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Emerging role of urinary micro RNA-21 and microRNA-342 in arsenic-induced toxicity

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Abstract: Arsenic (As) is a toxic substance that can cause damage to liver and kidney tissues. Many studies showed that exposure to small amounts of this substance induces oxidative stress and causes kidney injury through the formation of free radicals. Accumulation of these non-essential metals can occur over many years, especially in the kidneys. Extracellular vesicles (EVs) facilitate intercellular communication and include exosomes, ectosomes, and apoptotic bodies. EVs are secreted through endosomes and carry genetic materials and proteins. Recently, EVs have become the focus as a novel molecular target in diagnosis and treatment. EVs cargo, including mRNA and miRNA, have a significant function in stabilizing and the identification of exosomes and their function. miRNA is a post-transcriptional gene suppressor that may act as an indicator and early warning in developing pathogenesis in heavy metal toxicity. Kidney EVs are execrated from podocytes. We collected random urine samples from 20 rats which were exposed orally to Arsenic^{V} and evaluated the universal kidney function, including a novel molecular target in the diagnosis of glomerulonephritis. Results showed that medium to high doses of arsenic, prior to kidney damage, could elevate exosome-containing mRNA and miRNA. The primary effect of arsenic at the cellular level could increase oxidative defense gene expression, including nuclear factor erythroid 2–related factor 2 (NRF2) antioxidant system, which increases the novel miRNA biomarker miR-21 and miR-342. In conclusion, our findings shows that exposure to arsenic with initial effects can elevate exosomal miR-342 as well as miR-21.

INTRODUCTION

Chronic exposure to inorganic arsenic (iAs) through contaminated water supplies worldwide produces a wide range of adverse health effects. Epidemiological studies of chronic arsenic poisoning in Asian countries reveal serious health hazards in Bangladesh, Western India, and China. Severe keratosis is common on the palms, the soles of the feet, and elsewhere, and occasionally results in skin cancer in some patients [1]. Exposure to As can alter protein expression, deoxyribonucleic acid (DNA) methylation, DNA damage repair, and chromosome structure and produces a potential mechanism in oxidative stress, altered cell proliferation, co-carcinogenesis, and tumor promotion. The metabolism of arsenic includes reduction to trivalent state and with oxidative methylation to a pentavalent state [2]. The final form of As can accumulate in many organs, such as the kidneys. In kidney nephrons, the caspases cascade will increase together with interleukin 6 and interleukin 9 but, on the other hand, glutathione and other antioxidants will decrease, leading to an increase in free radicals and oxidative stress. As a consequences of multiple cascades, the proximal tubular will be damaged. Oxidative damage can cause prenatal toxicity from arsenic, that metals have an affinity to thiol groups as well as glutathione. The thiol group with tripeptide provides antioxidant protection. At the cellular level, there is significant transformation and carcinogenic actions in which iAs intermediates methylation and the final outcome has adverse effects on cell structure and function [3].

Urine is a waste fluid that can pickup wastes from specific organs, and is therefore ideal for several analysis and biomarker determinations. In the transferring system, blood and its components are filtered, and the output is secreted including salts, urea, proteins, metabolites and carbohydrates that may vary physiologically and pathologically and associated with various diseases, especially with regard to renal status. Renal biopsy is the gold standard method used for most renal disease diagnosis; it is an invasive procedure with limited options to repeat, and is often used in cases of irreversible kidney damage. The urinary tract is generally

sterile above the urethral meatus, indicating that an effective system maintains urine sterility by antibacterial activity [4]. Thus, urine has been the focus through the years as an ideal bodily fluid to revealthe physiology of the kidney and urinary tract. As new biomarkers in this biological fluid came to be defined, a number of studies were conducted into the possibility of using the so-called urinary extracellular vesicle (uEV). The estimation of uEVs was around 3% of urine proteins. Therefore, potentially interesting biomarkers contained in EVs may go undetected due to their extreme dilution [5].

Cox2 plays a major regulatory role in response to alterations in intravascular and renal blood flow. It can be mediated in renin release and regulate sodium excretion. Cox2 expression is localised in the kidney cortex in the macula densa, the thick ascending limb of Henle. Previous studies revealed that patients with Bartter syndrome had been shown with high expressionsofCox2. In contrast, the researcher found an increase of macula densa Cox2 in elderly patients and decreased basal renin production, and also noted the elevation of the gene in podocytes of the kidneys[6].

MicroRNAs play a crucial role as mediators of post-transcriptional gene regulation, which are involved in cellular proliferation, apoptosis, migration, and aging [7]. Several studies have suggested that some miRNAs were upregulated in AKI, such as microRNA-21 (miR-21). The specific mechanism of action of miR-21 expression is still unclear, which has led to many studies focusing on the study of the role ofmiR-21 in carcinogenesis arsenic induction. Previous studies revealed that miR-21 is elevated in kidney injury cases which lead to lipopolysaccharide-induced sepsis, which indicates that mir-21 is effective in acute kidney injury yet its mechanism is still unclear [8]. The potency of miR-21 showed properties in anti-apoptosis, which has the capability to promote proliferation and is engaged in mechanisms of renal healing and apoptosis arrest [9].

The miR-342 is a non-coding RNA (~17-23 nucleotides) that bind to the 3' untranslated region (3'-UTR) of target genes [10], which has negative regulation of the expression of the target genes by stimulating degradation of the messenger RNAs (mRNAs) and could inhibit protein translation. Previous studies have revealed that miR-342-3p is overexpressed in mouse kidney tissues; however, whether miR-342-3p is associated with the progression of kidney diseases, and through which mechanism, remains unknown. Indeed, it was found that the upregulation in miR-342-3p can extend cell proliferation, and prevent cellular fibrosis and apoptosis [11]. The targets for studying arsenic-induced kidney toxicity and fibrogenesis aremiR-21 andmiR-342-3p.

2. MATERIAL AND METHODS

Sodium arsenate dibasic heptahydrate, of an analytical grade ($Na_2HAsO_4 \cdot 7H_2O$, >98% purity) (Sigma Aldrich Company, St. Louis, Missouri, USA) was used on twenty male Wistar albino rats, weighing between 150g and 200g. These were divided into four groups (Control, Group2, Group3, Group4) containing five rats in each group. The groups were dosed over a period of 28 days as follows;

- Control: normal saline solution;
- Group2:$NaAsO_2$ 1 mg/kg orally;
- Group3:$NaAsO_2$ 5 mg/kg orally, the dose used in[12];
- Group4:$NaAsO_2$ 10 mg/kg orally,the dose used in[13].

Determination of LD50 of $NaAsO_2$ = 5 mg/kg it is 1/8 of the LD50 = 40 mg/kg for LD50 for albino rats[12]. After the acclimatization period, the rats will be controlled with a 12-hour light/dark cycle at the Faculty of Science and had access to high protein (26%) food and water.

2.1. BLOOD AND URINE COLLECTION

Two blood specimens were collected from the retro orbital plexus vein at the end of the experiment before rat sacrifice. Samples were collected in test tubes without anticoagulant. The serum was separated by centrifugation (1600 × g) for 10 minutes and kept in small aliquots at −20 °C until biochemical analysis could be carried out. The procedure for collecting urine samples involves holding the rat over a collection plate or a plastic disposable weigh boat and encouraging it to micturate [14].Then, protease inhibitor (Thermo Scientific) was added and kept in two small aliquots, at -20°C until biochemical analysis could be carried out, which the other aliquot went through exosomes isolation step immediately.

2.2. EXOSOMES ISOLATION

Fresh urine (1ml) was prepared for exosomes isolation as protocol in miRCURY® Exosome (cat. no. 76743, Qiagen, Germany). Urine samples were obtained from a cell-free specimen by centrifugation at 3000 x g (~2000 rpm) for 5–10 min to pellet cells and debris. The supernatant was transferred, as the fraction of interest, into a new tube. Then, 1ml of each sample was gently mixed with precipitation Buffer B and vortexed in order to mix thoroughly. These were then incubated for 60 min at 2–8°C, and then centrifuged at 10,000 x g for 30 min at 20°C, then the supernatant was removed and discarded. After that, the sample was centrifuged again for 5 seconds to remove any residual supernatant. As a final step, the samples sample were suspended in 100µl Re-suspension Buffer andstored at-80°C.

2.3. RENAL MARKER

The serum creatinine and urea were determined as specific renal markers. The biochemical parameters were determined by a spectrophotometer (5010, RIELE Co., Berlin, Germany), according to the manufacturer’s instructions, in ready-made kits (Speanreact Co., Spain).

2.4. REVERSE TRANSCRIPTION (RT) OF MIRNA

After RNA extraction, cDNA was made for miRNA (Applied Biosystems,USA) and used as manufacturer's instruction protocol.

2.5.REVERSE TRANSCRIPTION (RT) OF MRNA

After total RNA extraction, it was reversed transcripted to cDNA through a ready-made kit provided by Applied Biosystems, according to prescribed protocol.

2.6. PERFORM QPCR TO CDNA RETRIEVED FROM TOTAL RNA

Urinary exosomal RNA expression was quantified using ready-made kits provided by Applied Biosystems, which used the specific primers presented in Table 2.

2.7. PERFORM QPCR TO CDNA RETRIEVED FROM MIRNA

Urinary exosomal miRNA expression was normalised to RNU6 then quantified using ready-made kits provided by Applied Biosystems, which used the specific primers presented in Table3.

2.8. STATISTICAL ANALYSIS

The data foreach group were analysed using analysis of variance ONE-WAY ANOVA coupled with the Statistical Package for the Social Sciences (SPSS) program. The data were expressed as arithmetic mean and standard deviation of the mean (SD). Differences between groups were analysed for parametric parameters using one-way where< 0.05 was considered significant.

3. RESULTS

3.1. EVALUATE RENAL FUNCTION TEST IN SERUM

The parameter was measured in serum to evaluate the differences between the Control Group and the three groups of different arsenic doses: Group2, Group3, and Group4 (1mg/kg B.W., 5mg/kg B.W., and 10mg/kg B.W. respectively). No significant effect on serum creatinine levels were detected (Figure 1),although an estimation of serum BUN was determined and shows elevation in Group3 and Group4 when compared to the Control Group (Table 1).

3.2. EXOSOMES ANALYSIS

Urinary exosomes as a novel biomarker for kidney toxicity carry specific information related to the status of original cells. The size of the exosomes is between20-150nm. After exosomes isolation, the size was determined by zetasizer from Malvern Panalytical(Figure2). The size of the urinary exosomes found were between 100 – 200nm.

3.3. GENE EXPRESSION ANALYSIS FOR OXIDATIVE STRESS

Elevation of urinary exosomal NRF2 expression was found in Group3 and Group4 when compared to the Control Group, while Group3 shows a slight increase in both NRF2 and Cox2genes. A slight elevation of Cox2 expression was found in Group2 when compared to the other group but there were no significant differences in Nrf2 compared to the Control Group (Figure 3).

3.4. MIRNA EXPRESSION ANALYSIS

With regard to the expression of miR-21 prognosis to kidney fibrosis as a novel biomarker, an increase in ex-pression was found in Group4, and a slight increase in Group2 and Group3, which could be an indicator of the level of arsenic toxicity and an early marker for fibrosis. Furthermore, miR-342 expression was elevated in Group4. While there was a slight expression in Group3, there was no significant expression detected in Group2 and Control (Figure 3).

2.2. Figures and Tables

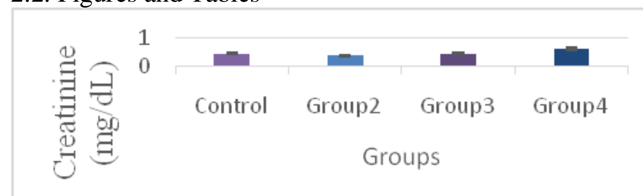

Figure 1: Effect of different treatments of Arsenic on serum level of serum creatinine (mg/dL) in experimental rat groups. Control: Control adult rat group givin Normal Saline; Group2: Rat was administrated with 1 (mg/kg) Na_3AsO_4; Group3 Rat was administrated with 5 (mg/kg) Na_3AsO_4; Group4 Rat was administrated with 10 (mg/kg) Na_3AsO_4.

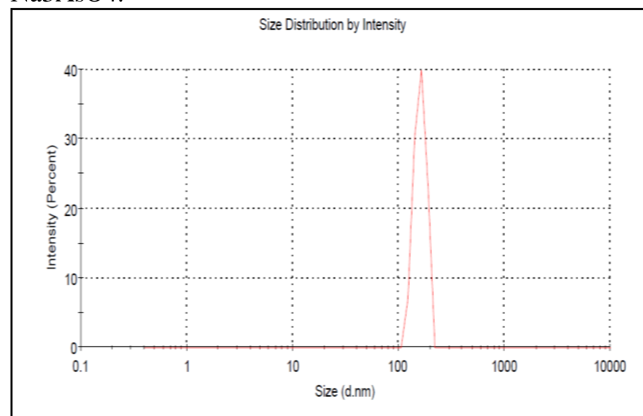

Figure 2: Sample size-concentration distribution of urine exosomes isolated from Groups was analyzed in Nanosight

NTA. Exosomes were resuspended in 100 uL Resuspension Buffer. The sample measured concentration to fall within the dynamic range of the instrument.

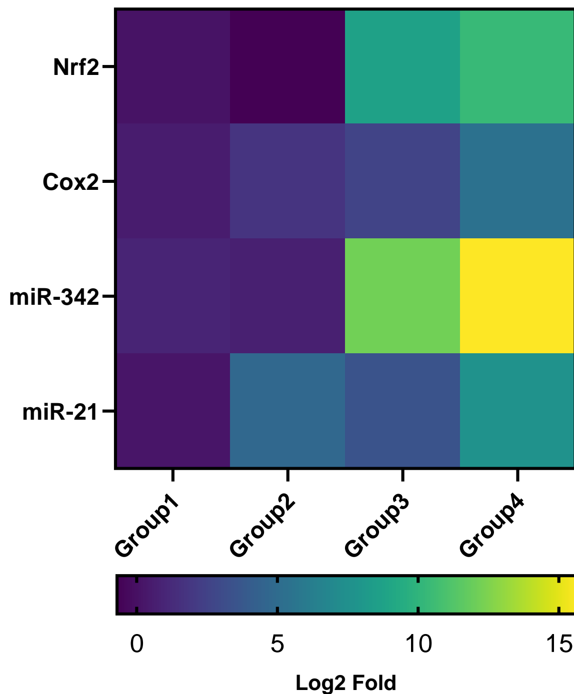

Figure 3: Effect of different treatments of Arsenic on serum level of serum creatinine (mg/dL) in experimental rat groups. Control: Control adult rat group givin Normal Saline; Group2: Rat was administrated with 1 (mg/kg) Na3AsO4; Group3 Rat was administrated with 5 (mg/kg) Na3AsO4; Group4 Rat was administrated with 10 (mg/kg) Na3AsO4

Table 1Results of creatinine and urea in different groups (n=5/group)

Parameter	Control Group	1 mg/kg (Group2)	5mg/kg (Group3)	10 mg/kg (Group4)
Serum Creatinine (mg/dL)	0.45±0.05	0.35±0.05	0.45±0.05	0.6±0.1
Serum Urea (BUN)(mg/dL)	27±3	22±2	31±4	41±1

Results were represented as means ± SD, n=5, means bearing the same alphabets in the same row are significantly different at ($p \leq 0.05$)

Table 22: Messenger RNA Primer Used in qPCR

Gene	Forward	Reverse
miRNAU6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
Nrf2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
Cox2	GATTGACAGCCCACCAACTT	CGGGATGAACTCTCTCCTCA

Table 3: MicroRNA Primer Used in qPCR

MiRNA	Forword	Reverse
miR-21	TAGCTTATCAGACTGATGTTGA	Universal RT provided with kit
miR-342	TCTCACACAGAAATCGCACCCGT	

4. DISCUSSION

The current study is aimed at testing the hypothesis that urinary exosomes can be a potential biomarker for urinary system damage, especially for kidney diseases, when exposed to arsenic as a cause of kidney toxicity and damage. This study investigated the effects of different doses of arsenic-induced toxicity in albino Wistar rats atthe biochemical level. The study was designed across four groups, namely Control (saline solution), Group2 (1 mg/kg), Group3 (5 mg/kg), and Group4 (10 mg/kg), alladministrated orally. The results showed that serum creatinine was slightly elevated in Group4 when compared to the other groups, which can be explained as an early indication of the nephrotoxicity effects of arsenic. These findings are in line with previously published work showing that administration of arsenic orally for 4 weeks resulted in kidney damage and could increase serum creatinine and serum urea [15], [16]. Arsenic toxicity could be reduced by the consumption of high protein and anti-oxidant vitamins which reduce or delay renal injury. Sharma et. al. demonstrated that the alternative therapy of arsenic-induced toxicity to have a high protein diet, and could reduce its toxic effects [17].

Exosomes and their cargo are produced in different contexts as a result of cellular responses to external and internal factors. Exosomes are 20-150 nm in size [18]. In this study, rats were exposed to sodium arsenate which can generate reactive oxygen species (ROS) that lead to oxidative stress. Nrf2 under normal conditions is regulated via ubiquitination and proteasomal degradation of cullin-based E3 ubiquitin ligase [19]. Under oxidative stress or Nrf2 activation conditions, Nrf2 detaches from Keap1 binding due to the thiol modification which prevents Nrf2 proteasomal degradation. Our results show elevation of Nrf2 which can be explained as a response to ROS production. Arsenic toxicity could be attenuate by an Nrf2-dependent antioxidant with enzymes to promote the detoxification of ROS [20]. Exosomal miRNA, which is made up of 19-23 nucleotides, plays several regulatory roles in gene expression. Exosomal miRNA-21, which is highly conservative to nucleic acid, has been found as dysregulated in the genitourinary system [21]. Previous studies demonstrate the high correlation between kidney fibrosis and inflammation to miRNA-21 in acute kidney injury [22]. The potential mechanism of miRNA-21 is still unclear, although Loboda et al. revealed that miRNA-21 upregulated at the posttranscriptional level [23]. Davis et al. suggested the association of Drosha/DGCR8/p68 microprocessor complex after it is transferred to the nucleus, that encourages the cleavage of pri miRNA to pre miRNA [24]. Regulation of posttranscriptional miRNA is about the direct binding of so-called Smad proteins. Regarding miR-21, Smad via posttranscriptional regulation significantly elevates the expression of miRNA-21. Many studies consider whether or not acute kidney injury or chronic kidney disease models show high expression of miRNA-21 in tissue samples, which are expressed in the cortex [25]. Smad is a posttranscriptional regulation protein which mainly elevates the expression of miR-21 in many conditions that are localised in the cortex, such as acute

kidney injury and chronic kidney disease [26]. Previous studies have revealed that, following injury, miR-21 was expressed at greatly elevated levels, mainly localised in the epithelium of the tubular kidney [26]. Furthermore, Chau et al., successfully generated miR-21 in knockout mice [27]. After kidney injury of two famous models of renal fibrosis, miR-21 was changed as presupposed [23], [28]. On the other hand, there is evidence of the dysregulation of miR-21 in a patient with sepsis which has a mediating role in cellular apoptosis in acute kidney injury. The mechanism of miR-21 related-sepsis is unclear, though further studies demonstrate that, in septic kidney damage, there is an elevation of miR-21 expression. Moreover, the septic and viral acute kidney damage have shown a significant increase in plasma miR-21, which suggests a clinical correlation to septic acute injury. In contrast, the HK-2 cell line was damaged in concert with a high expression of miR-21, which was suggested to be destructive to the septic kidney. On the other hand, some reports revealed the inducing of miR-21 could reduce renal injury for some abnormal conditions, such as atherosclerosis [22]. Some studies have proven that elevation of miR-21 decreases chemotherapeutic effects of curcumin when injected into breast cancer cell lines[29]. The knockdown of miR-21 expression inhibits the cytotoxicity of septic kidney injury, as well as the elevated expression of miR-21 enhancing the cytotoxic effect in septic kidney injury [22]. Another study showed the key role of miR-21 in pituitary tumor cells [30]. In this study, the elevation of miR-21 shows the emphasis of the urinary exosomal miR-21 correlated to arsenic exposure. Arsenic-induced toxicity can cause injury to the renal tubule macular densa cells, which also has relevance to kidney fibrosis as shown in the investigation [31].

Many micro-RNAs have associated kidney diseases, starting with acute kidney injuries and kidney toxicity, through chronic kidney diseases and renal fibrosis, to diabetic kidney diseases and lupus nephritis. The urinary exosomal miR-342 is one of the novel miRNAs that has been associated with kidney diseases. Tang et al. suggested that miR-342 has a high correlation to renal fibrosis. The mechanisms of renal fibrosis are still unclear, and the role of miRNAs in pathogenesis is not well understood [32]. The miR-342 has been considered as a candidate in the progression of renal fibrosis. Extracellular matrix receptors have been shown to interact with miR-342 in renal fibrosis. The production of extracellular matrix proteins show mechanisms for tissue repair after renal injury, and these proteins could elevate the miR-342 and that was found in renal cell carcinoma and adjacent non-tumor renal parenchyma [33], [34]. Jiang et al. revealed that the inhibition of renal fibrosis in diabetic nephropathy has been associated with miR-342-5p by diminishing several biomarkers of renal fibrosis [35]. Focusing on miR-342-3p that is relevant to urinary exosomes and novel therapeutic target of diabetic kidney disease, the correlation of high glucose levels and miR-342-3p expression in diabetic kidney disease has been revealed. Jiang et al. demonstrate that miR-342-3p promotes cell proliferation and inhibit the apoptosis of renal mesangial cells. Increased levels of miR-342-3p has been reported in renal fibrogenic changes [36]. Suppression of high glucose-induced renal fibrosis can be done with miR-342-3p. On the other hand, a novel urinary exosomal miR-342-3p can be related to the urinary albumin creatinine ratio [37]. It was reported that miR-342 has been changed in expression with diabetic nephropathy patients. The pathophysiology of miR-342-3p has an important role on Growth Factor Beta1 transformation, which acts as a regulator in diabetic renal disease. In this study, arsenic increased the risk of fibrogenic changes to the kidney and showed upregulation of urinary exosomal miR-342-3p as adefense mechanism.

5. CONCLUSIONS

This study represents the urinary exosomes which identified in water transporter and kidney solute, increased the capability to use these amino phospholipids as a biomarker for renal-related diseases. The benefit of using urinary exosomes may reflect the physiological state of the kidney and can solve the issues around non-invasive nephrotoxicity biopsy. The present study shows the onset of nephrotoxicity could be detected by a molecular test, which shows the onset of toxicity is regulated by exosomal Nrf2 expression, which is the first line of the anti-oxidant defense system. In addition, urinary exosomes have the advantage of being derived directly from the cells of the kidney. Urinary exosomal nucleic acid is a high preservative that is surrounded by a lipid bilayer. Many miRNAs could increase in dose-dependency were this study focused on miR-21 and miR-342. The miRNA is one candidate that is comparable to the standard clinical kidney function test. These miRNAs show different expressions in arsenic exposure and are targeted to regulate many processes and signals. Exosomal miR-21 is upregulated due to the fibrogenic effect of arsenic on the kidneys, which suggestsit maybe an inhibitor of cancer-causedarsenic toxicity. The miR-342 has potential mechanisms in the arsenic-induced fibrogenic kidney, which need further study to identify the novel pathway of the miR-342 pathogenesis inhibitor.

Supplementary Materials: This study includes experiments with Wistar albino rats. The ethical permission for this study was granted by the ethical review committee of Medicine Faculty, King Abdulaziz University. All national and institutional guidelines were followed for the care and use of animal models.

Author Contributions: AY, FA, and SH contributed to acquisition and analysis of the data. AY drafted the manuscript. AY, FA, and SH contributed to technical and material support. AY, FA, SH and MB contributed to critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

Institutional Review Board Statement: All samples were anonymized and used based on ethical approvals obtained from the Unit of Biomedical Ethics in King Abdulaziz

University Hospital (Reference No 159-20), with informed consent obtained from all participants.

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Conflicts of Interest:The author(s) declare that there is no conflict of interest regarding the publication of this article.

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