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As_2O_3 oxidation by vitamin C: cell culture studies

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Abstract The ability of As_2O_3 to induce apoptosis in various malignant cell lines has made it a potential treatment agent for several malignancies. In this study the chemical stability of As_2O_3 (As(III)) in cell-free growth media with various compositions was studied (MEM with different amount of amino acids and DMEM). Special attention was given to evaluate the influence of serum (FBS; fetal bovine serum) absence and vitamin C addition on the oxidation of As(III) to As(V) in cell-free growth media. FBS is an important source of antioxidants and vitamin C (ascorbic acid) is acting as a prooxidant in millimolar concentrations. Media were incubated with As(III) (0.6, 2 and 7 μmol l^{-1}) up to 72 h. Experiments were performed at 37°C in light or/and in the dark, with or without added serum (10%) or vitamin C (1.4, 0.14 mM). Metabolites were followed with high-performance liquid chromatography directly coupled to a hydride generation-atomic fluorescence spectrometry system. After 72 h up to 30% of As(III) was transformed into As(V) in MEMs and up to 35% in DMEM when exposed in dark. Light had no influence on transformations in MEMs, but changed the situation dramatically in DMEM where almost all As(III) was oxidized to As(V) after 72 h when exposed to light. Except for some faster oxidation rate the absence of FBS had little effect on the transformation rate in all media. The most visible impact on As(III) oxidation was observed by addition of vitamin C. Addition of vitamin C (1.4 mM) transformed almost all As(III) to As(V) within 72 h. In lower concentrations (0.14 mM) a pro-oxidative effect was still observed reaching approximately 60% oxidation of As(III) during 72 h. All oxidation processes could be explained by pseudo first order reaction kinetics, yielding reaction rates increasing with initial As(III) concentration and vitamin C concentration whereas the FBS content additionally increased the As(III) oxidation rate in the DMEM (light). The temporal oxidation of As(III) to As(V) in various cell-free growth media necessitates routine checking of the valence state of arsenic during cell culture experiments and the results of biological effects attributed to As(III) should be interpreted with caution. Special attention is needed particularly in cases with vitamin C which was acting pro-oxidatively in all conditions examined.

Keywords Arsenic trioxide · Vitamin C · Serum · Oxidation · Cell culture

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Introduction

Due to its known therapeutic effect, As_2O_3 is the arsenical of choice in distinct cancer research work. During the last decade arsenic trioxide (As_2O_3; TRISENOX®) has been used to treat patients with acute promyelocytic leukaemia (APL). APL is often cured by As_2O_3 when patients do not respond to, or have relapsed from, retinoid and anthracycline chemotherapy (first-line therapy) and whose APL is characterized by the presence of the t(15;17) translocation or PML/RAR-alpha gene expression (Cell therapeutics 2002; Cephalon 2008). As As_2O_3 has been shown to induce cell death in different malignant cell lines, many clinical trials were/are performed to evaluate its usefulness in the treatment of other cancers as well (Murgo 2001; Dilda and Hogg 2007); among them multiple myeloma (Berenson et al. 2007; Qazilbash et al. 2008) and solid tumours like glioblastoma (Grimm et al. 2008). Treatment strategies include the sole use of As_2O_3 or in combination with potential synergistic agent such as vitamin C (Vit. C) or other anticancer agents (Dilda and Hogg 2007; Qazilbash et al. 2008).

Arsenic metabolism and toxicity have been reviewed in many publications (Del Razo et al. 2001; Aposhian and Aposhian 2006; Cui et al. 2008; Drobna et al. 2009). After entering the human body arsenic undergoes metabolic transformations depending on its oxidation state. Both As(III) and As(V) are actively transported into the cells. As(III) is an analogon of glycerol and uses aquaglyceroporin channels for transport. As(V) is an analogon of phosphate, thus it probably enters the cell through non-specific anion transporters and other phosphate transporters, followed by reduction to As(III) by mitochondria. As(III) gets methylated to methylarsonic acid (MA(V)) or dimethylarsinic acid (DMA(V)) in the presence of As-methyltransferase, processes which mainly occur in the liver although to a lesser degree also in other tissues. Trivalent methylated intermediates are believed to be present in low amounts immediately forming conjugates through thiol groups on proteins. Arsenic toxicity depends highly on the oxidation state and type of arsenic compound, with trivalent species being more toxic than pentavalent ones. Trivalent arsenic toxicity is triggered either by attacking –SH (and –SeH) groups of important cellular components or through the generation of reactive oxygen species. Pentavalent arsenic causes competitive substitution of phosphate with arsenate which may lead to rapid hydrolysis of the high-energy bonds in compounds such as ATP. Nevertheless, the exact mechanisms of the therapeutic use of arsenic and its cytotoxicity are still in research (Del Razo et al. 2001; Miller et al. 2002; Lu et al. 2007; Cui et al. 2008; Yang et al. 2008; Wondrak 2009). As an antineoplastic chemotherapeutic agent it is involved in inhibition of proliferation and angiogenesis, induction of apoptosis and/or autophagy and induction of differentiation. The biological effects may be mediated by reactions with –SH or –SeH groups of critical proteins and through enhanced oxidative stress.

The above mentioned mechanisms of arsenic action in a living organism can be studied in an isolated environment. For this purpose cells are grown in nutrient media under suitable conditions and with all nutrients needed for cell development and multiplication. To keep cells alive for longer periods of time and to evaluate proliferation, migration and differentiation, a basal medium must be augmented with several supplements. The main supplements are amino acids, fetal bovine serum (FBS) or its substitutes, glutamine, antibiotics, etc. FBS is a natural complex mixture of different factors and contains a large number of components like growth inducers, proteins, vitamins, trace elements, hormones, etc., essential for the growth and maintenance of cells (Van der Valk et al. 2010). FBS addition is also important for cellular antioxidative protection (Lewinska et al. 2007). In its absence the total antioxidant capacity of cell culture media can be diminished. Anyway reactive oxygen species (ROS) can be generated in different cell-free culture media even in its presence, with or without the influence of light (Grzelak et al. 2000, 2001).

Vitamin C, which might have synergistic effects in combination with arsenite, has a controversial history in cancer treatment (Padayatty and Levine 2000) and paradoxical effects on the viability of cell cultures (Duarte et al. 2007). Depending on its physiological ($\mu mol\ l^{-1}$) or pharmacological ($mmol\ l^{-1}$) concentration it can act as an antioxidant or prooxidant (in vivo). In aqueous media vitamin C oxidizes to dehydroascorbate (DHA) in the presence of oxygen, generating reactive intermediates such as H_2O_2 (Duarte et al. 2007) to which the cytotoxic effect of vitamin C has been attributed. The formation of H_2O_2

is mediated by transition metal ions present in cell media, although autooxidation in the presence of oxygen only is also possible (Duarte et al. 2007). The usefulness of vitamin C as a synergistic agent in chemotherapy was under question, since it did not generate H_2O_2 in systems such as a simple buffer or human plasma. However, recent results are confirming the extracellular formation (intersticial) of H_2O_2 in animals exposed to vitamin C at doses that achieve mmol l^{-1} peak plasma concentrations (Chen et al. 2007). H_2O_2 formation was also detected within tumour interstitial fluids in mice (Chen et al. 2008). Its usefulness in combination with arsenic is intriguing since the proposed generation of H_2O_2 could oxidize As(III) to less effective As(V). Such almost complete vitamin C-induced As(III)/As(V) conversion has already been observed in media of multiple myeloma (MM) cells after three days exposure to arsenic (7 μmol l^{-1}) and vitamin C (5.7 mmol l^{-1}) (Falnoga et al. 2007). Its addition in lower (physiological) concentrations is believed to be beneficial by acting in favour of As(III) forms. Grad et al. (2001) demonstrated that extracellular vitamin C (100 μmol l^{-1}) enhances As_2O_3-induced cytotoxicity in four MM cell lines and Michel et al. (2003) reported that extracellular vitamin C synergizes with As_2O_3 to induce apoptosis in Sézary cells. In both studies vitamin C was added in low doses what is not the case in clinical trials.

The data on actual arsenic compounds in cell growth media are scarce (Bertolero et al. 1987; Falnoga et al. 2007). In vitro observations show that under some conditions the more toxic trivalent arsenic species might be oxidized to As(V) (Aposhian and Aposhian 2006; Falnoga et al. 2007). To further investigate these observations this study aims to perform temporal chemical stability tests for As(III) in various cell-free media commonly used in cell culture experiments. Special attention is given to the effect of FBS and vitamin C on the As_2O_3 stability.

Materials and methods

As_2O_3 in aqueous solution at physiological pH is present as arsenious acid (H_3AsO_3). The completely deprotonated form of arsenious acid is arsenite (AsO_3^{3-}) (ATSDR 2007). In this paper As_2O_3 is termed arsenite or As(III) whereas for the pentavalent form the term arsenate or As(V) is used. Although the chemical forms of As(III) and As(V) (degree of protonation) depend on the pH, the terminology mentioned embraces all these forms and allows simple comparison of concentration data when expressed as total As.

Chemicals

All chemicals were of at least analytical reagent grade. Millipore (Milford, MA, USA) Milli-Q Plus water (18.2 MΩ cm) was used for all solution preparations. The compounds of cell growth media are given in Table 1. As(III), As(V), MA(V) and DMA(V) stock solutions (10 μg ml^{-1}) were prepared as described previously and were kept at 4°C (Šlejkovec et al. 2001). Vitamin C (ascorbic acid, AA) was taken from ampoules with injection solution (500 mg/5 ml; Galenika, Serbia).

Cell-free media and arsenite exposure

Three cell-free media with different compositions were used for arsenic exposure. They were assigned as MEM1, MEM2 and DMEM, representing: (i) Minimum essential medium with Earl's Salts without L-glutamine, (ii) Minimum essential medium with Earl's Salts, added L-glutamine and non essential amino acids and (iii) Dulbecco's modified Eagle's medium with High Glucose without L-glutamine. Detailed composition is given in Table 1. Exposure was performed in all three media with and without FBS (10%). Additionally the media MEM2 and DMEM were used with and without vitamin C coadministration (0.14 and 1.4 mmol l^{-1}). As(III) concentrations were 0.6, 2 and 7 μmol l^{-1} whereas the batch without addition of As(III) served as a control (procedural blank). 5 ml of the selected media was added to a 15 ml test tube and shaken in a water bath (SW22, Julabo) at 37°C under air atmosphere for up to 72 h (3, 24, 48, 72 h). Arsenic species in media were determined immediately after incubation to prevent changes in arsenic speciation. Experiments were performed on light and on dark to evaluate the impact of light (UV) and tested with few exposures under the same conditions as cells are normally grown (classic incubator, 5% CO_2 atmosphere, 37°C, 72 h).

Table 1 Media composition

Ingredients	MEM1	MEM2	DMEM
Inorganic salts (mg l^{-1})			
$CaCl_2$	200	200	200
KCl	400	400	400
$MgSO_4$	97.7	97.7	97.7
NaCl	6,800	6,800	6,400
$NaH_2PO_4 \cdot H_2O$	140	140	125
$NaHCO_3$	2,200	2,200	3,700
$Fe(NO_3)_3 \cdot 9H_2O$	/	/	0.1
Amino acids (mg l^{-1})			
L-Arg · HCl	126	126	84
L-Cys	24	24	48
L-His · HCl · H_2O	42	42	42
L-Ile	52	52	105
L-Leu	52	52	105
L-Lys · HCl	72.5	72.5	146
L-Met	15	15	30
L-Phe	32	32	66
L-Thr	48	48	95
L-Trp	10	10	16
L-Tyr	36	36	72
L-Val	46	46	94
Gly	/	/	30
L-ser	/	/	42
Vitamins (mg l^{-1})			
D-Calcium-pantothenate	1.0	1.0	4.0
Choline chloride	1.0	1.0	4.0
Folic acid	1.0	1.0	4.0
Myo-inositol	2.0	2.0	7.2
Nicotinamide	1.0	1.0	4.0
Pyridoxal · HCl	1.0	1.0	4.0
Riboflavin	0.1	0.1	0.4
Thiamine · HCl	1.0	1.0	4.0
Other components (mg l^{-1})			
D-Glucose anhydrous	1,000	1,000	4,500
Phenol red	11	11	15
Additions (%)			
Penicillin/streptomycin	1	1	1
L-Gln	1	2	1
Non essential amino acids	/	1	/
FBS	10	10	10

MEM 1 Minimum essential medium with Earl's Salts without L-Glutamine (PAA; Lot No. E02410-0609), *MEM 2* MEM 1 with added L-Glutamine (PAA) and non essential amino acids (Sigma), *DMEM* Dulbecco's modified Eagle's medium with high glucose (4.5 g l^{-1}), without L-Glutamine (PAA, Lot. No. E00910-0142), *FBS* Fetal bovine serum (PAA, Lot No. A10707-1153)

Arsenic (species) determination by HPLC-HG-AFS and FI-UV-HG-AFS

For the determination of arsenic species in media high performance liquid chromatography was coupled to hydride generation-atomic fluorescence spectrometry (HPLC-HG-AFS) (Šlejkovec et al. 2001). For As(III) and As(V) chromatographic separation a Hamilton PRP-X100 column was used with a mobile phase consisting of 50 mmol l^{-1} KH_2PO_4 (pH 6.0), while for As(III), DMA(V), MA(V) and As(V) determination, 15 mmol l^{-1} $NH_4H_2PO_4$ (pH 6.0) was used. An on-line hydride generation (HG) step (4 mol l^{-1} HCl, 3 ml min^{-1} and 1.5% $NaBH_4$ in 0.1% NaOH, 3 ml min^{-1}) was applied to convert non-volatile arsenic compounds into their corresponding volatile hydrides, which were detected in an Excalibur atomic fluorescence spectrometer (PS Analytical, Kent, UK). For the determination of total arsenic in liquid samples a similar setup in flow-injection mode (column omitted, FI-UV-HG-AFS) with a UV-decomposition unit (3.1 m long, 0.5 mm i.d. FEP Teflon tubing coiled around an 8 W Camag UV lamp, 254 nm with addition of 4% $K_2S_2O_8$ and 4% NaOH) prior to the HG step was used.

Results and discussion

Incubation of cell-free media exposed to arsenite in presence or absence of FBS

To evaluate the stability of arsenite in cell-free growth media, incubation took place with different concentrations of arsenite and for varying incubation times. The influence of external growth conditions was first checked by exposing 2 and 7 µmol l^{-1} As(III) in media MEM1/MEM1^{-FBS} and MEM2/MEM2^{-FBS} under the same conditions cells are normally grown (incubator, 5% CO_2 atmosphere, 37°C, 72 h) and in a water bath shaker (air atmosphere, 37°C, 72 h). No differences were observed between the two exposure procedures (data not shown) so that all further

experiments were performed in a water bath, which is much simpler to operate than an incubator. On contrary the third medium, DMEM, showed higher As(III) instability for water bath conditions than for conventional incubation conditions, most probably due to light exposure as commented below. After incubation, arsenic speciation analysis of media was performed so that the arsenic mass balance could be established and the degree of conversion by oxidation in different cell-free growth media calculated. Average values for two experimental parallels, measured either in duplicate or in triplicate, were used in the figures (throughout the text). The standard deviations were below 5% for As(III) measurements in all media, while As(V) showed higher standard deviations, up to 20%, in the measurements at the beginning of exposure (after 0 and 3 h), when As(V) concentrations were still close to the detection limit (ca. 1 ng ml^{-1}).

During incubation a time-dependent transformation of As(III) to As(V) was observed in all three basic media–MEM1, MEM2 and DMEM (Fig. 1); longer incubation in all cases yielded higher amounts of As(V). The highest degree of transformation of As(III) to As(V) was observed in the medium DMEM: 77–91% in 72 h (depending on the initial As(III) concentration) when experiments were performed in the presence of light, and less than 35% for exposure in an incubator or in test tubes covered by aluminium foil (data not shown). In MEM2 the transformation to As(V) was from 23 to 27% and in MEM1 10 to 19% after 72 h. Shorter exposure times in all cases yielded lower transformation of As(III) to As(V). In search for an explanation the reduction potential (Eh), also known as the redox potential or oxidation–reduction potential (ORP), was measured in media at physiological pH. The Eh for MEM1, MEM2 and DMEM ranged from 180 to 200 mV; very precise readings were not possible due to matrix effects. In this Eh range the predominant arsenic species (>95%) in aqueous solution is As(V) as calculated (analogous to van Elteren et al. 2002) from thermodynamic equilibrium parameters (acid–base and redox equilibria) at pH 6.0 (Fig. 2). However, this only indicates the potential for redox conversion and not the time-path towards equilibrium which is governed by unknown kinetic parameters.

According to the composition of the media in Table 1, the obvious difference between the media DMEM, MEM1 and MEM2 is the presence of 0.1 mg l^{-1}

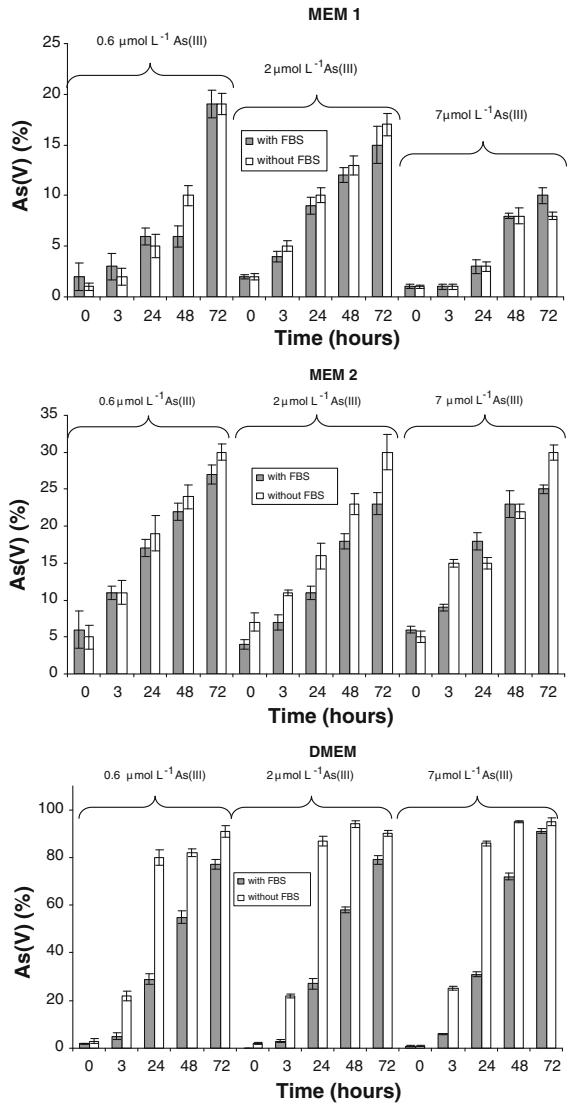

Fig. 1 Percentage of arsenite conversion to arsenate in cell free growth media with and without addition of FBS, exposed under light conditions at 37°C for up to 72 h

$Fe(NO_3)_3 \cdot 9H_2O$ in DMEM, which might trigger the observed rapid As(III) oxidation in DMEM medium. The generation of ROS can also be attributed to riboflavin (Grzelak et al. 2001) which is present at much higher levels in DMEM (0.4 mg l^{-1}) than in MEM1 or MEM2 (0.1 mg l^{-1}). As riboflavin is very sensitive to light exposure (autooxidation and generation of ROS) it is probably responsible for the high As(III) oxidation rate in DMEM water bath experiments on light.

To evaluate whether the absence of FBS has an influence on the transformation of arsenite, the same

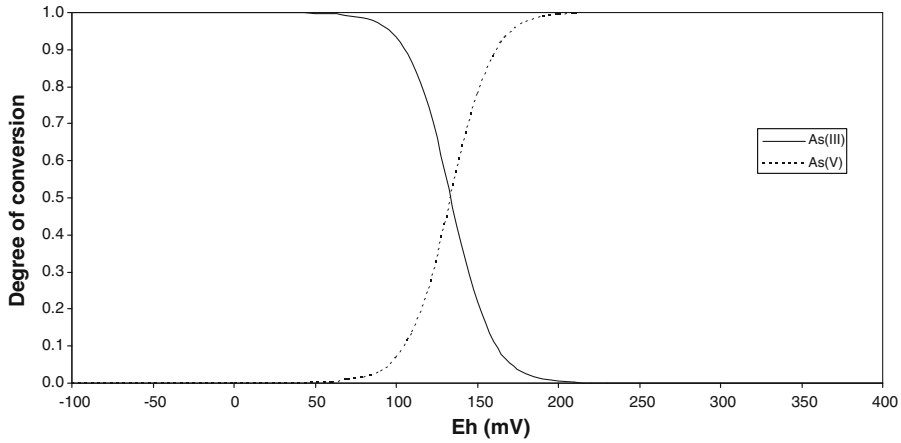

Fig. 2 Calculation of As(III/V) interconversion at physiological pH for varying Eh values

experiments were performed as mentioned above, without addition of FBS to cell free growth media. The highest transformation to As(V) was again observed in the medium DMEM^{-FBS}, where after 72 h 90–95% of As(III) was transformed to As(V). In comparison to DMEM the transformation was faster (Fig. 1c). In MEM2^{-FBS} the transformation was 30% after 72 h. In comparison to MEM2 (with FBS) the transformation was only marginally higher, so the absence of FBS had very little effect (Fig. 1b). In MEM1^{-FBS} the transformation was 8–19% after 72 h. In comparison to MEM1 (with FBS), the absence of FBS also had no significant influence on the transformation (Fig. 1a). Although the composition of FBS is not exactly known, it is a natural material (plasma from blood drawn from a bovine fetus) and as such a complex mixture of different factors containing a large number of components like growth factors, proteins (albumin, transferrin, selenoprotein P), vitamins, trace elements, hormones etc. (Van der Valk et al. 2010). It also shows seasonal and continental variations in the serum composition so that batch-to batch variations are well documented (Van der Valk et al. 2010). As its addition is important for cellular antioxidative protection (Lewinska et al. 2007), its absence could diminish the total antioxidant capacity of the media, what is particularly important in the case of DMEM due to its high iron and riboflavin content (Table 1). Iron can undergo redox cycling (ferrous–ferric conversion), triggering H_2O_2 generation, depending on the balance between oxidizing and reducing agents in the media, whereas riboflavin may also induce the formation of ROS on its own way (see above).

Incubation of cell-free media exposed to arsenite in presence or absence of vitamin C

To evaluate the influence of vitamin C on arsenite transformation, the similar experiments as discussed above were performed with addition of varying doses of vitamin C (0, 0.14 and 1.4 mmol l^{-1}) to cell free growth media MEM2 and MEM with 7 µmol l^{-1} As (III) for 3, 24, 46 and 72 h. Experiments were performed in dark. Significant As(III) to As(V) transformation was observed as shown in Fig. 3. In DMEM$^{+1.4\ mmol/l\ Vit.C}$ and MEM2$^{+1.4\ mmol/l\ Vit.C}$ all As(III) was transformed to As(V) after 72 h. Without vitamin C only 25% of As(III) was transformed to As(V) in MEM2 and 35% in DMEM. The observed conversion was also considerably faster in experiments with vitamin C. Results with ten times lower doses of vitamin C (0.14 mmol l^{-1}) still trigger some arsenit conversion although in much lesser amount.

Mechanisms can be related to in vitro experiments performed by Ahmad et al. (2000) who showed that arsenic (in different forms) or vitamin C or both together, are able to activate protein-bound iron (ferritin) by its detachment. The aerobic autooxidation of vitamin C—in the presence but also absence of trace levels of free metal ions (iron, copper)—can generate H_2O_2 and ascorbate radicals. Karasavvas et al. (2005) found that vitamin C generates cytotoxic concentrations of H_2O_2 in culture media without cells and Clement et al. (2001) are also warning that about the ability of vitamin C to produce H_2O_2 at different rates in different culture media being the highest in DMEM. Since As(III) is easily oxidized by H_2O_2, a high degree of oxidation of As(III) in growth media

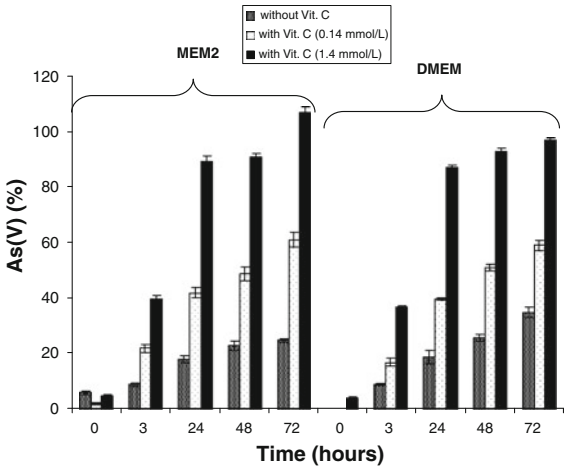

Fig. 3 Percentage of arsenite transformation to arsenate in cell free growth media MEM2 and DMEM after addition of 7 μmol l^{-1} As(III), with varying concentrations of vitamin C exposed under dark conditions at 37°C for up to 72 h

in the presence of vitamin C as observed in our experiments can be explained by the mechanisms given in literature.

Although H_2O_2 is a poorly reactive molecule, in the presence of transition metal ions it can be converted to the highly reactive hydroxyl radical. (Halliwell and Gutteridge 1999). The toxic effects of vitamin C are attributed to the generation of ROS and the consequent depletion of GSH in the cells. Since As(III) is easily oxidized by H_2O_2, a high degree of oxidation of As(III) in growth media in the presence of vitamin C is expected. We have observed previously that addition of 5.7 mmol l^{-1} vitamin C to 7 μmol l^{-1} As(III) in media with MM BM cells (multiple myeloma, bone marrow) converted almost all arsenite to the pentavalent form (Falnoga et al. 2007). The generation of H_2O_2 was attributed to oxidative stress in cells caused by As_2O_3 and to vitamin C addition. From the results of the present experiments the As(III)-to-As(V) conversion should also be also attributed to extracellular H_2O_2 generation in media. The therapeutic synergism of vitamin C and arsenic is regulated by increased ROS production, but in the meantime the effectiveness of As(III) through thiol- and selenol-redox pathways (Miller et al. 2002, Holmgren and Lu 2010) is diminished and combination of compounds in clinical trials questionable.

Kinetics of As(III) transformation in cell growth media

Oxidation rates of As(III) in all cell culture media (see Figs. 1 and 3) were used to determine the rate equations and quantify the rate constants and partial reaction orders. For a second order reaction A + B → C, the rate equation is given by

$$r = k[A][B] \tag{1}$$

with k denoting the rate constant and $[A]$ and $[B]$ expressing the concentrations of the species A and B, respectively. If $[B]$ remains constant due to a large excess or behaviour as a catalyst, Eq. 1 reduces to a pseudo first order rate equation with a rate constant k' which incorporates $[B]$, and potentially more reactants for complex systems as in our case:

$$r = k'[A] \tag{2}$$

depending on the concentration of reactant A only. The rate equation for Eq. 2 can be written as

$$r = -\frac{d[A]}{dt} = k'[A] \tag{3}$$

and integration leads to

$$\ln[A] = -k't + \ln[A]_0 \tag{4}$$

with $[A]_0$ the initial concentration. All As(III) oxidation processes occurring in Figs. 1 and 3 could be satisfactorily described by pseudo first order rate equations assuming As(III) to be the only reactant (see Fig. 4). This resulted in all cases in better fits to experimental data than presuming zero or second order rate kinetics. Oxidation rate constants were retrieved from numerical least squares fitting of the experimental data points on all initial concentrations simultaneously resulting in a single rate constant k' for the media MEM1, $MEM1^{-FBS}$, MEM2, $MEM2^{-FBS}$, DMEM, $DMEM^{-FBS}$ (see Figs. 4a–c). The kinetic parameters retrieved for these media are summarized in Table 2, together with the half-lives $t_{1/2}$ defined by

$$t_{1/2} = \frac{[A]_0}{2k'} \tag{5}$$

which are independent of the initial As(III) concentration $[As(III)]_0$. The oxidation rate constants increase in the following order: $MEM1^{-FBS} \approx MEM1 >$

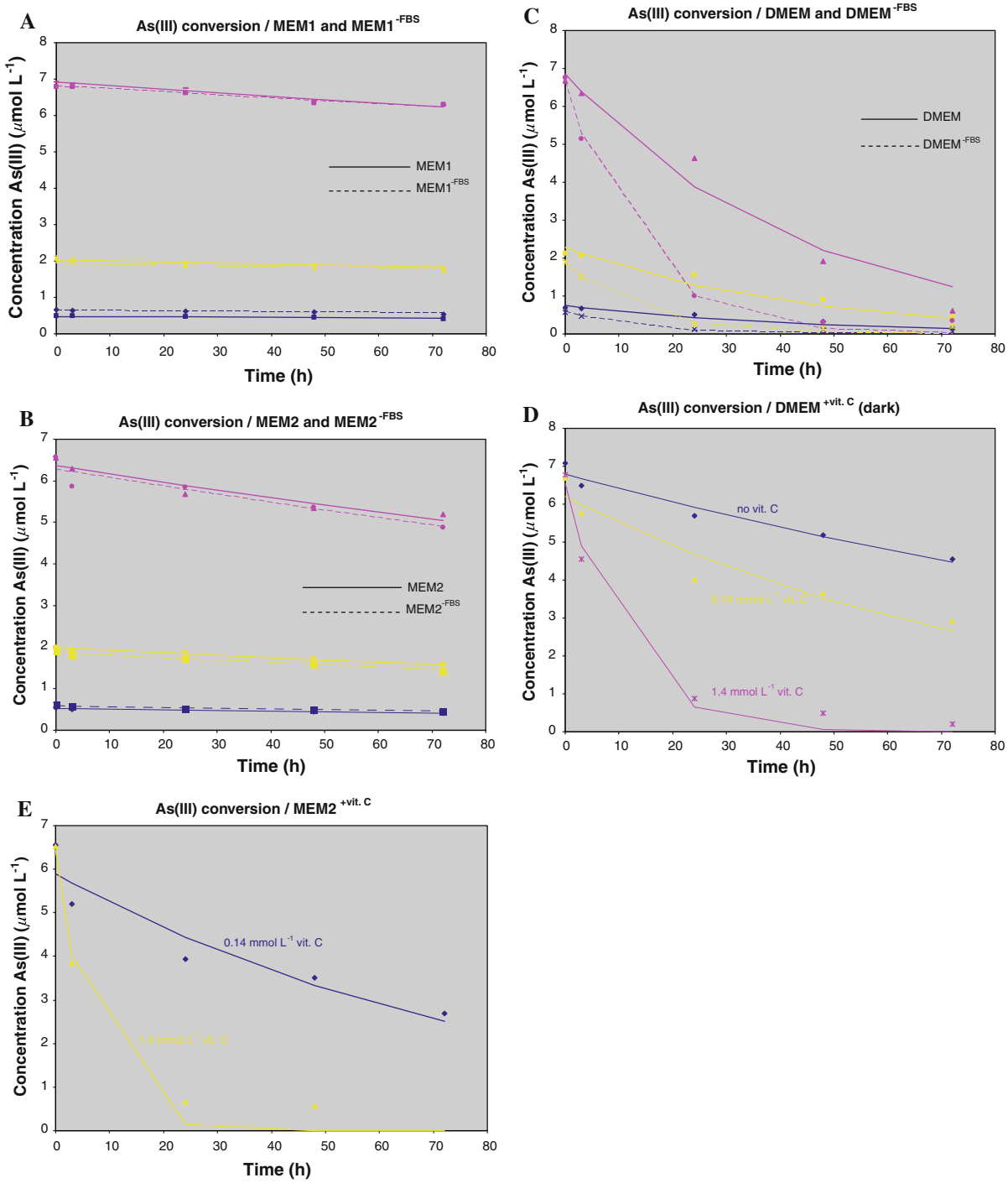

Fig. 4 As(III) concentration in time as a function of the medium composition [initial As(III) concentration (0, 0.6 and 7 μmol l^{-1}) and presence of FBS or vit. C (0.14 and 1.4 mmol l^{-1})]

$MEM2^{-FBS} \approx MEM2 > > DMEM > DMEM^{-FBS}$; similar As(III) concentrations in $DMEM^{-FBS}$ are oxidized 55 times faster than in $MEM1^{-FBS}$.

The presence of vit. C (0.14 and 1.4 mmol l^{-1}) in MEM2 and DMEM (see Fig. 4d) on the stability of As(III) (7 μmol l^{-1}) is especially detrimentous in

Table 2 Kinetic parameters retrieved from fitting the experimental As(III) oxidation data to pseudo first order rate equations as defined in Eq. 4; for MEM1, $MEM1^{-FBS}$, MEM2, $MEM2^{-FBS}$, DMEM and $DMEM^{-FBS}$ the three initial As(III) concentrations $[As(III)]_0$ were fitted simultaneously to yield one rate constant k'

	k' (h^{-1}) fitted	$[As(III)]_0$ (μmol l^{-1}) fitted			$t_{1/2}$ (h)
		$[As(III)]_{0,exp} = 0.6$	$[As(III)]_{0,exp} = 2$	$[As(III)]_{0,exp} = 7$	
MEM1	1.454E−03	0.476	2.03	6.92	477
$MEM1^{-FBS}$	1.234E−03	0.634	1.93	6.81	562
MEM2	3.268E−03	0.523	1.99	6.37	212
$MEM2^{-FBS}$	3.466E−03	0.587	1.86	6.28	200
DMEM	2.374E−02	0.753	2.28	6.86	29.2
$DMEM^{-FBS}$	7.918E−02	0.588	1.90	6.67	8.75
$MEM2^{0.14\ mM\ vit.\ C}$	1.189E−02			5.89	58.3
$MEM2^{1.4\ mM\ vit.\ C}$	1.572E−01			6.43	4.41
$DMEM^{no\ vit.\ C\ (dark)}$	5.818E−03			6.79	119
$DMEM^{0.14\ mM\ vit.\ C\ (dark)}$	1.180E−02			6.20	58.8
$DMEM^{1.4\ mM\ C\ vit.\ (dark)}$	9.650E−02			6.55	7.18

MEM2 medium; oxidation rates are 3.6 and 48 times higher with 0.14 and 1.4 mmol l^{-1} vit. C, respectively, than without vit. C. Comparison of the DMEM media with and without vit. C is not so straightforward since in the vit. C experiment (see Fig. 4e) the samples were kept in the dark whereas all other experiments were performed under light conditions. This results in $DMEM^{no\ vit.\ C\ (dark)}$ having a 4.1 times slower As(III) oxidation rate than $DMEM^{no\ vit.\ C\ (light)}$. This suggests that light-sensitive compounds such as riboflavin in DMEM may indeed be responsible for autooxidation or generation of reaction oxygen species. The addition of 0.14 and 1.4 mmol l^{-1} vit. C to DMEM under dark conditions yields 2.0 and 16.6 times higher As(III) oxidation rates compared to $DMEM^{no\ vit.\ C\ (dark)}$ showing the prooxidant behaviour of vit. C in millimolar concentrations.

Conclusions

Arsenite is not stable in any growth medium implying that in cell experiments the cells are not only exposed to As(III) but also to a certain percentage of As(V), depending on the medium composition and the exposure conditions. Consequently the biological affects attributed to As(III) should be interpreted with caution. Also cell studies using different cell media are hardly comparable.

The presence of FBS in media influences the transformation of As(III) in relatively small degree depending on the medium type and exposure conditions.

Vitamin C (pharmacological levels, mM) can oxidize almost all arsenite to arsenate in 72 h regardless to the type of media. If the same is occurring in vivo, in extracellular fluids, the proposed therapeutic synergism between vitamin C and arsenic trioxide should be questioned, especially since substantial therapeutic effects are related to As(III) interactions with thiol and selenol ligands of essential compounds. As recently the selenocysteine-dependent thioredoxine reductase (TrxR enzyme), known to be inhibited by arsenite, has emerged as an important molecular target for anticancer drug development, it is supposed that arsenite-TrxR interaction is a basis for cancer therapy by arsenic trioxide (Lu et al. 2007; Holmgren and Lu 2010).

Summarizing we can say that arsenite is not stable in cell free media which are able to generate ROS, despite the presence of FBS and particularly by addition of vitamin C in high concentrations. From these observations it is clear that routine checking of the arsenic speciation during cell culture experiments is necessary, especially for long-term exposures.

Regarding the interpretation of expected As(III) biological effectiveness in clinical trials, the situation is even more difficult as redox states in organism are variable and unpredictable, different among cell

compartments and outside the cells and influenced by many pathological states like inflammation, cancer ect.

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