In utero arsenic exposure increases DNA damage and gene expression changes in umbilical cord mesenchymal stem cells (UC-MSCs) from newborns as well as in UC-MSC differentiated hepatocytes

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1. Introduction

Cumulative evidence revealed that arsenic is a transplacental carcinogen [1]. Arsenic exposure can cause detrimental health effects, particularly during pregnancy, which is a sensitive period for the developing fetus [2]. In utero arsenic exposure is associated with various types of genetic damage in the newborn such as 8-hydroxydeoxyguanine (8-OHdG), 8-nitroguanine, and micronuclei, which may contribute to the development of a variety of diseases including cancer later in life [3, 4]. In addition, epidemiological evidence from northern Chile shows that in utero and early life arsenic exposure through drinking water increased the risk of noncancerous and cancerous effects and increased the mortality rate from myocardial infarction in young adults [5]. A study in the same region found that exposure to arsenic during gestation increased the risk of childhood liver cancer mortality [6]. However, the mechanisms through which in utero and early life arsenic exposure leads to liver disease and cancer later in life are not fully understood.

In general, gestation is a period of extremely rapid organogenesis, proliferative growth, cell differentiation, and metabolic imprinting of the fetus, all of which are processes that largely involve stem cells [7]. It
has been proposed that exposure to chemicals during pregnancy alters the number of stem cells during embryonic or fetal development and this may affect stem cell-based developmental processes that increase the risk of disease development later in life [8]. The human umbilical cord is a tissue connecting the fetus and mother, and is a source of stem cells. The umbilical cord contains a substance composed of mucus and connective tissue called Wharton’s jelly, which is a rich source of mesenchymal stem cells (MSCs). MSCs are multipotent stem cells that originate in the mesoderm at early development and migrate through the developing umbilical cord during embryogenesis [9]. Wharton’s jelly-derived umbilical cord MSCs possess characteristics of stemness, i.e. a high capacity for self-renewal and a multi-directional differentiation potential that can give rise to multiple lineages including bone, cartilage, adipose tissue and neuronal cells as well as other tissues such as hepatocytes [10]. MSCs also support haemopoiesis in the first trimester of pregnancy [11]. Increasing evidence indicates that arsenic exposure targets stem cells and causes disruption of stem cell differentiation [12] thereby promoting the development of cancer and other diseases. Such changes alter stem cell adaptability and induce normal stem cells to acquire a cancer stem cell (CSC) phenotype, which is linked to both arsenic-associated cancer and prenatal arsenic exposure-associated CSC emergence [12]. Arsenic is clearly a transplacental carcinogen and fetal stem cells may be the key targets of arsenic during transplacental carcinogenesis [13,14]. Supportive studies in animals also showed that in utero arsenic exposure in mice enhanced the formation of skin cancer in offspring, possibly by targeting stem cells [15]. Transplacental arsenic exposure in C3H mice resulted in significantly higher liver tumor incidence in adult offspring [13,16]. Arsenic may target and alter stem cells

Fig. 1. Immunohistochemical detection of MSC markers and DNA damage (8-OHdG and 8-nitroguanine) in paraffin-embedded umbilical cord sections. Paraffin-embedded sections of umbilical cord samples from 100 arsenic-exposed newborns were subjected to immunohistochemical detection of MSC markers and 8-OHdG and 8-nitroguanine. The drawing picture of umbilical cord composition and the staining procedure for MSC markers and 8-OHdG and 8-nitroguanine in umbilical cord cross-sections were modified from a study by Shugar et al. [29] (A) (WJ: Wharton’s jelly). Representative images showing localization of MSC markers in Wharton’s jelly; CD73 (red), CD90 (yellow), and CD105 (orange) in umbilical cord tissue as judged by co-localization of DAPI (blue) as indicated in merge (white) (B-upper). Representative images showing localization of 8-OHdG (green), and 8-nitroguanine (pink) in umbilical cord MSCs as judged by co-localization of DAPI (blue) as indicated in merge (white) (B-lower). Cells were viewed at 200× magnification.
The mechanism of arsenic-induced toxicity and disease development involves the generation of oxidative stress due to the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated during arsenic biotransformation. Unstable free radicals derived from ROS and RNS react with guanine nucleobases to form mutagenic oxidative DNA lesions such as 8-OHdG and 8-nitroguanine, respectively. These mutagenic DNA lesions lead to G:C to T:A transversion mutations, which play an important role in carcinogenesis [19].

Generation of ROS/RNS initiates intracellular signaling pathways that stimulate increases in the levels of inflammatory cytokines such as IL-6, IL-8, and IL-10, encoded by CXC chemokine ligand 6 (CXCL6), and IL-8, encoded by CXC chemokine ligand 8 (CXCL8), which promotes inflammation [20]. Chronic inflammation is an important cause of cancer and promotes a cancer microenvironment. IL-6 and IL-8 have been shown to play roles in the carcinogenic process [21]. Moreover, oxidative stress also activates the nuclear factor, erythroid 2-related factor 2 (Nrf2) encoded by NFE2L2, which is a transcription factor that controls cellular defense mechanisms through the regulation of oxidative stress defense genes [22]. However, constitutive activation of Nrf2 confers growth advantages to tumors by creating favorable microenvironments [23]. Arsenic is known to disrupt DNA damage repair by altering the expression of DNA repair genes; for example, mutagenic DNA lesions such as 8-OHdG are mainly repaired by 8-oxoguanine DNA glycosylase 1 (OGG1), which recognizes and cleaves 8-OHdG. Impairment of DNA repair combined with increased DNA damage could lead to initiation of carcinogenesis [24]. Our earlier studies demonstrated that in utero arsenic exposure increases mutagenic DNA damage, as indicated by increased urinary 8-nitroguanine levels of the newborn, which was significantly correlated with increased levels of inflammatory gene transcripts in the cord blood [3]. A follow-up study in the same cohort of children who were exposed to arsenic in utero showed increased salivary 8-OHdG that was associated with decreased expression of its DNA repair gene, OGG1, throughout early life [25]. In addition, in another birth cohort study in Vietnam, prenatal arsenic exposure was associated with multiple types of genetic damage in the newborns, including 8-OHdG, 8-nitroguanine, and micronuclei that were positively correlated with arsenic concentrations in maternal toenails [4].

In the present study, we further explored the effects of in utero arsenic exposure on mutagenic DNA damage in fetal stem cells by determining 8-OHdG and 8-nitroguanine in umbilical cord MSCs, which are multipotent stem cells. In addition, a series of in vitro studies were designed using an MSC cell line derived from Wharton’s jelly in umbilical cord tissue (UC-MSCs) to ascertain whether the effects observed in fetal stem cells were due solely to arsenic exposure and to gain insight into the mechanism involved by investigating the expression of genes related to DNA damage in UC-MSCs and hepatocytes differentiated from UC-MSCs.

Lastly, the cell transformation ability of hepatocytes differentiated from UC-MSCs that had been exposed to arsenic during differentiation was determined.

### 2. Materials and methods

#### 2.1. Umbilical cord tissue collection and arsenic exposure in the mother

Umbilical cord tissues were collected from 100 newborns prenantly exposed to arsenic in the same cohort described in our earlier study in Hanam province, Vietnam [4]. The pregnant subjects were healthy volunteers, aged 20–40 years and all babies were delivered naturally without birth stimulation or anesthesia. This study was conducted according to the recommendations of the Declaration of Helsinki [26]. Informed consent was obtained from all subjects and the study protocol was approved by the local ethics committee (IRB approval code 013/2552). The umbilical cord tissues were collected after birth and stored at –80 °C until analysis. Maternal arsenic exposure was measured and stratified by maternal toenail concentration into three groups including low exposure (<0.5 µg/g), medium exposure (0.5–1 µg/g) and high exposure (>1 µg/g) as previously described [4].

#### 2.2. Simultaneous detection of umbilical cord MSCs and mutagenic DNA damage in MSCs by immunohistochemistry

MSC markers and mutagenic DNA damage, 8-OHdG and 8-nitroguanine, were determined in paraffin-embedded umbilical cord tissue. Sections were cut at a thickness of 3 µm and mounted onto slides coated with the adhesive, 3-aminopropyltriethoxysilane, and dried in a 60 °C oven for 4 h to ensure maximum adhesion. For subsequent analyses, sections were deparaffinized in xylene and washed in a decreasing gradient of ethanol before blocking nonspecific binding sites with 1% normal goat serum for 1 h (Cell Signaling Technology, USA). All antibodies were diluted in antibody dilution buffer (Cell Signaling Technology) at the indicated dilution factor. Firstly, rabbit monoclonal anti-human CD90 antibody, clone D3V8A (1:50, Cell Signaling Technology) and mouse monoclonal anti-8-nitroguanosine antibody, clone NO2G52 (1:250, Cosmo Bio, Japan) were added to the sections and incubated 1 h at 37 °C. Secondly, Alexa Fluor®750 goat immunoglobulin G (IgG) anti-rabbit secondary antibody (1:500, Abcam, UK), Alexa Fluor®594 goat IgG anti-mouse secondary antibody (1:500, Molecular Probes, USA), mouse monoclonal anti-human CD73 conjugated to PE (1:50, Abcam), mouse monoclonal anti-human CD105 conjugated to PerCP-Cy5.5 (1:50, BD Pharmigen™, USA), and mouse monoclonal anti-8-OHdG conjugated to Alexa Fluor®488 (1:100, Santa Cruz Biotechnology, USA) were incubated for 1 h in the dark at 37 °C. Finally, the slides were mounted with Fluoromount-G™ mounting medium with DAPI (Invitrogen, UK). For negative controls, the primary antibody was replaced with antibody diluent. The surface markers used to identify MSCs were CD73, CD90, and CD105. The cells expressing MSC markers were assayed for mutagenic DNA damage in the form of 8-OHdG and 8-nitroguanine. Tissue slice images were captured and analyzed with an Axio Imager Z2 Epi-Fluorescence microscope (Zeiss GmbH, Jena, Germany) equipped with a digital camera and the Tissue FAXIS® Version 6.0 image analysis system and viewed at 200× magnification. One thousand cells were counted and classified in terms of MSC surface markers (CD73, CD90 and CD105) present based on fluorescence staining. Positive cells that expressed all three surface markers of MSCs were chosen for...
determination of the levels of 8-OHdG and 8-nitroguanine by quantification of their fluorescence intensity of staining.

2.3. Cell culture and sodium arsenite treatment

A human UC-MSC cell line was obtained from ATCC and cultured in alpha minimal essential medium (Gibco, USA) supplement with 10% fetal bovine serum (FBS, EmbryoMax®, Merck Millipore, USA), 1% of 200 mM L-Glutamine (Gibco), and 1% of 10,000 U Penicillin/Streptomycin (Gibco). For cytotoxicity, DNA damage, and DNA damage-related gene experiments, UC-MSCs were treated with varying concentrations of sodium arsenite (NaAsO$_2$) for 24 h (0–5 µM). For hepatocyte differentiation studies, UC-MSCs were treated at 0–0.5 µM NaAsO$_2$ for 21 days during differentiation into hepatocytes, comparison to the control.

2.4. Determination of cell viability

UC-MSCs were treated with varying concentrations of arsenite from 0.5 to 25 µM for 24 h and 0.1–5 µM for 21 days. Time-course studies were done with 0.5–5 µM arsenite for 24, 48, and 72 h. Cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay (Sigma-Aldrich, USA) and were reported as a percentage (%) of the control.

2.5. Determination of 8-OHdG and 8-nitroguanine in cells

Fluorescence immunocytochemistry was used for measuring the levels of 8-OHdG and 8-nitroguanine in cells. UC-MSCs (2 × 10$^4$ cells) were cultured in a 4-chambered slide (Millicell EZ, USA), treated with arsenite for 24 h (0–5 µM) and 21 days (0–0.5 µM) at 37 °C with 5% CO2. After treatment, cells were fixed and double fluorescence
immunocytochemistry staining was performed according to the Trevigen manufacturer’s protocol with modifications. The immunocytochemical detection of DNA damage in the form of 8-OHdG and 8-nitroguanine was performed as described in the immunohistochemical detection.

2.6. Determination of gene expression

Total RNA was isolated from cells treated with arsenite using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s protocol. All mRNA expression levels were analyzed using a Light Cycler 480 real-time RT-PCR machine (Roche Diagnostic, Germany) and the one-step QuantiTect SYBR® Green RT-PCR Kit (Qiagen). The primers used were as follows: CXCL6 (F) 5’-TGATGATTTTACCCAGGC-3’ (R) 5’-CGGTACATCCCTCGACGG-3’, CXCL8 (F) 5’-CTCTTGACGCCCTCCCTGATT-3’ (R) 5’-TATGCACCTGACATCAAGTCTTCCAGGC-3’, NFE2L2 (F) 5’-AAACGATGATCTGCCAAC-3’ (R) 5’-AGCATCTGATTGGAATGTG-3’, OGG1 (F) 5’-ATGGGGCATCGTACTCTAGC-3’ (R) 5’-CTCCCTCCACCGGAAAGAT-3’, ALB (F) 5’-TGCAACTCTTGAGAAACCTATG-3’ (R) 5’-ACATCAACCTCTGGTCTCACC-3’, SOX9 (F) 5’-AGCGAA. CGCACATCAAGAC-3’ (R) 5’-CTGTAGGCTGATCTGTTGGGG-3’, and the reference gene GAPDH (F) 5’-TCTCCACGAGCGAGATCC-3’ (R) 5’-TTGTGATGATGACCCCTGGC-3’. The reaction mixture for each target gene contained 0.25 µM primer, QuantiTect SYBR® Green RT-PCR Master Mix, and QuantiTect RT mix with total RNA (20 ng) samples. The thermal cycling conditions were 95 °C for 15 min; followed by 40 cycles of 95 °C for 15 s, annealing at 60 °C for 45 s (CXCL6 and CXCL8), 60 °C for 15 s (NFE2L2 and OGG1), and 60 °C for 30 s (ALB and SOX9), and finally at 72 °C for 30 s. The levels of mRNA expression were normalized to those of the reference gene and quantified using the comparative Ct method [27], and reported as % of the control.

2.7. Differentiation and characterization of hepatocyte-like cells

Hepatogenic differentiation of UC-MSCs was carried out using a modified two-step hepatic standard protocol [28]. Briefly, UC-MSCs (1.5 × 10⁴ cells) were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco) without FBS, supplemented with 10 ng/mL basic fibroblast growth factor (bFGF, Merck, USA) and 20 ng/mL epidermal growth factor (Merck) for 48 h. Subsequently, cells were induced to differentiate into a hepatic lineage via a further 2-step differentiation protocol. Step 1 (the differentiation step), UC-MSCs were cultured in IMDM supplemented with 40 ng/mL hepatocyte growth factor (Merck), 10 ng/mL bFGF and 5 mM nicotinamide (Sigma-Aldrich) for 7 days. In step 2 (the maturation step), UC-MSCs were cultured in IMDM supplemented with 1 × Insulin-Transferrin-Selenium (Sigma-Aldrich), 1 µM dexamethasone (Merck) and 20 ng/mL oncostatin M (Sigma-Aldrich) for 7 days. Cell viability was determined using MTT assay. Each point represents the mean ± SE from three independent experiments. *, **, and *** represent a statistically significant difference from untreated cells at p < 0.05, 0.01, and 0.001, respectively.
For each step, the medium was changed twice weekly. The differentiated hepatocytes were characterized by determining the transcript levels of the albumin (ALB) and SRY-related HMG-Box 9 (SOX9) genes and the biochemical functions of hepatocytes were assessed by measuring albumin production and glycogen storage. For albumin production, the cell supernatants were collected weekly to quantify albumin concentrations using a human albumin ELISA kit (Abcam). Glycogen storage in differentiated hepatocytes was analyzed using Periodic Acid-Schiff staining at day 21 of differentiation.

2.8. Soft agar colony formation assay for detecting cell transformation

During differentiation of UC-MSCs into hepatocytes, the cells were treated with arsenite 0.5 μM for 21 days before monitoring for cell transformation using the CytoSelected™ 96-well Cell Transformation, Cell Recovery Compatible Kit (Cell Biolabs, Inc., USA) according to the manufacturer’s protocol. Briefly, the assay medium consisted of two layers: a base agar matrix and a cell suspension matrix. The treated cell suspensions (5 × 10^3 cells/well) were applied to the matrix layers and the top layer of agar matrix was overlaid with medium that replaced every three days. Cells were incubated at 37 °C in 5 % CO_2 for 8 days. The number of viable cells within the colonies of transformed cells was measured using the MTT assay and the A_{570 nm} was measured using a SpectraMax microplate reader (Molecular Devices, USA). The percent viable transformed cells in UC-MSCs treated with arsenite during differentiation were compared with those in untreated conditions.

2.9. Statistical analysis

Data were expressed as the mean value ± the standard error (SE). Results were the average of at least three independent experiments. The Kruskal Wallis Test was used to determine statistically significant differences in the levels of 8-OHdG and 8-nitroguanine in human umbilical cord tissue samples. A Spearman rank correlation was used to determine correlation in the human study. In vitro studies, a one-way ANOVA was used to determine statistically significant differences between control
and treatment groups. A p-value of < 0.05 was considered as a statistically significant difference.

3. Results

3.1. Mutagenic DNA damage (8-OHdG and 8-nitroguanine) in umbilical cord MSCs from prenatally arsenic-exposed newborns

Given that our earlier study showed that prenatal arsenic exposure increased the levels of genetic damage in the cord blood of the newborns [4], we further investigated mutagenic DNA damage, in the form of 8-OHdG and 8-nitroguanine, in umbilical cord MSCs from newborns of the same birth cohort which were used as a representative for fetal stem cells. The levels of DNA damage in umbilical cord MSCs were analyzed in relation to maternal arsenic exposure, which was determined by maternal toenail arsenic concentrations and stratified as low (< 0.5 µg/g), medium (0.5–1 µg/g), and high (> 1 µg/g). Immunohistochemical analyses of umbilical cord MSCs were performed simultaneously with the DNA damage assays. Initially, MSCs in umbilical cord tissue mostly in Wharton’s jelly were identified by the presence of MSC-specific cell surface markers (CD73, CD90, and CD105) prior to the determination of 8-OHdG and 8-nitroguanine levels (Fig. 1A). The results of image analyses confirming the expression of MSC markers, including CD73 (red), CD90 (yellow), and CD105 (orange), in umbilical cord tissue along with the localization of 8-OHdG (green) and 8-nitroguanine (pink) are shown in Fig. 1B. DNA damage, measured as fluorescence intensity of 8-OHdG and 8-nitroguanine, increased with increasing levels of arsenic exposure in a dose-dependent manner as shown in Table 1. The mean level of DNA damage detected in umbilical cord MSCs grouped by maternal arsenic exposure levels showed that the intensity of 8-OHdG in stained MSCs was increased by 12.85 % in the medium-exposure group (67.60 ± 2.05) and 50.07 % in the high-exposure group (89.89 ± 6.43; p < 0.001), when compared to the low-exposure group (59.90 ± 3.68). Consistent with 8-OHdG formation, the mean intensity of 8-nitroguanine was increased by 8.87 % in the medium-exposure group (69.07 ± 2.29) and 49.57 % in the high-exposure group (94.89 ± 5.77; p < 0.001), when compared to the low-exposure group (63.44 ± 3.58).

3.1.1. Association of DNA damage with arsenic exposure in umbilical cord MSCs

The association between DNA damage in umbilical cord MSCs and arsenic exposure determined by arsenic concentrations in cord blood and maternal arsenic exposure is illustrated in Fig. 2. The level of 8-OHdG and 8-nitroguanine in umbilical cord MSCs showed a significant positive association with arsenic exposure in newborns measured in terms of the arsenic concentration in umbilical cord blood (r = 0.175, p < 0.05 for 8-OHdG and r = 0.174, p < 0.05 for 8-nitroguanine) (Fig. 2A). Maternal arsenic exposure, measured as arsenic concentrations in toenails, was also significantly positively associated with 8-OHdG (r = 0.401, p < 0.01) and 8-nitroguanine (r = 0.408, p < 0.01) (Fig. 2B).
3.2. Investigation of the mechanisms involved in arsenite-induced DNA damage using a UC-MSC cell line

To ascertain whether the observed increase in 8-OHdG and 8-nitroguanine in umbilical cord MSCs of the newborns was due to arsenic exposure as well as to gain insights into the associated mechanisms, a series of experiments were conducted using a UC-MSC cell line exposed to arsenite in vitro.

The effects of arsenite exposure on DNA damage and the expression of genes involved in the inflammatory response, the oxidative stress response, and DNA repair in the aforementioned stem cells were investigated.

Initially, the dose-response relationships and time-course effects of arsenite treatment on cytotoxicity in UC-MSCs were determined in order to establish the optimal non-cytotoxic concentration and duration of exposure for subsequent experiments. UC-MSCs were treated with various concentrations of arsenite (0, 0.5, 1, 5, 10, and 25 µM) for 24 h. As shown in Fig. 3, arsenite treatment decreased the viability of UC-MSCs in a concentration and time dependent manner. Cell viability of UC-MSCs treated with arsenite decreased significantly (p < 0.01) by 28% at 10 µM and (p < 0.001) 35% at 25 µM (Fig. 3A). However, arsenite treatment of 0.5, 1 and 5 µM did not have a significant cytotoxic effect on UC-MSC viability over the 24 h test period and were therefore
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3.3. Effect of arsenite treatment during cell differentiation on DNA damage and cell transformation ability of hepatocytes differentiated from UC-MSCs

MSCs are known to differentiate into multiple cell lineages, including hepatocyte-like cells. The liver is one of the target organs for arsenic toxicity and epidemiological studies have reported that in utero arsenic exposure increased the incidence of various types of tumors including liver cancer. To further study the consequences of exposure to arsenite during UC-MSCs differentiation, the formation of 8-OHdG and 8-nitroguanine, as well as cell transformation ability, were investigated in hepatocytes differentiated from exposed UC-MSC. Initially, the potential for UC-MSC differentiation into hepatocyte-like cells was studied by determining of the presence of hepatocyte markers including morphology, mRNA expression of ALB and SOX9 as well as functional tests of albumin production and glycogen storage as shown in Fig. 6. The morphological changes during UC-MSCs differentiation into hepatocytes are shown in Fig. 6A. The contraction of the cytoplasm progressed and most differentiated cells became dense and formed an ovoid shape with a polygonal structure. The expression of hepatic-specific gene markers including ALB and SOX9 was observed (Fig. 6B). The transcript level of ALB significantly increased over the differentiation period from day 7 to day 21 with 2.22-fold (p < 0.05) and 3.53-fold (p < 0.001) increases by days 14 and 21, respectively. In contrast, the transcript level of SOX9, a stemness marker, was significantly decreased by 2.58-fold (p < 0.05), 5.59-fold (p < 0.001), and 5.51-fold (p < 0.001) at days 7, 14, and 21, respectively. Moreover, hepatocyte functions determined by albumin production confirmed upregulation of ALB expression. When compared to the control, albumin level increased by 1.26-fold (p < 0.01) and 4.20-fold (p < 0.001) at days 14 and 21, respectively. The albumin levels in differentiated hepatocytes were highest at day 21. Additionally, on day 21 the differentiated hepatocytes showed a marked increase in glycogen storage in the cytoplasm when compared to that in undifferentiated UC-MSCs (Fig. 6B). Thus, 21 days is the optimal period for differentiation, which is consistent with the results reported in a previous study [28].

3.3.2. Oxidative and nitrosative DNA damage and expression of DNA-damage related genes in differentiated hepatocytes derived from arsenite-treated UC-MSCs

A dose-response study was performed in UC-MSCs treated with varying concentrations of arsenite (0.1, 0.5, 1, 2.5, and 5 µM) for 21 days to determine an optimum, non-cytotoxic concentration. As shown in Fig. 7, arsenite treatment up to 0.5 µM did not significantly affect the viability of UC-MSCs, while 1 µM up to 5 µM significantly decreased cell viability. Thus, non-cytotoxic concentrations between 0.1 and 0.5 µM were selected for these experiments. As reported earlier, arsenite treatment increased 8-OHdG and 8-nitroguanine formation in UC-MSCs. In order to determine if these types of mutagenic DNA damage persist upon differentiation into hepatocytes, 8-OHdG and 8-nitroguanine levels were determined in UC-MSCs treated with arsenite (0-5 µM) during differentiation into hepatocytes (21 days). Fluorescence images of 8-OHdG (green) and 8-nitroguanine (pink) showed that both adducts were selected for the subsequent time-course study up to 72 h. As observed in the dose-response study, arsenite treatment at these concentrations did not significantly affect the viability of UC-MSCs during the initial 24 h period of treatment, but significant decreased cell viability at 48 h and 72 h (Fig. 3B). Accordingly, treatment with non-cytotoxic concentrations of arsenite at 0.5, 1, and 5 µM for 24 h were chosen as the treatment conditions for the subsequent in vitro studies.

3.2.1. Mutagenic DNA damage in arsenite-treated UC-MSCs

To investigate whether arsenic can induce DNA damage in fetal stem cells, UC-MSCs were treated with arsenite (0–5 µM) for 24 h, then the 8-OHdG and 8-nitroguanine levels were determined. Fluorescence images of 8-OHdG (green) and 8-nitroguanine (pink) stained UC-MSCs are shown in Fig. 4A. The fluorescence intensity of 8-OHdG in UC-MSCs significantly increased with increasing concentrations of arsenite treatment by 1.51-fold (p < 0.01), 1.58-fold (p < 0.01), and 1.69-fold (p < 0.001), at 0.5, 1 and 5 µM, respectively. Moreover, treated UC-MSCs also showed significant dose-dependent increases in 8-nitroguanine by 1.33-fold (p < 0.01), 1.42-fold (p < 0.001), and 1.60-fold (p < 0.001) at 0.5, 1, and 5 µM, respectively above those of the untreated cells (Fig. 4B). These results strongly support the observation that arsenic exposure in utero increases 8-OHdG and 8-nitroguanine in umbilical cord MSCs of exposed newborns.

3.2.2. Alterations in the expression of genes related to DNA damage

In order to gain insight into the mechanism associated with arsenite-induced 8-OHdG and 8-nitroguanine formation in UC-MSCs, the transcript levels of genes associated with the cause of these types of DNA damage such as inflammation (CXCL6 and CXCL8), and the oxidative stress response (NFE2L2), as well as a DNA repair gene (OGG1) were studied. The effect of arsenite treatment (0-5 µM, 24 h) in UC-MSCs showed that the expression of inflammation-related genes, including CXCL6 and CXCL8, was significantly increased at 0.5–1 µM and then plateaued at 5 µM. Expression of CXCL6 significantly increased by 1.29-fold (p < 0.001), 1.38-fold (p < 0.001), and 1.32-fold (p < 0.01) in UC-MSCs treated with arsenite at 0.5, 1, and 5 µM, respectively (Fig. 5A). Consistent with CXCL6 expression, CXCL8 mRNA expression significantly increased by 2.27-fold, 2.51-fold, and 2.94-fold in treated UC-MSCs at 0.5, 1, and 5 µM, respectively (Fig. 5B). Moreover, in UC-MSCs treated with 1 and 5 µM arsenite, a significant increase in the expression of NFE2L2 was observed (Fig. 5C), while the expression of OGG1 decreased (Fig. 5D). Alteration of the transcript expression of NFE2L2 and OGG1 appeared to reach a maximum at 1 µM arsenite.

Fig. 7. Dose-response study of the viability of UC-MSCs treated with arsenite for 21 days. UC-MSCs were treated with various concentrations (0.1, 0.5, 1, 2.5, and 5 µM) of arsenite for 21 days. Cell viability was determined using MTT assay. Each point represents the mean ± SE from three independent experiments. * and *** represent statistically significant difference from untreated control at p < 0.05 and 0.001, respectively.
hepatocytes differentiated from UC-MSCs and undifferentiated UC-MSCs. At 0.5 μM arsenite treatment, the fluorescence intensity of 8-OHdG and 8-nitroguanine was significantly \( p < 0.05 \) higher in hepatocytes differentiated from UC-MSCs compared to that in undifferentiated UC-MSCs (Fig. 9A and B). Alterations in the expression of genes related to DNA damage, including \( \text{CXCL6} \), \( \text{CXCL8} \), \( \text{NFE2L2} \), and \( \text{OGG1} \), were also investigated in UC-MSCs treated with arsenite (0–0.5 μM) during differentiation into hepatocytes for up to 21 days. In undifferentiated UC-MSCs, the expression of \( \text{CXCL6} \) increased by 1.76-fold (0.1 μM arsenite, \( p < 0.05 \)) and 1.72-fold (0.5 μM arsenite, \( p < 0.05 \)) when compared to untreated cells. By contrast, increases of 2.04-fold \( (p < 0.05) \) and 4.14-fold \( (p < 0.001) \) were observed in hepatocytes differentiated from UC-MSCs treated with arsenite at 0.1 and 0.5 μM, respectively. In addition, expression of \( \text{CXCL6} \) in hepatocytes differentiated from UC-MSCs was significantly \( p < 0.01 \) higher than that of undifferentiated UC-MSCs (Fig. 9C). Consistent with \( \text{CXCL6} \) expression, arsenite treatment (0.5 μM) increased \( \text{CXCL8} \) mRNA expression by 2.91-fold \( (p < 0.001) \) in undifferentiated UC-MSCs and 3.09-fold \( (p < 0.05) \) in differentiated hepatocytes treated with arsenite 0.5 μM (Fig. 9D). Expression of \( \text{NFE2L2} \) was less responsive to arsenite treatment. It increased by 1.24-fold \( (p < 0.05) \) in hepatocytes differentiated from UC-MSCs treated with arsenite at 0.5 μM, with no significant change in undifferentiated UC-MSCs (Fig. 9E). As shown in Fig. 9F, treatment of hepatocytes differentiated from UC-MSCs with arsenite at 0.5 μM significantly decreased \( \text{OGG1} \) expression, but caused no significant change in undifferentiated UC-MSCs. These results indicated that increasing levels of 8-OHdG and 8-nitroguanine in UC-MSCs treated with arsenite, which continued through differentiation into hepatocytes, are related at least in part to increased expression of the inflammatory gene, \( \text{CXCL6} \), and decreased expression of the DNA repair gene, \( \text{OGG1} \).

3.3.3. Cell transformation in hepatocytes differentiated from arsenite-treated UC-MSCs

In order to assess the neoplastic potential of hepatocytes differentiated from arsenite exposed UC-MSCs during differentiation, the transformation ability of these cells was investigated. Subsequent to arsenite exposure during differentiation (0.5 μM, 21 days), the aforementioned hepatocytes were allowed to grow in soft agar for 8 days, after which colonies of transformed cells were counted and compared with those found in the untreated group. As shown in Fig. 10A, higher numbers of
transformed colonies were observed in arsenite-treated cells relative to the untreated group. As shown in Fig. 10B, arsenite treatment during hepatocyte differentiation significantly increased cell transformation, increasing the number of viable cells in transformed colonies with anchorage-independent growth capacity by 1.21-fold (p < 0.01) when compared with untreated cells. This result indicated that hepatocytes differentiated from arsenite-treated UC-MSCs had increased cell transformation ability which may also increase the potential of these cells to develop further to cancer cells.
4. Discussion

Accumulated evidence indicates that prenatal and early life arsenic exposure can cause a variety of detrimental health effects during childhood and increase the risk of developing diseases such as bladder, lung, kidney, and liver cancer in adulthood [2]. Our previous findings provide supportive evidence that arsenic exposure in utero was associated with various types of genetic damage in the newborn that may potentially contribute to increased risk of cancer development later in life. In this study, we explored the effects of prenatal arsenic exposure on mutagenic DNA damage in fetal stem cells by determining 8-OHdG and 8-nitroguanine in umbilical cord MSCs collected from the birth cohort in Vietnam [4]. The effects of arsenite exposure on DNA damage as well as alterations in the expression of inflammation-related and DNA repair genes in an umbilical cord-derived MSC cell line and in hepatocytes differentiated from this cell line were further investigated in vitro. Moreover, the transformation ability of these hepatocytes that were exposed to arsenite during differentiation was also investigated.

In this study, Wharton’s jelly-derived MSCs from the umbilical cord of newborns that had been exposed to arsenic in utero were used as a representative for fetal stem cells because Wharton’s jelly-derived MSCs have a high expansion potential and can differentiate into multiple germ lineages as well as other cell types such as hepatocytes [30]. Umbilical cord MSCs can differentiate toward low immunogenic and functional hepatocytes easily [31]. MSCs develop during the eleventh week of gestation. They can differentiate into multiple lineages and play an important role in haemopoiesis [10,11]. In terms of MSC characterization, we were able to identify MSCs in umbilical cord tissue that were confirmed by immunophenotyping for the expression of CD73, CD90 and CD105, which are surface markers of MSCs [32].

The present study clearly revealed that arsenic exposure in utero significantly increased mutagenic DNA damage in the form of 8-OHdG and 8-nitroguanine in umbilical cord MSCs, and that the levels of DNA damage were positively associated with the levels of cord blood arsenic as well as maternal arsenic exposure. These types of mutagenic DNA damage play an important role in carcinogenesis [33]. One possible mechanism of arsenic-induced 8-OHdG and 8-nitroguanine formation could be through the generation of ROS/RNS during arsenic metabolism concurrent with the impairment of the oxidative stress response system resulting in overproduction of ROS/RNS. It has been reported that 8-OHdG and 8-nitroguanine can lead to G:C to T:A transversions, which accumulate and cause genetic instability that might contribute to disease development and carcinogenesis later in life [19]. In addition, it has been reported that DNA damage alters gene function in stem cells resulting in aberrant expression of genes that control stem cell differentiation and self-renewal, including inactivation of tumor suppressors and activation of oncogenes [34]. Thus, increased mutagenic DNA damage in fetal stem cells may lead to mutations in fetal stem cells and...
predispose increased cancer risk later in life.

The *in vitro* study conducted in a UC-MSC cell line showed that arsenite at non-cytotoxic concentrations (0.1–5 µM) significantly increased 8-OHdG and 8-nitroguanine formation. The arsenite concentrations used (equivalent to 7.5–375 µg/L) are within the range of arsenic concentrations found in the drinking water of subjects in this cohort study (0.9–298 µg/L) [35]. The *in vitro* study therefore confirmed that the observed increase in mutagenic DNA damage in umbilical cord MSCs of the newborn is the result of arsenic exposure *in utero*.

It is known that inflammatory cytokines can indirectly induce DNA damage by increasing free radical generation, which is one of the key factors promoting malignant transformation of cells and carcinogenesis [20]. Under inflammatory conditions, ROS and RNS generated from inflammatory cells are capable of causing DNA damage such as 8-OHdG and 8-nitroguanine leading to genomic instability and ultimately carcinogenesis [21]. The results in this study showed that UC-MSCs exposed to arsenite (1 µM for 24 h) also had increased expression of the inflammatory genes, CXCL6 and CXCL8, which encode IL-6 and IL-8 as well as NFE2L2, which encoded Nrf2, a transcription factor known to be involved in the oxidative stress response [22]. Although arsenic is known to induce oxidative stress through various mechanisms [33], increased NFE2L2 expression suggests the notion that arsenic-induced oxidative stress should be the likely cause of the increased levels of 8-OHdG in UC-MSCs treated with arsenite. Interestingly, the transcript levels of the DNA repair gene, OGG1, a base excision repair enzyme that recognizes and cleaves 8-OHdG [36], was decreased in arsenite-treated UC-MSCs. Down-regulation of OGG1 would slow down the repair of 8-OHdG and thus contribute to the persistence of 8-OHdG in the DNA of arsenite-treated UC-MSCs. Thus, increased transcript levels of CXCL6, CXCL8, and NFE2L2 along with decreased levels of OGG1 mRNA in UC-MSCs are associated with the oxidative and nitrative DNA damage observed in arsenic-exposed fetal stem cells.

In this study, the effect of arsenite exposure in UC-MSCs as they differentiated into hepatocytes was followed because the liver is one of the target organs known to be susceptible to arsenic-induced carcinogenesis in both humans and animals [37]. A study in Antofagasta, Chile reported that a population exposed to high levels of arsenic during gestation and early childhood had higher rates of liver cancer mortality in young adults [5]. Supportive evidence from animal studies also suggested that exposure of mice to arsenic during gestation resulted in the development of various types of tumors in the offspring when they reached adulthood, including liver cancer [38]. Moreover, *in utero* arsenic exposure in mice was found to dysregulate the liver’s developmental program [39]. There are a variety of potential mechanisms involved in arsenic-induced hepatocarcinogenesis such as impairment of DNA repair, oxidative DNA damage, genomic instability, and apoptosis tolerance [37]. UC-MSCs are able to differentiate into multiple lineages, including hepatocytes. As shown in this study, differentiated hepatocyte-like cells derived from UC-MSCs displayed morphological changes and showed increased hepatic markers such as increased levels of ALB transcripts, the presence of albumin at levels comparable to those in functionally mature hepatocytes, and glycogen storage. These hepatocytes differentiated from UC-MSCs also exhibited decreased stemness as indicated by decreased transcript expression of SOX9 which was in line with the results in a previous study showing that increases in SOX9 mRNA levels reduces the proliferation rate in differentiated hepatocytes [40].

Interestingly, our study also demonstrated that arsenite treatment of UC-MSCs at non-cytotoxic concentrations (0.1–0.5 µM, 21 days) increases mutagenic DNA damage (8-OHdG and 8-nitroguanine) in differentiated hepatocytes to levels that are significantly higher than those in undifferentiated UC-MSCs, suggesting mutagenic DNA damage occurs at a greater level and persists during hepatocyte differentiation. This study indicates that stem cells are a target of arsenic toxicity and that accumulation of mutagenic DNA damage in stem cells may lead to the increased risk of liver cancer later in life.

Increased mRNA levels of the inflammatory genes, CXCL6 and CXCL8, were also observed in both undifferentiated UC-MSCs and hepatocytes differentiated from UC-MSCs as a result of arsenite treatment (0.5 µM) and coincided with the increase in 8-OHdG and 8-nitroguanine. However, arsenite treatment significantly increased NFE2L2 transcript levels only in differentiated hepatocytes. This is in agreement with the notion that chronic arsenic exposure may overwhelm arsenic-mediated Nrf2-dependent protection mechanism(s) leading to the over-stimulation of Nrf2 regulated gene expression [41]. The over-expression Nrf2 may be related to carcinogenesis [42]. In addition, a significant decrease in OGG1 transcript levels was also observed in hepatocytes differentiated from UC-MSCs, which coincided with significantly increased formation of 8-OHdG. The changes in gene expression observed in this study may contribute to arsenite-induced DNA damage in the aforementioned hepatocytes and eventually make them more susceptible to liver cancer development later in life.

Another important finding in this study was that arsenite (0.5 µM) treatment in UC-MSCs significantly increased cell transformation in differentiated hepatocytes. This is in agreement with the observation that increased cell transformation occurred in arsenic-treated mouse embryonic fibroblasts deficient in OGG1 [36]. Activated Nrf2 also plays an important role in arsenic-induced transformation of normal human prostate stem-progenitor cells [43]. Accumulated DNA damage causes mutations in cells that lead to cell transformation. This may be due to the fact that mutated genes confer the ability to survive and escape from cell apoptosis, eventually leading to cell transformation [44]. This study suggests that increased mutagenic DNA damage and decreased expression of OGG1 in hepatocytes differentiated from arsenite-treated UC-MSCs can lead to transformation to malignant cells. The results indicate the potential consequences of arsenic exposure *in utero* and its effects on fetal stem cells, which may contribute to development of liver cancer.

5. Conclusion

This study reports for the first time that *in utero* arsenic exposure induces mutagenic DNA damage, in the form of 8-OHdG and 8-nitroguanine, in fetal MSCs. *In vitro* studies also confirmed that arsenite induces DNA damage in UC-MSCs and in hepatocytes differentiated from UC-MSCs as well as increased cell transformation. Increased mutagenic DNA damage in arsenite-treated UC-MSCs is in part related to the increases in the expression of genes related to inflammation (CXCL6 and CXCL8), the oxidative stress response (NFE2L2), as well as decreased expression of a DNA repair gene (OGG1). Taken together, this study provides strong evidence indicating that arsenite-induced DNA damage in fetal stem cells and in hepatocytes differentiated from the stem cells increases the risk for liver disease development. Our findings highlight the importance to minimizing arsenic exposure during gestation to prevent risk of disease development later in life.

Author contributions

SK performed analysis of DNA damage formation in umbilical cord MSCs from newborn and UC-MSC cell line, gene expression, hepatocytes differentiation, and colony formation assay. PN coordinated and designed laboratory experiments, data analysis, and manuscript preparation. KC, PH, and VP prepared data for experiment part of the manuscript. MR, as the principal investigator, conceived and designed the whole study and experiments, sought funding supports as well as wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence
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Data availability

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