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The Activity of AQP9 Is Mediated MAPK in Arsenic-Treated Mouse Model of Hepatocellular Carcinoma

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Abstract

Background: Arsenic metabolism is primarily undergoing in hepatocytes, but the underlying mechanisms are to be defined. It is essential to study the response of aquaporin AQP9, protein kinase p38, and JNK with the stimulation of arsenic in mouse models of hepatocellular carcinoma. **Methods:** Mouse model of hepatocellular carcinoma (HCC) was induced with H22 cells injected subcutaneously on the lateral side of each right axilla in C57BL/6 mice. Then, western blotting and co-immunoprecipitation were used to detect the protein expression and phosphorylation of molecules AQP9, p38 and JNK in mouse models of hepatocellular carcinoma, respectively. The hepatocellular distribution of AQP9 was examined by the immunofluorescent method. **Results:** In both wild mice and a mouse model of liver tumor, there was no significant difference in the expressions of AQP9, protein kinase p38, and JNK after arsenic treatment, but the phosphorylation expression levels of the three were significantly increased to varying degrees, and the tumor model Compared with the wild-type group, the expression increased. Laser confocal experiments showed that in HepG2 cells, phosphorylated AQP9 was mainly distributed on the cell membrane under the stimulation of arsenic. **Conclusion:** Arsenic stimulation can increase the phosphorylation of AQP9, p38, and JNK in both wild-type C57 mice and liver tumor mice models. Arsenic stimulation facilitates phosphorylated-AQP9 predominantly distributed on the cell membrane of hepatoma cells HepG2.

Keywords

AQP9, Phosphorylation, P38, JNK, Arsenic

1. Background

Arsenic trioxide (As_2O_3, more commonly known as arsenic), recorded in both traditional Chinese and ancient Greek medicine, has been applied to the care of various disorders such as psoriasis, syphilis, and rheumatism for more than 1500 years. In the 1970s, Chinese scholars first applied it to acute promyelocytic leukemia (APL) and achieved a significant curative effect [1], later to gynecological tumors [2], liver cancer [3], lung cancer [4], and pancreatic cancer [5]. Since then, the anti-tumor effect of arsenic has attracted more and more attention. The mechanistic study demonstrated that arsenic achieves the therapeutic purpose by degrading PML/RARA fusion protein, down-regulating BCL2 gene expression, and inducing apoptosis of leukemia cells [6]. In this way, the simple understanding of "fighting poison with poison" in traditional Chinese medicine has been bridged to modern medical molecular biology. Arsenic-induced apoptosis has been elucidated from the perspective of genetics, making arsenic one of the first cell apoptosis-inducing drugs. However, tumor cells are often desensitized to arsenic along with repeated treatment, this drug resistance phenomenon will hinder its therapeutic effect, there are few studies on the resistance mechanism of arsenic agents so far. Three arsenic-resistant tumor cell lines [7] were established including HepG2/AS, SGC7901/AS, and SMMC7721/AS. The known ABC transporter family members ABCB1, ABCC1, and ABCC2 are found increased in these resistant cell lines. Our previous work also found that AQP9, a membrane transporter, plays an important role in the cellular uptake of arsenic [8] [9], affecting the accumulation of arsenic in cells.

AQP (Aquaporin) is a member of the major intrinsic protein (MIP) family. Eleven AQP isoforms, AQP0 - AQP10, have been identified in mammals by far [10]. They are basically involved in the transport of intracellular and extracellular fluids as channel proteins. In the previous studies in our laboratory, we found that arsenic can regulate the phosphorylation of the aquaporin AQP9 (aquaglyceroporin9, AQP9), thereby affecting the quantity of arsenic entering cells [8] [9]. However, the mechanism of phosphorylation regulation of AQP9 is still unclear. In the study of arsenic tolerance in yeast [11], Hog1p (p38 homolog, whose gene sequence is 52.3% similar) can regulate the phosphorylation of AQP9 homologous analog Fps1p, thereby affecting the entry of arsenic into cells.

Previous studies in our laboratory also showed that p38 protein kinase has a certain regulatory effect on AQP9 phosphorylation in normal hepatocyte L-02 [12]).

Mitogen-activated protein kinases (MAPKs), as a family of signaling pathways, mediate extracellular stimulation to intracellular responses, which regulate cell proliferation and differentiation, apoptosis, and the interaction between cells [13]. It is mainly divided into extracellular-signal-regulated protein kinase (ERK), c-Jun amino-terminal kinase (JNK), and P38 [14] [15]. Studies have shown that MAPK signaling is involved in the regulation of ROS on carcinogenesis, cancer cell apoptosis, and cancer metastasis [16] [17] [18] [19]; many an-

ticancer drugs also play a therapeutic effect by increasing reactive oxygen species and inducing the corresponding tumor cell apoptosis. Therefore, it is very meaningful to explore the mechanism of the MAPK signaling pathway to control the accumulation of arsenic in cells by regulating AQP9 to achieve an anti-tumor effect or generate drug resistance. Studies had shown that the MKK3/6-p38 signaling cascade is initiated by arsenic trioxide (As_2O_3) to control the anti-leukemia effect mediated by As_2O_3 in a negative feedback regulation manner [16].

A clinic study [17] showed that inhibition of p38 MAPK could enhance the efficacy of the combined use of As_2O_3 and thalidomide (bortezomib) in the treatment of myeloma, suggesting the p38 MAPK signaling pathway is involved in arsenic-induced apoptosis. In recent years, many studies have confirmed that arsenic *in vivo* induces apoptosis through the JNK signaling pathway [19] [20]. In addition, it was [18] found that JNK kinase inhibitor can significantly reduce the phosphorylation level of Bcl-2 induced by arsenic, which leads to mitochondrial apoptosis in cervical cancer cells. In short, P38 and JNK play important roles in the ROS-induced apoptosis signaling pathway.

This study will focus on the alteration in the expression and phosphorylation of AQP9 and p38 and JNK in mouse models of hepatocellular carcinoma by arsenic, and further, reveal that AQP9 phosphorylation mediated by MAPK-related signaling pathways is resistant to arsenic antitumor resistance. In addition, the localization and distribution of phosphorylated AQP9 in HepG2 cells will be revealed by laser confocal experiments.

2. Material & Methods

2.1. Animal and Cell Lines

C57 mice were purchased from Shanghai Southern Model Biotechnology Co., Ltd. Human hepatoma cell line HepG2 is cryopreserved in our laboratory. $NaAsO_2$ (Sigma, USA) Cell culture HepG2 cell line was cultured in RPM1640 medium; containing 10% fetal bovine serum, 100 $U{\cdot}mL^{-1}$ penicillin and streptomycin) (GIBCO, USA), placed in a saturated humidity incubator with 5% CO_2 volume at 37˚C. Mouse model of hepatocellular carcinoma (HCC) was induced with 5×10^6 H22 cells injected subcutaneously on the lateral side of each right axilla in C57BL/6 mice. When the tumor grows to about 100 mm^3, the modeling is successful.

2.2. Western Blotting

C57BL/6 wild-type mice and tumor model mice were selected as a group of three mice, and each group was intraperitoneally injected with $NaAsO_2$ at a concentration of 8 μmol/L at a dose of 10 mL/kg. The non-$NaAsO_2$ group was set as the normal control group. It was injected once a day, and after ten consecutive days, the liver tissues of normal mice and model mice were taken and stored in liquid nitrogen for freezing. After grinding, the cell lysate was made by RIPA method,

then subjected to quantification, SDS-PAGE, transferred to nitrocellulose membrane. Blotting were performed with appropriate diluted primary antibodies (mouse anti-human AQP9 antibody, mouse anti-human GAPDH antibody (Santa Cruz company), rabbit anti-human p38, JNK antibody, mouse anti-human p38, JNK phosphorylated antibody and mouse anti-pan-phosphorylated antibody (abcam company), followed by horseradish peroxidase (HRP) labeling secondary antibody, visualized by ECL kit (Amersham Bioscience company). Here GAPDH functions as an internal control. Immunoprecipitation was performed using protein A + G agarose with Anti-AQP9 as described previously.

2.3. Plasmids Construction

The full-length AQP9 cDNA was subcloned into pEGFP-N3 (Invitrogen) to obtain the pEGFP-N3-AQP9 plasmid. In addition, site-directed mutagenesis pEGFP-N3-AQP9 S11A (phosphorylated expression inhibition) and pEGFP-N3-QP9 S11D (phosphorylated constitutive expression) plasmids were constructed accordingly.

2.4. Confocal Immunofluorescence

Confocal immunofluorescence was performed on HepG2 transfected with different constructs of plasmids treated with 8 μmol·L^{-1} of $NaAsO_2$ for 12 with a confocal microscope (Carl Zeiss AG, German).

2.5. Statistical Analysis

SPSS13.0 statistical software was used to analyze the data, and one-way analysis of variance (ANOVA) was used for multiple comparisons between means. GraphPad Prism software draws the graphs.)

3. Results

3.1. Arsenic Stimulates AQP9 Phosphorylation in Mice Model of Liver Tumor

The C57BL/6 wild-type mice and tumor mice were intraperitoneally injected with 8 μmol/L of $NaAsO_2$, and the liver tissues of the mice were collected after ten consecutive days, and the unmedicated group was used as the control. Immunoprecipitation and Western blot were used to detect AQP9 expression and phosphorylation status. The results showed that there was no significant difference in the expression of AQP9 among the groups (**Figure 1(a)** and **Figure 1(b)**). The phosphorylation of AQP9, however, was significantly increased in the arsenic treatment group and tumor model group (**Figure 2(a)** and **Figure 2(c)**).

3.2. Arsenic Stimulates P38 Phosphorylation in Liver Specimen from C57 Tumor Model Mice

Since arsenic affects p38 expression and phosphorylation, this study also detected the changes of arsenic on p38 and its phosphorylation in the liver of C57

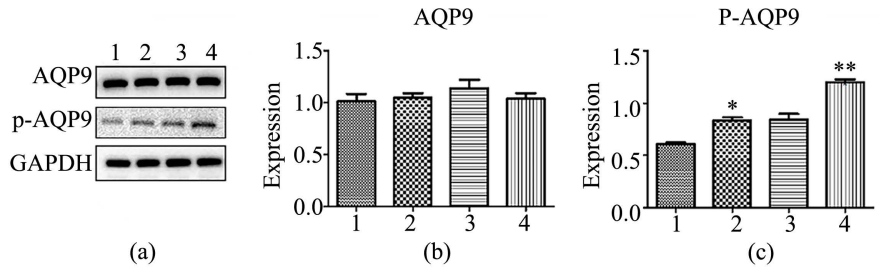

Figure 1. AQP9 expression and phosphorylation in livers of mice treated with $NaAsO_2$. (a) 1 WT mice; 2 WT mice treated with $NaAsO_2$; 3 Hepatoma mouse models; 4 Hepatoma mice treated with $NaAsO_2$. The graph represents the densitometry measurement of the AQP9 and P-AQP9 ((b), (c)). *p < 0.05, **p < 0.01.

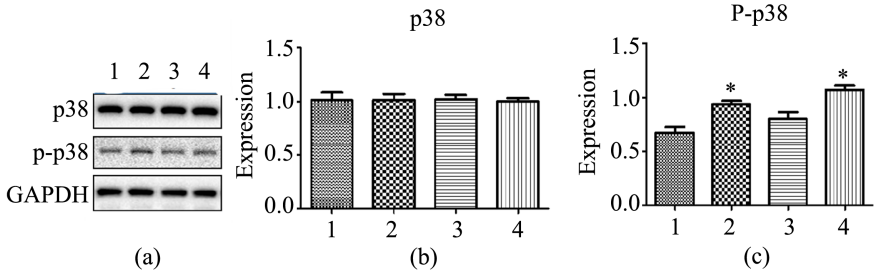

Figure 2. p38 expression and phosphorylation in livers of mouse treated with $NaAsO_2$. (a) 1 WT mice; 2 WT treated with $NaAsO_2$; 3 hepatoma mouse model; 4 hepatoma mice treated with $NaAsO_2$. The graph represents the densitometry measurement of the p38 and p-p38 ((b), (c)). *p < 0.05.

tumor model mice. Western blotting experiments showed that p38 protein expression was not significantly different among the groups (**Figure 2(a)** and **Figure 2(b)**). The expression level of P-p38 was significantly increased in the arsenic-treated groups (**Figure 2(a)** and **Figure 2(c)**).

3.3. Arsenic Increases JNK Phosphorylation in C57 Tumor Model Mice

The changes of JNK expression and its phosphorylation with arsenic treatment in the liver of C57 tumor model mice were further detected. Western blotting experiments showed that there was no significant difference in JNK protein expression among the groups (**Figure 2(a)** and **Figure 2(b)**) just like p38. But p-JNK was significantly increased in the arsenic-treated groups (**Figure 2(a)** and **Figure 2(c)**). Compared with the wild type, the tumor model group had increased expression (**Figure 3(a)** and **Figure 3(c)**).

3.4. Active AQP9 Is Predominantly Expressed in the Membrane in HepG2 Cells

The constructed pEGFP-N3-AQP9, pEGFP-N3-AQP9 S11A (phosphorylation inhibition), and pEGFP-N3-AQP9 S11D (phosphorylation constitutive expression) were transfected into HepG2 cells and treated with 8 μmol·L^{-1} $NaAsO_2$ 12 h, the fluorescence of HepG2 cells transfected with the wild-type vector was

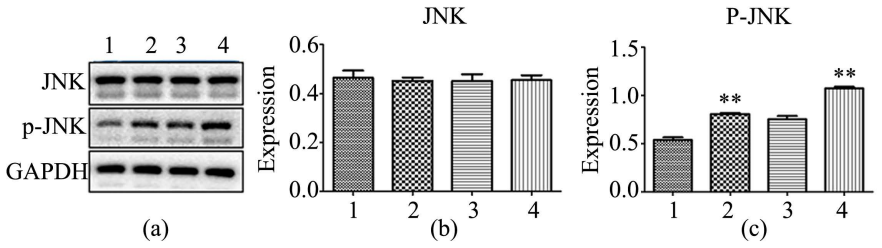

Figure 3. JNK expression and phosphorylation in livers of mouse treated with $NaAsO_2$. (a) 1 WT wild group; 2 WT + $NaAsO_2$ treatment; 3 tumor model group; 4 tumor model treated with $NaAsO_2$ group. The graph represents the densitometry measurement of the JNK and P-JNK ((b), (c)). *$p < 0.05$.

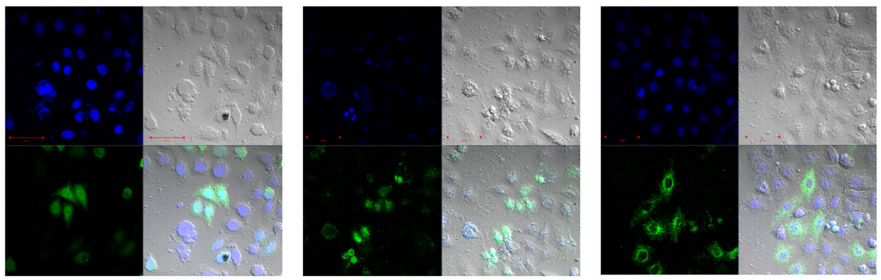

Figure 4. AQP9 localization in HepG2 cells. (a) HepG2 cell transfected with pEGFP-N3-AQP9; (b) HepG2 cell transfected with pEGFP-N3-AQP9-S11A; (c) HepG2 cell transfected with pEGFP-N3-AQP9-S11D. All of cells were treated with 8 μmol/L $NaAsO_2$ for 12 h.

mainly distributed in the cell membrane under the laser confocal fluorescence microscope (**Figure 4(a)**). The fluorescence of HepG2 cells transfected with the pEGFP-N3-AQP9 S11A mutant vector was mainly in the cytoplasm as seen (**Figure 4(b)**), while the fluorescence of HepG2 cells transfected with pEGFP-N3-AQP9 S11D mutant vector is mainly distributed on the cell membrane, and compared with the cells transfected with the wild-type vector, it shows stronger fluorescence on the membrane (**Figure 4(c)**).

4. Discussion

In the current study, using the C57BL/6 mouse model of hepatoma, we found that arsenic can activate aquaporin AQP9 by phosphorylation, and translocate predominantly its expression to the cell membrane; and further, arsenic could stimulate the phosphorylation of kinases p38 and JNK.

Previous studies have shown that AQP9 is the channel for arsenic to enter/exit cells, and its phosphorylation is the main way to regulate its entry into cells [8] [9]. The hypothesis that protein kinases p38, JNK, etc. may regulate the phosphorylation of AQP9 is proposed accordingly. Preliminary experiments show that p38 has a certain regulatory effect on arsenic intake [12]. However, there are few related research reports on animal experiments. In one study in a mouse model with type 1 diabetes, arsenic uptake was recorded significantly higher in

the liver in comparison with normal literate, in concert with higher expression of AQP9 and Glucose transport 1 (GLUT1) in the liver [21]. This paper focuses on the effect of arsenic on the protein expression and phosphorylation expression level of AQP9 and protein kinase p38, JNK in animal experiments.

The experiments showed that under arsenic stimulation, the expression of AQP9 protein in the liver of normal wild-type mice did not increase significantly, but the phosphorylation level increased significantly; the phosphorylation of AQP9 also increased in the liver tumor model in mice (**Figure 1**), and their phosphorylation levels were higher than those in wild-type mice, which is in parallel with the previous findings [8]. In addition, the expression of kinase p38 did not change significantly after arsenic treatment, but the phosphorylation level increased (**Figure 2**), *in vitro* hepatocyte L-02 model also witnessed the similar findings [8] [12]. The expression and phosphorylation pattern is similar in another protein kinase JNK with increased phosphorylation but no change in protein expression (**Figure 3**). In short, phosphorylation of AQP9 may be a major way to affect the intake of arsenic, while the phosphorylation of AQP9 may be affected by the phosphorylation of protein kinases p38 and JNK. This requires more experiments to further verify.

In addition, in the laser confocal experiment, we observed that the phosphorylated AQP9 was more localized on the cell membrane of HepG2 cells after being stimulated by arsenic. Due to the common characteristics of organisms seeking advantages and avoiding disadvantages, when they are stimulated by the outside world, they generally choose to fight and block harmful substances or expel them. Therefore, we believe that the increased expression of AQP9 phosphorylation on the cell membrane should be related to the inhibition of arsenic entry into cells or expel it. This implies that phosphorylation of AQP9 is a way for this channel protein to transport arsenic into and out of cells. AQP9 may act as a two-way channel for arsenic into or out of cells, in which its phosphorylation may be the main regulatory mode that determines whether it allows arsenic into or out of cells. This also lays the foundation for the later research work.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Zang, T. (1998) Study on the Treatment of Leukemia with Arsenic-Containing Traditional Chinese Medicine-Talk about the Treatment of Leukemia with Ailing No. 1 Injection. *China Journal of Integrated Traditional Chinese and Western Medicine*,

18, Article 581.

[2] Yue, C., Feng, X., Sun, M., *et al.* (2010) Research on the Prevention and Treatment of Cervical Precancerous Lesions with Gongbao Powder. *China Medical Innovation*, **7**, 74-75.

[3] Chen, F. and Chen, X. (2005) Arsenic Trioxide Injection in the Treatment of 15 Cases of Advanced Primary Liver Cancer. *Modern Oncology Medicine*, **13**, 665-666.

[4] Chibhabha, F., Li, Y., Hao, Y., *et al.* (2016) Aquaporin 5 Expression and Its Relationship to Apoptosis in Different Grades of Differentiated Non-Small Cell Lung Carcinoma. *Advances in Lung Cancer*, **5**, 1-12. https://doi.org/10.4236/alc.2016.51001

[5] Liu, J., Qin, S., Qian, J., *et al.* (2005) Clinical Observation of Arsenic Trioxide Injection in the Treatment of Advanced Pancreatic Cancer. *Journal of Clinical Oncology*, **10**, 117-119.

[6] Chen, G., Chen, S., Wang, Z., *et al.* (1998) Mechanism and Prospect of Arsenic Oxide Injection in the Treatment of Promyelocytic Leukemia. *China Journal of Integrative Medicine*, **18**, 581-582.

[7] Chen, X. (2010) The Mechanism of Arsenic Trioxide Resistance in Hepatocellular Carcinoma and the Research on Anti-Resistance. Harbin Medical University Dissertation, Harbin.

[8] Cao, Y., Yang, L., Deng, L., *et al.* (2012) Effects of Aquaporin 9 Phosphorylation on Arsenic Uptake in Mammalian Cells. *Chinese Journal of Pharmacy*, **47**, 679-683.

[9] Cao, Y., Tan, X., Huang, X., *et al.* (2013) Relationship between Phosphorylation of Aquaporin 9 and Arsenic Tolerance in Human Umbilical Vein Endothelial Cells. *Chinese Journal of Pharmacology and Toxicology*, **27**, 240-247.

[10] Verkman, A.S. and Mitra, A.K. (2000) Structure and Function of Aquaporin Water Channels. *American Journal of Physiology-Renal Physiology*, **278**, F13-F28. https://doi.org/10.1152/ajprenal.2000.278.1.F13

[11] Thorsen, M., Di, Y., Tangemo, C., *et al.* (2006) The MAPK Hog1p Modulates Fps1p-Dependent Arsenite Uptake and Tolerance in Yeast. *Molecular Biology of the Cell*, **17**, 4400-4410. https://doi.org/10.1091/mbc.e06-04-0315

[12] Deng, L., Xia, S., Pan, F., *et al.* (2015) Protein Factors Regulating AQP9 Phosphorylation in Mammalian Cells and Their Effects on Arsenic Intake. *Chinese Journal of Pharmacy*, **50**, 339-343.

[13] Santarpia, L., Lippman, S.M. and Ei-Naggar, A.K. (2012) Targeting the MAPK-RAS-RAF Signaing Pathway in Cancer Therapy. *Expert Opinion on Therapeutic Targets*, **16**, 103-109. https://doi.org/10.1517/14728222.2011.645805

[14] Wyler. M.R., Smith, D., Cayanis, E., *et al.* (2009) Cell-Based Assays to Probe the ERK MAP Kinase Pathway in Endothelial Cells. In: Clemons, P., Tolliday, N. and Wagner, B., Eds., *Cell-Based Assays for High-Throughput Screening. Methods in Molecular Biology*, Humana Press, Totowa, 29-41. https://doi.org/10.1007/978-1-60327-545-3_3

[15] Zohrabian, V., Forzani, B., Chau, Z.A., *et al.* (2009) Rho/ROCK and MAPK Signaling Pathways Are Involved in Glioblastoma Cell Migration and Proliferation. *Anticancer Research*, **29**, 119-123.

[16] Giafis, N., Katsoulidis, E., Sassano, A., *et al.* (2006) Role of the p38 Mitogen-Activated Protein Kinase Pathway in the Generation of Arsenic Trioxide-Dependent Cellular Responses. *Cancer Research*, **66**, 6763-6771. https://doi.org/10.1158/0008-5472.CAN-05-3699

[17] Wen, J., Feng, Y., Huang, W., *et al.* (2010) Enhanced Antimyeloma Cytotoxicity by the Combination of Arsenic Trioxide and Bortezomib Is Further Potentiated by p38 MAPK Inhibition. *Leukemia Research*, **34**, 85-92. https://doi.org/10.1016/j.leukres.2009.05.024

[18] Kang, Y. and Lee, S. (2008) The Role of p38 MAPK and JNK in Arsenic Trioxide-Induced Mitochondrial Cell Death in Human Cervical Cancer Cells. *Journal of Cellular Physiology*, **217**, 23-33. https://doi.org/10.1002/jcp.21470

[19] Wang, G., Zhang, T., Sun, W., Zhang, T., Sun, W., *et al.* (2017) Arsenic Sulfide Induces Apoptosis and Autophagy through the Activation of ROS/JNK and Suppression of Akt/mTOR Signaling Pathways in Osteosarcoma. *Free Radical Biology and Medicine*, **106**, 24-37. https://doi.org/10.1016/j.freeradbiomed.2017.02.015

[20] Mao, J., Yang, J., Zhang, Y., *et al.* (2016) Arsenic Trioxide Mediates HAPI Microglia Inflammatory Response and Subsequent Neuron Apoptosis through p38/JNK MAPK/STAT3 Pathway. *Toxicology and Applied Pharmacology*, **303**, 79-89. https://doi.org/10.1016/j.taap.2016.05.003

[21] Wei, H., Hu, Q., Wu, J., *et al.* (2018) Molecular Mechanism of the Increased Tissue Uptake of Trivalent Inorganic Arsenic in Mice with Type 1 Diabetes Mellitus. *Biochemical and Biophysical Research Communications*, **504**, 393-399. https://doi.org/10.1016/j.bbrc.2018.06.029