb-β, hemoglobin-β.

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exposure [6]. Proteomics and related omics technologies are ideal platforms for the identification of biomarkers which not only reflect the physiological, pharmacological, or disease processes associated with arsenic toxicity but also be useful for developing therapeutic interventions [7].

One widely used approach for the identification of biomarkers is the analysis of the serum or plasma protein complement [7]. As plasma circulates through tissues it picks up proteins released from cells due to physiological events including tissue remodeling and cell death. Thus, specific disease processes can often produce distinctive plasma proteome signatures [8]. Moreover, variations in the plasma proteome have been linked with various physiological and pathophysiological conditions [8]. Some progress has been made in defining changes in the human serum proteome following environmental exposure to arsenic [9].

A number of animal models, including fish, have been used to understand how arsenic exposure causes the diverse disease outcomes [10–18]. In experimental studies, arsenic concentration varying from 10 to 100 ppm has been used in rat model [19,20]. Similarly, concentrations ranging from 1 ppb to 50 ppm have been used in fish models [12–18], which includes both model (zebrafish, *Danio rerio*) [12–14] and some non-model but commercially important fish species including salmonids and cyprinids [15–18]. The carp *Labeo rohita* has been used as the experimental animal in this study. It is an important food fish in the Indian subcontinent and is widely consumed in As-hit areas. Moreover; it is a cyprinid, like the model organism zebrafish, for which the blueprint of complete genome is available [21]. As our earlier studies on *L. rohita* fingerlings showed development of skin lesions and cataract [22] and generalized immune suppression at 15 ppm arsenic concentration [23], in this preliminary study we investigated the plasma proteomic changes in *L. rohita* following arsenic exposure at 0.5–15 ppm concentration to identify potential biomarkers of arsenicosis, which could be extrapolated in other organisms including humans for diagnosis/prognosis purposes.

2. Materials and methods

2.1. Experimental animals

Healthy *Labeo rohita* fingerlings (n = 70; length 13–15 cm, average weight 24–25 g) were procured from a local hatchery and acclimatized in the laboratory for 15 days before the exposure. Fishes were fed with a commercially available feed at 3% of body weight daily. The study including sample collection, animal experimentation and sacrifice meet the ethical guidelines, including adherence to the legal requirements, of the study country.

2.2. Experimental arsenic exposure

An exposure study was carried out with different concentrations of arsenic for 12 days, since the adverse effects of acute exposure are manifested within a short time (up to 14 days) after administration of a given concentration of toxicant like arsenic [24]. Based on our previous work [22,23], arsenic (sodium meta arsenite, $NaAsO_2$) was added at doses of 0.5-, 1-, 2.5-, 5-, 10- and 15 ppm, to different glass aquaria containing 10 fishes each in 30 l of water. A group of 10 fishes with no arsenic was taken as control. The water of each aquarium was exchanged daily with fresh water containing arsenic at the required dosage. Aerators were connected to all the aquaria to maintain the dissolved oxygen level in the water at around 6 ppm.

2.3. Collection of plasma

The fish were anaesthetized using MS 222, (Tricaine methanesulfonate; 200 mg/L) and the blood from the caudal vein was collected into heparinized microfuge tubes. The blood samples were centrifuged at 10,000 rpm for 10 min at 4 °C in a microcentrifuge (Biofuge *FRESCO,* Heraeus), the plasma samples collected and stored in aliquots at −40 °C for later analysis. The protein concentration of the plasma was determined using the Bradford protein assay with BSA (A8002, Sigma) as the standard [25].

2.4. Arsenic accumulation

Accumulation of arsenic in different fish tissues (Blood plasma, liver, gill, scale, muscle) (n = 10 for both control and 15 ppm arsenic exposed group) were assayed using atomic absorption spectrometry (AAS) (Varian Spectra-220 AA, Australia). Pre weighed tissues were taken in a conical flask and a digestion mixture (3 ml 70% $HClO_4$ + 21 ml 65% HNO_3 + 1.5 ml 98% H_2SO_4) was added and incubated overnight at room temperature. Further, it was heated for 2–3 h or until colorless. It was then filtered with Whatman paper no. 42 making the volume up to 100 ml with 2% HNO_3 [26]. The arsenic contents were expressed in mg/kg (ppm).

2.5. 2D GE

To separate plasma proteins by 2D GE, isoelectric focusing was performed using a Protean IEF Cell (Bio-Rad,UK) with immobilized pH gradient (IPG) strips (7 cm, pH 5–8, Bio-Rad) and second dimension SDS-PAGE was performed using 12% separating gel with 5% (w/v) stacking gel in a mini- Protean 3 electrophoresis cell (Bio-Rad) following standard protocol, as described earlier [27]. In many studies involving serum/plasma proteomics, depletion of high abundant proteins are done during sample preparation as high abundance proteins pose a major problem in resolving other low abundance proteins. However, these high abundant proteins act like molecular sponges and readily bind a wide variety of low abundance proteins (341 different proteins and peptides bound to albumin only) [28]. Depleting the high abundance proteins results in removal of these low abundance proteins also [28,29]. Therefore, out of the two options available, we opted for non-depletion of high abundant proteins in the present study.

2.6. Gel image analysis

Gel images were acquired using an ImageScanner III (GE Healthcare) running LabScan version 6.0. Image analysis of the 2D GE protein profiles was carried out using PD Quest (version 7.2.0) (Bio-Rad) [27]. Each experimental condition was represented by three independent biological replicates. Student's *t*-test was performed with a 95% significance level to identify those proteins with significant differences in abundance between the control and exposed fish plasma proteomes.

2.7. MALDI-TOF/TOF MS

Protein spots of interest were cut from the 2-D polyacrylamide gel and the resulting peptides were extracted following standard protocols and analyzed by MALDI-TOF/TOF MS using a 5800 Proteomics Analyzer (AB Sciex,USA) [30]. The MS proteomics data have been deposited to the ProteomeXchange Consortium [31] via the PRIDE partner repository with the dataset identifier PXD003404. For protein identification, peptide masses from trypsin digests derived using the MALDI-TOF/TOF MS were used to search against Ludwig NR database and taxonomy set to Actinopterygii (ray-finned fishes) using the MASCOT sequence matching software (Matrix Science, www.matrixscience.com). The MASCOT search parameters were set following standard published protocol [27].

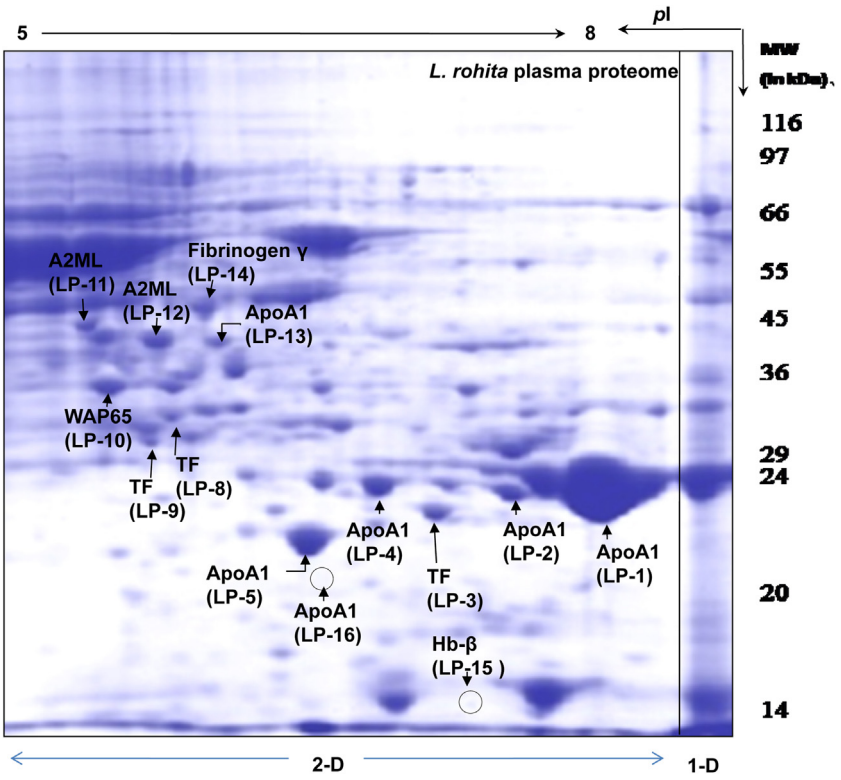

Fig. 1. Representative 2D GE protein profile of plasma proteins recovered from *Labeo rohita* fingerlings exposed to 15 ppm arsenic. Arrows indicate protein spots that exhibit significant differences in abundance between control and treated fish.

2.8. Tryptic peptide analysis by LC–MS/MS

On the basis of 2-D Gel image analysis, 14 protein spots were found to be significantly altered in abundance and all of them were subjected to identification by MALDI-TOF/TOF MS analysis. 11/14 proteins identified had significant scores when searched in MASCOT whereas rest three did not give any significant scores. Thus these 3 spots were subjected LC–MS/MS analysis. Further, 6 more spots were randomly selected out of the spots successfully identified by MALDI-TOF/TOF MS for validation of the results. LC–MS/MS analysis was carried out following previously documented protocols [32]. Briefly, protein spots were analyzed by LC–MS/MS using a HCT Ultra PTM Discovery System (Bruker Daltonics, Coventry, UK) coupled to the UltiMate 3000 LC System (Dionex, Camberley, Surrey, UK). Peptides were separated on the Monolithic Capillary Column (200 mm id, 65 cm; Dionex part no. 161409).

2.9. Pathway analysis

To infer the tentative interaction maps associated with arsenic induced stress response, proteomic data were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com). Each gene identifier was mapped to its corresponding human homolog in the Ingenuity Pathways Knowledge Base.

2.10. RNA extraction, cDNA synthesis and PCR for transcript analysis

Total RNA was extracted from liver using RNA Express reagent (Himedia, India) according to manufacturer's instructions and was reverse transcribed into cDNA, following previously published protocol [23]. Polymerase chain reaction (PCR) was performed on a Veriti 96 well Thermocycler (Applied Biosystem) [23] for the amplification of target cDNAs using primer pairs available in literatures. Predicted amplicon sizes and the cycling conditions for each gene are given in Supplementary Table S1. Constitutive expression of housekeeping gene beta-actin was used as both positive control and for sample normalization. PCR products (8 μl) were visualized on a 1.6% agarose gel pre-stained with EZ-Vision (Amresco) using ImageQuant LAS4000 (GE Healthcare). Densitometric analysis of gene expression was carried out in Image J gel densitometry software to calculate the band intensities of target genes in comparison to beta-actin bands. Statistical differences in gene expression between control and treated samples were assessed by the Student's two-tailed non-paired *t*-test, irrespective of the normality of distribution. $P \leq 0.05$ was considered statistically significant.

2.11. Sequencing of amplicons, sequence homology and alignment

PCR product of respective genes was electrophoresed on 1.6% agarose gels, and bands of appropriate sizes were purified from the gels using gel extraction kit (Himedia Laboratories, India). They were sequenced using Sanger's dideoxy sequencing protocol (ABI 3730 XL). To confirm the identity of the consensus genes, the sequences were subjected to Blastn and Blastx [33] for comparing with the GenBank nucleotide and protein database, respectively. The relevant sequences were retrieved from GenBank for multiple sequence alignment using ClustalW within molecular evolutionary genetics analysis (MEGA 6.0) package with respect to the nucleotide sequence of different genes. Data were analyzed using poisson correction, and gaps were removed by complete deletion. Partial sequences of the respective genes were submitted to GenBank and the consensus sequence was used for further analysis.

Table 1
Protein spots in *Labeo rohita* plasma proteome identified by MALDI-TOF/TOF MS.

Gel spot No.[a]	Identified protein	Thr. pI; mass	MASCOT Score	Seq. Cov. (%)	Acc. no	Function[b]	Species	Fold change	t-test (p-value)
LP-1	Apo-A1	6.37;30098	110	4%	D6R712	lipid transport	*Hypophthalmichthys molitrix*	↑10.45	0.004
LP-2	Apo-A1	6.37;30098	95	4%	D6R712	lipid transport	*Hypophthalmichthys molitrix*	0	0.38
LP-4	Apo-A1	6.37;30098	84	4%	D6R712	lipid transport	*Hypophthalmichthys molitrix*	↑3.11	0.009
LP-5	Apo-A1	5.25;30098	51	8%	F1ACC9	lipid transport	*Megalobrama amblycephala*	↑0.22	0.009
LP-8	TF variant F	5.91;73041	229	5%	B3GPN3	iron ion transport	*Cyprinus carpio*	↑1.09	0.005
LP-9	TF variant F	5.91;73041	151	3%	B3GPN3	iron ion transport	*Cyprinus carpio*	↑0.56	0.001
LP-11	leg 1A	5.09; 39906	55	3%	A5PF61	liver development	*Danio rerio*	↑0.22	0.004
LP-12	A2ML (Fragment)	5.30;159920	46	6%	A0JMP8	liver development	*Danio rerio*	↑7.93	0.004
LP-14	Fibrinogen gamma polypeptide	5.12;48733	628	13%	D6R707	platelet activation, signal transduction	*Hypophthalmichthys molitrix*	↓0.59	0.002
LP-15	Hb-β	7.70;16330	94	8%	B0BL35	oxygen transport	*Carassius auratus*	Absent in fish exposed to 15 ppm arsenic	0.008
LP-16	Apo-A1	6.37;30098	79	8%	D6R712	lipid transport	Hypophthalmichthys molitrix	Absent in fish exposed to 15 ppm arsenic	0.001

Protein spots of interest on the basis of alteration were cut from the 2-D polyacrylamide gel (Fig. 1) of arsenic exposed fishes/control and assigned spot no indicated in brackets. Identified protein name, calculated p*I*, MASCOT Score were obtained by Peptide mass fingerprinting. ↑ and ↓ arrow indicates up and down regulation of the protein spots. Fold change value was calculated by using 2D gel image analysis software PDQuest (Bio-Rad) version 7.2.0. A value of $p < 0.05$was considered statistically significant.

[a] Refers to protein spot numbers given in Fig. 1.

[b] Functions of individual proteins as predicted through Uniprot (http://www.uniprot.org/uniprot/).

Table 2
Protein spots in plasma proteome of *Labeo rohita* identified by LC–MS/MS.

Gel spot No.[a]	Protein ID	Protein Mass	Isoelectric point	No. of Unique peptides	No. of Unique Spectra	Identified protein	Function[b]	Species	Fold change	p-value
LP-3	gi|187340330|emb|CAM96565.1|	18797.35	8.04	2	3	TF	iron ion transport	*Labeo rohita*	↑1.15	0.001
LP-4	gi|267881846|gb|ACY82518.1|	29824.57	6.81	2	2	Apo-A1	lipid transport	*Cirrhinus molitorella*	↑3.11	0.009
LP-5	gi|295314922|gb|ADF97611.1|	30154.78	6.81	2	4	Apo-A1	lipid transport	*Hypophthalmichthys molitrix*	↑0.22	0.009
LP-9	gi|189473163|gb|ACD99641.1|	75036.33	6.24	3	9	TF variant F	iron ion transport	*Cyprinus carpio*	↑0.56	0.001
LP-10	gi|14388583|dbj|BAB60809.1|	50640.2	5.92	3	21	Wap65	metal ion binding	*Cyprinus carpio*	↑2.45	0.004
LP-11	gi|6009729|dbj|BAA85039.1|	157876.23	5.66	1	2	A2ML	liver development	*Cyprinus carpio*	↑0.22	0.004
LP-12	gi|116487878|gb|AAI25960.1|	161516.11	5.1	1	1	A2ML	liver development	*Danio rerio*	↑7.93	0.004
LP-13	gi|295314922|gb|ADF97611.1|	30154.78	6.81	3	35	Apo-A1	lipid transport	*Hypophthalmichthys molitrix*	↑1.05	0.002
LP-16	gi|295314922|gb|ADF97611.1|	30154.78	6.81	2	9	Apo-A1	lipid transport	*Hypophthalmichthys molitrix*	Absent in fish exposed to 15 ppm arsenic	0.001

Protein spots of interest on the basis of alteration were cut from the 2-D polyacrylamide gel (Fig. 1) of arsenic exposed/control fishes and assigned spot no indicated in brackets. Identified protein name, protein mass, isoelectric point, no. of unique peptides and spectrums, were obtained by LC–MS/MS analysis. ↑ and ↓ arrow indicates up and down regulation of the protein spots. Fold change value was calculated by using 2D gel image analysis software PD Quest (Bio-Rad) version 7.2.0. A value of $p < 0.05$ was considered statistically significant.

[a] Refers to protein spot numbers given in Fig. 1.

[b] Functions of individual proteins were obtained from the Uniprot database (http://www.uniprot.org/uniprot/).

3. Results

3.1. Accumulation of arsenic in different organs

Arsenic accumulation was estimated in different tissues of both the control and 15 ppm arsenic exposed group (n = 10). Total arsenic accumulation in blood plasma, liver, gill, white muscle and scale of fish exposed to 15 ppm arsenic was found to be 11.6 ± 0.7, 1.9 ± 0.16, 2.2 ± 0.07, 2.3 ± 0.21 and 5.1 ± 0.16 ppm, respectively after the exposure period. No arsenic was detected in the tissues of the control fish. The results are expressed as mean ± standard error.

3.2. 2D GE

A representative 2D GE protein profile of the plasma proteome of *Labeo rohita* exposed to 15 ppm arsenic is shown (Fig. 1). 150 discrete protein spots were resolved for the soluble plasma protein extracts within the pH range of 5–8 and molecular mass range of 14–70 kDa. Under the experimental conditions used, plasma 2D GE protein profiles of control fish and fish exposed to ≤5 ppm arsenic appeared similar. As the effects of 15 ppm arsenic were more conspicuous and drastic, so the latter was taken for 2-D gel image analysis and spot identification by MS.

Quantitative image analysis of the plasma proteomes of fish exposed to 15 ppm arsenic revealed that 10 protein spots were significantly increased in abundance and one protein spot was significantly reduced in abundance compared to the plasma proteomes of control fish (Fig. 1). Further two protein spots were detected in the plasma of control fish but not in plasma samples collected from fish exposed to 15 ppm arsenic (Fig. 1; marked as empty circles). One of the spots that did not change in abundance in both control as well as 15 ppm arsenic–exposed fish was also taken as an internal control for identification as in proteomic studies it is necessary to show proteins that do not undergo any change (significant) in abundance due to exposure or other such experimental conditions (Table 1).

3.3. Protein identification by mass spectrometry

3.3.1. MALDI-TOF/TOF MS

The fourteen protein spots described in Section 3.2 were excised from the 2-D gels for MALDI-TOF/TOF MS analysis. Eleven of the selected protein spots gave significant scores when searched using MASCOT (Table 1). The functions of the identified individual proteins were predicted using Uniprot (http://www.uniprot.org/uniprot/). Five of the protein spots (LP-1, 2, 4, 5, 16) were identified as apolipoprotein-A1 (Apo-A1). Two of these protein spots, were significantly higher in abundance (LP-1, 10.45-fold and LP-4, 3.11-fold) in the plasma of fish exposed to 15 ppm arsenic. LP-16 was only detected in the plasma of the control fish. The abundance of LP-2 was similar in the plasma of both arsenic-exposed and control fish (Fig. 1).

Two protein spots, LP-8 and 9, that showed modest (1.09-fold and 0.56-fold respectively) but significant increase in abundance in the plasma of arsenic exposed fish were identified as transferrin variant F. Protein spot LP-14 was identified as fibrinogen gamma polypeptide and showed a significantly lower abundance (0.59-fold) in the plasma of the arsenic exposed fish. The protein spot LP-15, identified as hemoglobin beta, was detected in the plasma of control fish but not in the plasma of fish exposed to 15 ppm arsenic.

Two protein spots, LP-11 and LP-12, were identified as liver-enriched gene protein1A (leg1A) and α-2 macroglobulin-like protein (A2ML), respectively; both these proteins are associated with liver development (http://www.uniprot.org/uniprot/) and both proteins spots increased in abundance in the plasma of arsenic

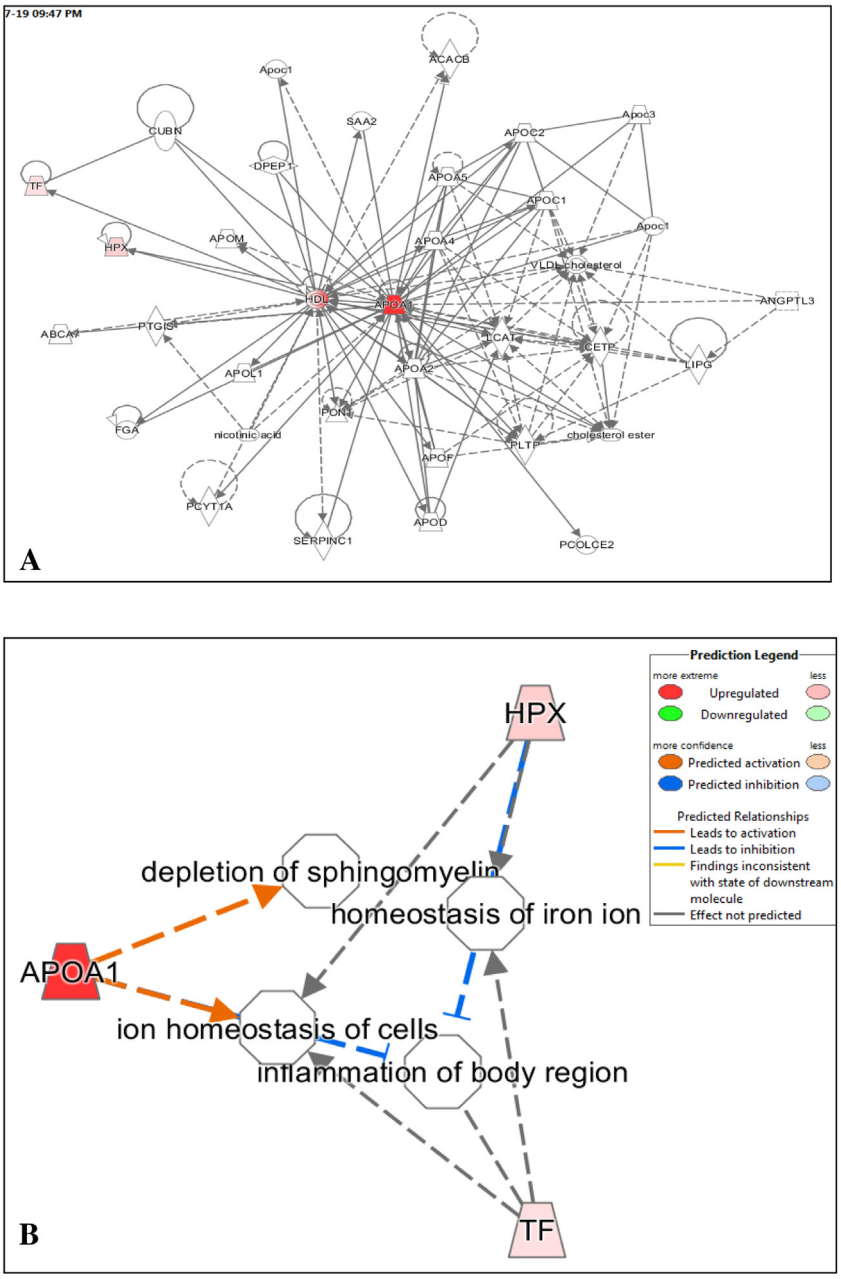

Fig. 2. (A) Putative biochemical networks affected by arsenic exposure, as revealed by Ingenuity Pathway Analysis (IPA). Differentially-expressed proteins were selected and entered into IPA Software to illustrate potential interactions. Solid arrows represent known physical interactions, dotted arrows represent indirect interactions. Red shapes indicate proteins in this network that were identified by 2D GE and mass spectrometric analysis. (B) The major pathways influenced by the up-regulated proteins in the arsenic-induced stressed fish. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exposed fish. Three protein spots, LP-3, LP-10 and LP-13, were increased in abundance in the plasma from fish exposed to 15 ppm arsenic but could not be identified using MALDI-TOF/TOF MS.

The peptide mass spectra of three Apo-A1 variants (LP-1, 2 and 4) identified from the plasma proteome of fish exposed to arsenic are shown in Supplementary Fig. S1. The MS/MS fragmentation of LMPILEAVR (Supplementary Fig. S1) showed differences in the spectral patterns for these three Apo-A1 variants. In case of LP-1 and LP-4,which increased in abundance following arsenic exposure, there were 2 peaks with *m*/*z* vales of 911.5019 and 928.5285 that were absent in the spectra of LP-2 (Supplementary Figs. S2–S4). The plasma proteomic information of rohu can be viewed at the PRIDE partner repository (http://www.ebi.ac.uk/pride) and also at fish proteomic database, FISHPROT (http://www.cifri.ernet.in/fishprot).

3.3.2. *LC–MS/MS*

Nine protein spots, which were processed for MALDI-TOF/TOF MS were also analysed by LC–MS/MS (Table 2). The identities of five of the protein spots identified as Apo-A1 (LP- 4, 5, 16), TF (LP- 9) and A2ML (LP-12) by MALDI-TOF/TOF MS were confirmed by LC–MS/MS. In addition, three protein spots that were not identified by MALDI-TOF/TOF MS were successfully identified using LC–MS/MS as TF (LP-3), Wap65 (LP-10) and Apo-A1 (LP-13) (Table 2). Another protein spot (LP-11) identified as leg1A by MALDI-TOF/TOF MS was identified as A2ML by LC–MS/MS (Table 2).

3.4. Pathway analysis-Biochemical networks affected by arsenic induced stress

Pathway analysis was carried out using IPA software to generate potential interaction maps of the identified proteins. Disease and function analysis showed that the identified proteins play role in cellular functions and maintenance, lipid metabolism and molecular transport of lipids. Lipid metabolism and cholesterol transport were the most inclusive pathways obtained from the proteomic data (Fig. 2A) which include three proteins viz. Apo-A1, HPX (hemopexin- human homolog of Wap65) and transferrin (TF) out of the four major up-regulated proteins identified. The proteins (Apo-A1, HPX and TF) were selected for further regulator effect analysis which indicates that both Apo-A1 and HPX inhibits inflammation under stress and Apo-A1 was found to be associated with activation of ion homeostasis of cells and depletion of sphingomyelin (Fig. 2B).

3.5. Transcript analysis to validate the proteomic data

Proteomic analysis showed high abundance of Apo-A1, A2ML, Wap65 and TF. To verify and validate the proteomic changes, transcript analysis of the respective genes were carried out (Supplementary Table S2). It was observed that the two isoforms of *Apo-A1* gene (*Apo-A1-1* and *Apo-A1-2a*) were down regulated (0.96 and 0.68 fold, respectively); however, another variant of *Apo-A1-2* (we named *Apo-A1-2b* (Accession no. KT634299); Supplementary Table S2) was found to be 3.79 fold up regulated (Fig. 3A–C) (Supplementary Fig. S5A–C). Besides, transcripts for *A2ML*, *Wap65* and *TF* were up regulated by 1.36, 1.89 and 1.56 folds, respectively in 15 ppm arsenic exposed fishes (Fig. 3D–F, Supplementary Fig. S5D–F).

4. Discussion

Arsenic is one of the environmental contaminants that has become the major cause of concern in recent times. Arsenic contamination in water has been reported from over thirty countries and river basins of the Ganga, Brahmaputra and Meghna in India and Bangladesh is one of the most affected zones [3,5]. About 25 million people in Bangladesh and 6 million people in West Bengal, India are exposed to arsenic contaminated ground water [5,35]. Arsenic is a highly carcinogenic metalloid and chronic arsenic toxicity also leads to cutaneous manifestations like melanosis, keratosis, and cutaneous cancer in humans [35]. Moreover, it has also been reported in fish models, arsenicosis can cause cataract and leads to generalized immunosuppression [22,23]. In arsenic affected areas people come across arsenic contamination through drinking water and also through food chain. Early diagnosis of arsenic contamination would be helpful in taking necessary actions to prevent the occurrence of the manifestations of arsenic toxicity. Therefore, in the present study, a comparative proteomic analysis of plasma of *Labeo rohita* experimentally exposed to arsenic (0.5–15 ppm) was carried out with the objective of identifying biomarkers of chronic arsenic toxicity. A dose of 15 ppm at the higher end was considered in the present study as arsenic concentration in some of the highly contaminated areas have been reported to be >10 ppm [3,4]. Further, as this is an experimental study and the dose of arsenic ranged up to 15 ppm, a short term exposure study was designed. Earlier, we have seen that at such dose gross pathological changes like skin lesions and cataract appear [22,23] and the aim of the study was to identify biomarkers that can be used for prognosis/diagnosis purposes before the onset of these manifestations.

The evolutionary position and the ability to adapt to a wide variety of environments have increased the utility of fishes in toxicological research, especially to detect the effects of environmental hazardous contaminants and toxicants [11–18]. Similarly accumulation of toxic materials in different tissues of fish is also studied to extrapolate the possible accumulation of arsenic in different organs in human [34,35]. Therefore, in the present study we estimated the accumulation of arsenic in various tissues of the fish. In this study, relatively lower amount of arsenic was detected in liver and gill tissues compared to the plasma and scales. Liver and gill are the main organs of detoxification and the lower level of arsenic in these two tissues could be due to its rapid detoxification and removal following short-term exposure [36]. Arsenic accumulation was much higher in the scale than muscle tissues which indicate that this could be one of the tissues where higher accumulation of arsenic occurs. Among all the tissues analyzed, the maximum concentration of arsenic was observed in blood plasma.

Out of the 14 spots picked for investigation, six protein spots (LP- 1, 2, 4, 5, 13, 16) were identified as isoforms of Apo-A1, which is the main protein constituent of high-density lipoprotein (HDL)

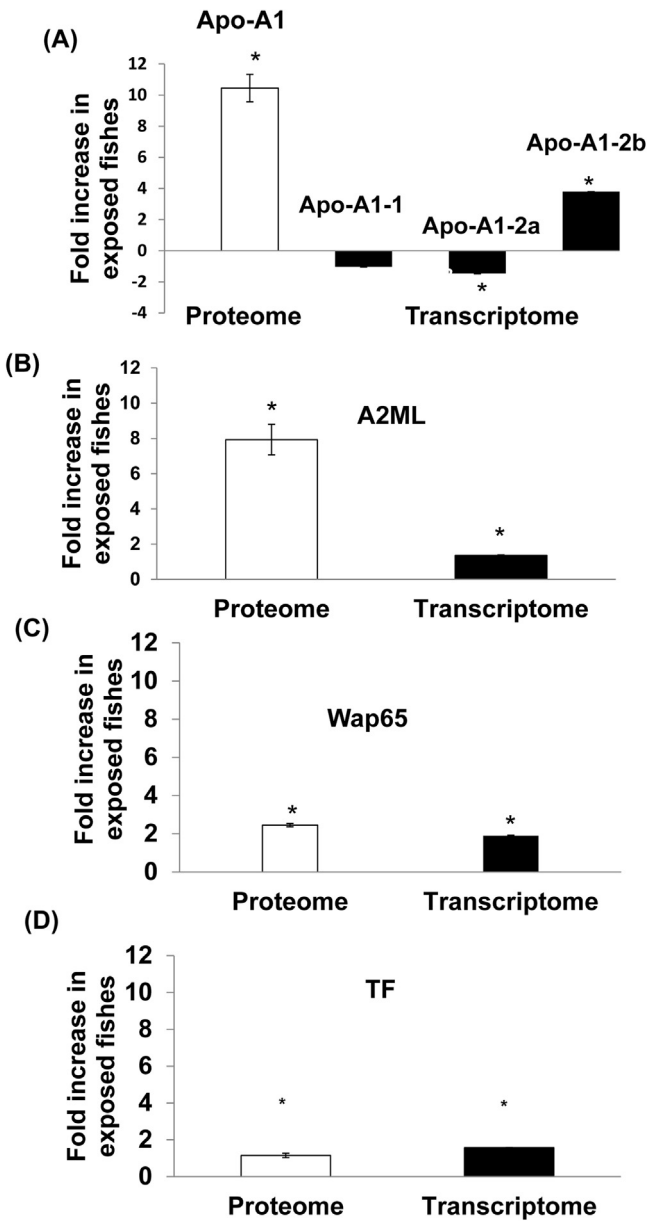

Fig. 3. Comparison of fold change for protein abundance observed by 2D GE gel analysis and mRNA expression obtained by RT-PCR for the (A) Apo-A1, (B) A2ML, (C) Wap65 and (D) TF genes in liver of arsenic exposed fishes in comparison to control. Error bars represent SEM. * Indicates significant difference relative to the control.

and is synthesized in the cells of the liver and small intestine of mammals [36] as well as a number of other tissues across species [37,38]. The primary role of Apo-A1 is in lipid metabolism [37] where it acts as a cofactor for lecithin-cholesterol acyltransferase (LCAT) (EC 2.3.1.43), that is involved in reverse cholesterol transport and confers water solubility to the lipoprotein complex to facilitate lipid transport and metabolism. In a previous study, it was found that hepatic *lcat* expression was markedly increased following arsenic exposure in rats, along with lysophosphatidyl cholines (LysoPC) in blood plasma, which indicated that arsenic exposure may disrupt the lipid signaling pathway [6]. Among the piscines, Apo-A1 is found to be a negative regulator of the C3 component of the complement system in cod (*Gadus morhua*) and acts as an inhibitor of the formation of membrane attack complex [39]. In *Labeo rohita* following arsenic exposure, C3a was found to be significantly down-regulated [23]. Besides, Apo-A1 could act as a significant determinant of epithelial integrity in rainbow trout gill cell cultures [38]. Similarly, in a previous study, expression of Apo-A1 protein increased in gills of Gibel carp following experimental exposure to copper indicating that it determines transcellular permeability and could therefore be important in maintaining intact cell membrane permeability [40]. Mass spectra of the positional variants of this protein (Supplementary Figs. S1–S4) exhibited specific peaks (*m*/*z* 911.5019 and 928.5285) in case of those spots (LP-1 and LP-4) which were significantly increased in abundance following arsenic exposure. It could be possible that these changes are due to some form of arsenic induced post-translational modification or alternatively, these could be markers for specific isoforms that are differentially synthesized in the presence of arsenic. This is a speculation which merits to be investigated.

Increase in abundance of A2ML proteins in the plasma of arsenic exposed fish was observed which is essential for liver growth and development [41]. Knocking down A2ML results in increased vacuolization of the liver together with a deficit in lipid metabolism [41]. Moreover, it is crucially involved in many immune responses against external threats showed previously in *Oryzias latipes* following exposure to bisphenol A [42]. In a recent study, proteomic analyses of plasma revealed conserved proteins between zebrafish and human plasma which interestingly consists of three homologs of ApoA1 and seven homologs of A2ML [43]. Phylogenetic analysis has shown that zebrafish and carp A2ML proteins are closely related to human A2ML [41]. Changes in the plasma abundance of A2ML and some isoforms of Apo-A1 have been linked to liver fibrosis due to chronic liver disease in humans [44].

Wap65 is also associated with the acute immune response pathway and its expression is modulated by heavy metal exposure in addition to its essential role in thermal acclimation [45]. The increase in abundance of this protein in this study agreed with the reported increase in Wap65 synthesis in liver of mud loach (*Misgurnus mizolepis*) exposed to cadmium, copper and nickel [45]. These heavy metals are known pro-oxidative agents, which cause oxidative/hypoxic stress. Thus the increased abundance of Wap65 suggested that it plays a protective role against cellular stresses arising from metal toxicity.

TF showed increased abundance in the plasma of arsenic exposed fish, which are iron-binding plasma glycoproteins that control the level of free iron in biological fluids. Approximately 18% of the total plasma arsenic is maximally bound to TF [46], which has been implicated to play an important role in arsenic detoxification. In humans, TF might serve as a biological storage site or reservoir in the blood to prevent arsenic affecting ATP synthesis prior to its methylation [46].

Arsenic exposure increases oxidative stress, inflammation and atherosclerotic lesion formation [47] and anti-inflammatory functions of HDL can be impaired under influence of stress [48]. The association of ApoA1/Hpx/TF with HDL may play an important role in maintaining the anti-inflammatory properties of HDL (Fig. 2A). Inflammation of organs and the body cavity is one of the major disease/function occurring during arsenic induced stress and the proteins like Apo-A1 and HPX inhibit the process of inflammation by maintaining ion homeostasis of the body [48] (Fig. 2B). These interesting possibilities are amenable to experimental testing in future studies.

Pathway analysis indicated lipid metabolism and transport as potential molecular targets of arsenic toxicity consistent with a previous study reporting increased fibrosis and decreased lipid accumulation and uptake in zebrafishes exposed to arsenic [13,14]. These data agree with the study on the plasma proteome described in this report. Specifically, the changes observed in the plasma proteome of *Labeo rohita* indicated that arsenic caused hepatotoxicity and liver damage under the experimental conditions used. Since the liver is not only the primary organ responding to arsenic toxicity, but also the major site of detoxification [13], the identification of the proteins indicative of liver damage in the plasma will be useful as diagnostic or prognostic biomarkers.

The two isoforms of *Apo-A1* gene, *Apo-A1-1* and *–2*, differ in only about 10% of the nucleotides in case of rainbow trout *(Oncorhynchus mykiss)* and *Apo-A1-1* has been reported as a constitutive form, whereas *Apo-A1-2* is selectively increased in hepatocellular carcinoma [49]. In this study, partial sequence homology of *Apo-A1-2a* and *Apo-A1-2b* showed 80% of homology and differences in few bases (Supplementary Fig. S5). A common feature of Apo-A1 protein is the presence of multiple repeats of amino acids which may have evolved due to internal gene duplication [49]. In a previous study, it has been found that partial gene duplication in *Apo-A1* gene caused a frame shift mutation and a truncated *Apo-A1* variant appeared in patients suffering from HDL deficiency [50]. The appearance of the additional variant of *Apo-A1-2* merits further investigation to find out if there is a specific linkage to arsenicosis. Transcript analyses of *A2ML*, *Wap65* and *TF* (Supplementary Fig. S5D–F) showed up regulation of these genes following arsenic exposure, suggesting that the increase in the protein abundance may be due to increased mRNA transcription (Fig. 3B–D).

The proteins found to be increased in abundance in the present study have also been reported to be present in high concentration in various other hepatic diseases like viral hepatitis, alcoholic liver disease, non alcoholic fatty liver disease etc. For example, Apo-A1 and A2ML are among the proteins, used in 'Fibrotest' to detect the extent of fibrosis and liver damage [51]. Similarly, Wap65 has been reported to have protective role in heavy metal (Cd, Cu, Ni) induced oxidative stress [45]. However, reports on the increase in abundance of these proteins in combination are scanty. Thus this study suggests that these proteins in combination could be used as biomarkers of arsenic induced hepatotoxicity and it would be interesting to study further, if this combination is specific to arsenic or not.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2017.04.054.

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