

# Ascorbate ameliorates *Echis coloratus* venom-induced oxidative stress in human fibroblasts

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Received March 21, 2017; Accepted May 2, 2017

DOI: 10.3892/etm.2017.4522

**Abstract.** Reports related to the effects of *Echis coloratus* venom (EcV) on the antioxidant capacity of human tissues is very scarce. The present study was undertaken to investigate the activities and gene expression levels of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT), as well as the levels of reduced glutathione (GSH), oxidized glutathione (GSSG) and the generation rates of superoxide anions (SOA), hydrogen peroxide ( $H_2O_2$ ) and lipid peroxides (LPO) in cultured human fibroblasts incubated with EcV, ascorbate (Asc) and EcV plus Asc at concentrations and incubation periods that maintained cell viability. Results indicated that the activities of all antioxidant enzymes and their corresponding transcripts underwent highly significant decreases and downregulation in EcV-treated cultures (0.5  $\mu$ g/ml medium for 4 h) compared to venom-free controls ( $P<0.001$ ). Additionally, there were concurrent equally significant increases in SOA,  $H_2O_2$  and LPO generation rates in the venom-incubated cultures compared to controls ( $P<0.001$ ). Results also indicated very significant decreases and parallel equally significant increases in GSH and GSSG levels respectively in the envenomed cultures compared to controls ( $P<0.001$ ) leading to a drastically lower GSH/GSSG ratio. However, further incubation of the EcV-treated cultures with Asc (400  $\mu$ M for 12 h) restored the activities and levels of all investigated parameters including the expression levels of the antioxidant genes to control venom-free values. It is concluded that Asc acted to neutralize the increased reactive oxygen species generation, thus ameliorating the EcV-induced

oxidative stress and alleviating the downregulation of antioxidant genes.

## Introduction

*Echis coloratus* is a venomous viper species native to several Middle Eastern countries including Saudi Arabia and Egypt (1). Its venom induces functional alteration of many systems and organs which may lead to death. Viper venoms contain an abundance of proteins that disrupt the coagulation cascade, the normal hemostatic system and tissue repair (2). Some of the enzyme proteins include serine proteinases,  $Zn^{+2}$ -metalloproteinases L-amino acid oxidase and group II phospholipases A2 (3). Such enzymes interfere in several physiological processes, induce a variety of pharmacologic effects and cause breakdown of intracellular organelles leading to necrosis and organs dysfunction (4-6). Human envenomation by *Echis coloratus* is manifested by local swelling and necrosis, pain, respiratory failure, arrhythmia, hypotension and circulatory collapse leading to loss of renal function and hepatocellular damage (5-9).

Limited concentrations of reactive oxygen species (ROS) including superoxide anions (SOA), hydrogen peroxide ( $H_2O_2$ ), lipid peroxides (LPO) and hydroxyl radicals are generated during normal cellular oxidative metabolism. This occurs as a result of the activity of the complexes of the mitochondrial respiratory chain and other enzymes and pathways (10). Although these activities consume most of the oxygen utilised by cells, about 2% undergoes reduction and results in ROS production. Normal baseline ROS levels are essential regulators of many cellular functions. They act as messengers for the activation of specific transcription factors and mediators of signaling transduction pathways in cell growth, proliferation and apoptosis (11). However, increased cellular ROS generation causes oxidative stress (OS) which results in damage of cellular organelles, structural changes of macromolecules including lipids, proteins and DNA and alteration in gene expression of apoptosis related genes resulting in cytotoxicity and cell death (11-14). To counteract OS cells synthesise antioxidant enzymes which neutralize ROS. These include superoxide dismutase (SOD) which transforms SOA to  $H_2O_2$  which along with LPO get converted to water by glutathione peroxidase (GPx) and catalase (CAT). GPx acts to transfer the energy of peroxides to reduced glutathione (GSH) thus

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**Key words:** ascorbate, *Echis coloratus*, venom, antioxidants, oxidants, fibroblasts

forming oxidized glutathione (GSSG) which is then reduced back to GSH by glutathione reductase (GR) (15).

Besides causing many human pathologies (16), OS seems to be a major causative factor of venom-induced toxicity and has been associated with renal failure, hepatic impairment and acute pancreatitis in viper and other envenomed experimental animals and humans (6,17-20). To this end, ROS generation has been demonstrated during scorpion envenomation (21). *Echis pyramidum* venom has also been shown to cause the formation of highly reactive LPO and OS in several mouse organs (22), and to significantly lower hepatic CAT and SOD activities in rats (23). Similarly, *Echis ocellatus* envenomed mice exhibited lowered serum GPx, SOD and CAT activities (24). In another study, whereas hepatic and renal H<sub>2</sub>O<sub>2</sub>, LPO and carbonyl proteins levels were significantly increased, CAT and SOD activities underwent pronounced decreases in *Naja Haje* envenomed mice (25).

The use of large amounts of ascorbate (Asc) was shown to provide protection against oxidative damage both *in vivo* (26) and *in vitro* using cultured human fibroblasts (27). The vitamin was shown to combat arsenic-induced OS in mouse liver (28), and provided protection against both metal ion-dependent oxidation of low density lipoproteins and lipids (29), and as a hepato and cardioprotective agent after carbon tetrachloride treatment (30). The use of mega Asc doses showed that it acted as a reducing agent, an oxidizing agent, an anti-histamine, anti-toxins and anti-infective agent (31). Treatment of snake envenomation using Asc was started by Klenner by administering 4 g of the vitamin intravenously (32). However, there is a distinct lack of reports related to the effect of Asc on venom-induced oxidative injury. Only one recent study (33), reported that administration of Asc (50 mg/kg body weight) to *Bitis arietans* envenomed rats improved the elevated serum AST, ALT, creatinine and BUN levels, reduced liver peroxidation levels and increased GPx, SOD and CAT activities.

Due to the paucity of data regarding the protective role of Asc against viper envenomation, the current comprehensive study was conducted to investigate the effect of Asc in combating OS induced by *Echis coloratus* envenomation of human tissue. The activities of several antioxidant enzymes including GPx, GR, glutathione S-transferase (GST), CAT and SOD, as well as GSH levels and the corresponding oxidant generation rates including H<sub>2</sub>O<sub>2</sub>, LPO, SOA and GSSG were assayed in venom-free cultures and in cultures incubated with a sub-lethal dose of crude *Echis coloratus* venom (EcV). In addition, the gene expression levels of the investigated antioxidant enzymes were studied in EcV-treated cultures in the presence of increasing Asc concentrations and incubation periods.

## Materials and methods

*Echis coloratus* crude venom was purchased from Latoxan, (Rosans, France). Fibroblast culture reagents including Eagle's Minimum Essential Medium (MEM), Hank's Buffered Salt Solution (HBSS), fetal calf serum, trypsin, and tissue culture flasks were obtained from Flow Laboratories, Inc. (McLean, VA, USA). Analytical grade chemicals and biochemical were purchased from Sigma Chemical Co., Poole, Dorset, UK.

**Preparation of human skin fibroblast cultures.** Primary human fibroblast cultures were established from ten epidermal forearm skin biopsies (~15 mg in weight) taken from healthy adult donors (average age, 25.9±1.73 years). Acquisition of the biopsies was approved by the Ethics Committee, College of Medicine and King Khalid University Hospital, King Saud University (CMIRB-KKU-H-KSU). Fibroblasts were cultivated in MEM (20 ml) containing 10% fetal calf serum and harvested by trypsinisation. The composition as well as procedures related the preparation of culture, trypsinisation and harvesting media and cells are as detailed by us elsewhere (27). Cells were cultured in 75 cm<sup>2</sup> flasks in a Gelare BSB 4A Laminar Flow cabinet (Sydney, Australia) in an atmosphere containing 18% O<sub>2</sub>. Confluent passage 5 fibroblasts at an early stage of their proliferative lifespan were used for investigation.

**Preparation of EcV and/or Asc treated media and experimental design.** The only source of Asc in normal growth MEM is fetal calf serum which gives it a 60-100 µM concentration of the vitamin depending on the batch of serum used. Hence, a serum-free medium will be devoid of Asc. In the present study four groups of triplicate 75 cm<sup>2</sup> flasks of ten passage 5 confluent fibroblast cultures were set up for investigation. Group I were control cultures grown to confluence in normal routine MEM. Group II consisted of EcV-incubated cultures where normal MEM was removed and replaced with serum-free MEM containing an aliquot of crude EcV (dissolved in HBSS, pH 7.4) to give a final venom concentration equivalent to 0.5 µg/ml, and cells further incubated in this medium for 4 h at 37°C. Group III were Asc-incubated cultures where normal MEM was replaced with serum-free MEM containing 400 µM Asc and cells further incubated for 12 h at 37°C. Finally, group IV consisted of confluent fibroblast cultures incubated with serum-free MEM containing 0.5 µg/ml EcV for 4 h then supplemented with Asc (400 µM) and cells further incubated for 12 h at 37°C. The use of the above concentrations and incubation periods of EcV and Asc were based on data obtained and presented later in the result section. Post-incubation cell cultures of all groups were harvested by trypsinisation, resuspended in harvesting medium, thoroughly washed and centrifuged at 2,000 x g for 5 min. The pellets were kept on ice and immediately sonicated for 20 sec in 0.1 M phosphate buffer (pH 7.0, 0.5 ml) using a Fisher Sonic Dismembrator Model 150 (Thermo Fisher Scientific, Waltham, MA, USA) at 50% of the power output equivalent to 1,000 Hz frequency. Appropriate sonicate aliquots were then used for the assay of various parameters.

**Determination of the viability of EcV and Asc incubated cells.** A modified MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay based on that documented by Mosmann (34) was used to establish EcV and Asc doses and incubation periods at which fibroblasts maintain normal metabolic activity and proliferation. Triplicate passage 5 fibroblasts were grown in 96-well microplates with 8x10<sup>4</sup> cells/ml initial concentration using routine MEM. At confluence, the medium was removed and replaced with serum-free MEM (100 µl) containing increasing amounts of EcV equivalent to 0.10, 0.25, 0.50, 1.00, 1.50, 2.50 and 4.00 µg/ml and cells incubated for either 4, 12 or 24 h at 37°C.

The EcV-containing medium was removed and replaced with buffered saline (pH 7.2) containing sterilized MTT (2.4 mM, 400  $\mu$ l). After a 2-h incubation, the MTT solution was removed and formazan crystals (formed as a result of the cleavage of MTT by succinate dehydrogenase of viable cells) were solubilized using acidified isopropanol (300  $\mu$ l/well). Finally, absorbance of all samples was measured at 570 nm using an EIA plate reader (model 2550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) against a background absorbance at 690 nm. The above experiment was repeated by incubating confluent fibroblast cultures in serum-free media containing increasing Asc concentrations equivalent to 200, 300, 400 and 500  $\mu$ M for 4, 12 and 24 h. The viability of either EcV or Asc-incubated cells was then expressed as mean  $\pm$  SD percentages at each venom or vitamin concentration against venom-free controls or controls cultured in normal MEM containing  $\sim$ 100  $\mu$ M Asc, both of which were considered to have absorbance values representative of 100% viability.

*Oxidative status of cultures with respect to EcV concentration and incubation time.* In this experiment routine MEM of confluent passage 5 cultures (n=10) was replaced with serum-free media containing 0.10, 0.25, 0.50 and 1.00  $\mu$ g EcV/ml and cells further incubated with these media for 4, 12 and 24 h. Fibroblasts were then harvested, pelleted and sonicated as described earlier and protein carbonyl content (PCC) were assayed as described later. PCC was chosen to serve as a biomarker of the oxidative status of cultures at increasing EcV concentrations and incubation periods.

*Antioxidant/oxidant status of EcV-treated cells with respect to Asc concentration and incubation time.* A pilot study was run to determine the Asc concentration and incubation period required to produce maximal change in marker antioxidant enzymatic activity and oxidant generation in viable EcV-treated cell cultures. For this purpose triplicates of the ten passage 5 cultures were grown to confluence in normal MEM which was then replaced with serum-free media containing 0.5  $\mu$ g EcV/ml and cells were incubated in these media for 4 h. Asc was then added to give final concentrations equal to 200, 300, 400 and 500  $\mu$ M and cells further incubated for 4, 12 and 24 h at 37°C. Fibroblasts were then harvested, pelleted and sonicated as described earlier, and SOD activity and the corresponding SOA generation rates were assayed in appropriate aliquots of the sonicates according to methodologies presented later. SOD was chosen for this pilot study since it has cytosolic, mitochondrial and other compartmental isoforms, thus allowing for variations in intracellular Asc transport the rate of which could be affected by its concentration and incubation time. Results were compared to those obtained for the control cultures grown in normal venom-free MEM containing an approximate 100  $\mu$ M Asc concentration contributed by fetal calf serum.

*Biochemical assays.* GPx, CAT, SOD and GR specific activities as well as the generation rates of H<sub>2</sub>O<sub>2</sub>, SOA and LPO and GSH and GSSG levels were spectrophotometrically assayed using appropriate volumes of fibroblasts sonicates according to the respective methodologies previously detailed and documented by us (35,36).

GST activity was measured according to Habig *et al* (37). The assay measures total GST activity and is based on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH. Fibroblast sonicates (50  $\mu$ l) were added to potassium phosphate buffer (2 ml, pH 6.5) containing 0.1% Triton X-100, CDNB (1.0 mM) and 5.0 mM GSH and incubated at 25°C in a cuvette. The increase in absorbance at 340 nm (the rate of which is directly proportional to GST activity) was monitored for 3 min in a recording thermostated spectrophotometer (Model UV-2401 PC; Shimadzu, Dubai, United Arab Emirates).

Total protein content of fibroblast sonicates (20  $\mu$ l) was assayed according to Bradford (38).

PCC was assayed using dinitrophenylhydrazine (DNPH) according to Reznick and Packer (39) with minor modifications. Fibroblast sonicates (100  $\mu$ l) were incubated with 10 mM DNPH (0.5 ml) dissolved in 2 M HCl and blanks using 2 M HCl only (1 ml) were run in parallel. Samples were left standing in the dark for 1 h accompanied by frequently mixing. Protein hydrazone derivatives were then precipitated with 20% TCA (0.5 ml) by centrifugation (12,000  $\times$  g for 5 min at 4°C) and pellets were washed three times using ethanol: ethylacetate (1:1, 1 ml). The final pellets were then dissolved in guanidine (6 M, 1 ml), centrifuged at 12,000  $\times$  g for 15 min and PCC (n mol/mg tissue) measured spectrophotometrically at 360 nm using an absorption coefficient of  $22 \times 10^3$  M<sup>-1</sup> cm<sup>-1</sup>.

*Gene expression profiling of hsGPx, hsGR, hsGST, hsCAT and hsSOD using real-time quantitative PCR (RT-qPCR).* Freshly collected pellets were stored in RNeasy<sup>®</sup> RNA stabilization solution at -80°C and homogenized using a Tissue Lyser LT (both from Qiagen, Hilden, Germany) in 1.0 ml TRIzol<sup>®</sup> Reagent (Invitrogen, Paisley, UK) and total RNA was extracted according to standard procedures. Genomic DNA was then eliminated and cDNA synthesized from RNA (1  $\mu$ g) in a final reaction volume (20  $\mu$ l) using the QuantiTect Reverse Transcriptase kit (Qiagen). RT-qPCR was subsequently performed as described by us earlier (40) using a QuantiTect SYBR-Green PCR kit (Qiagen) with the following gene primer assays for each antioxidant gene: GPx (QT00203392), GR (QT00038325), GST (QT00063357), CAT (QT000796764) and SOD (QT01664327) in a final reaction volume (25  $\mu$ l) containing the diluted cDNA sample (5  $\mu$ l), 2X SYBR-Green PCR Master mix (12.5  $\mu$ l), each forward and reverse primer (10  $\mu$ M stock, 2.5  $\mu$ l) and RNAase-free water (2.5  $\mu$ l). The amplification program and PCR amplicon specificity were performed and assessed as previously reported (40). Each fibroblast tissue sample was represented by biological replicas and three technical replicas, with the inclusion of a no-template control. Raw data were analysed using the Rotor-Gene cycler software 2.3 to calculate the threshold cycle using the second derivative maximum. The fold-change in each gene was determined after normalization to the expression levels of 18 S as a house-keeping gene.

*Statistical analysis.* Analysis of variance followed by post hoc Tukey HSD test were performed to evaluate statistical differences between mean  $\pm$  SD values of all parameters assayed in control venom-free cultures against those incubated with different concentrations of EcV, Asc and EcV plus Asc for different periods. This was done using the SPSS version 17.0

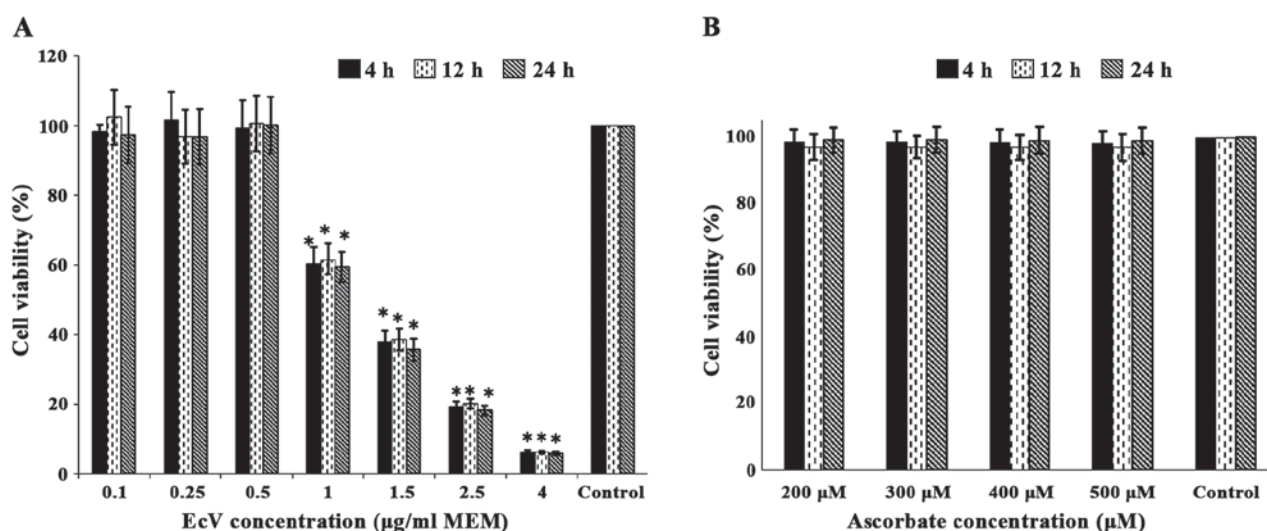


Figure 1. (A) Effect of incubation of fibroblast cultures with EcV (0.1-4.0  $\mu\text{g/ml}$  MEM) for 4, 12 and 24 h on percentage cell viability. Confluent passage 5 cultures were used. Control (venom-free) cultures were grown in routine MEM and were considered to be 100% viable. Values shown are means  $\pm$  SD of triplicate determinations for 10 cultures. \* $P < 0.001$  when percentage cell viability values for EcV-incubated cells were compared against controls. (B) Effect of incubation of fibroblast cultures with ascorbate (200-500  $\mu\text{M}$ ) for 4, 12 and 24 h on percentage cell viability. Confluent passage 5 cultures were used. Control (venom-free) cultures were grown in routine MEM containing  $\sim 100$   $\mu\text{M}$  ascorbate and were considered to be 100% viable. Values shown are means  $\pm$  SD of triplicate determinations for 10 cultures. EcV, *Echis coloratus* venom; MEM, Eagle's Minimum Essential Medium.

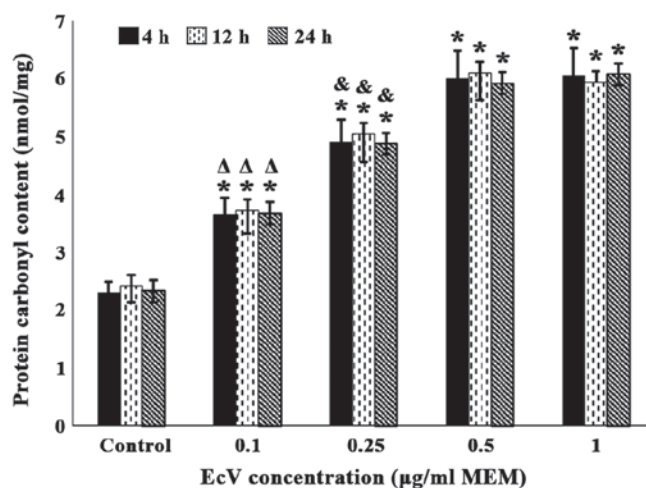


Figure 2. Effect of incubation of fibroblast cultures with EcV (0.1-1.0  $\mu\text{g/ml}$ ) for 4, 12 and 24 h on protein carbonyl content. Confluent passage 5 cultures were used. Control (venom-free) cultures were grown in routine MEM. Values shown are means  $\pm$  SD of triplicate determinations for 10 cultures. \* $P < 0.001$  when protein carbonyl content values for all EcV-incubated cells were compared against controls.  $\Delta P < 0.001$  when protein carbonyl content values for cells incubated with 0.10  $\mu\text{g/ml}$  EcV were compared to those incubated with 0.25  $\mu\text{g/ml}$  EcV at all incubation periods.  $\& P < 0.001$  when protein carbonyl content values for cells incubated with 0.25  $\mu\text{g/ml}$  EcV were compared to those incubated with 0.50 and 1.00  $\mu\text{g/ml}$  EcV at all incubation periods. EcV, *Echis coloratus* venom.

software (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to be statistically significant.

## Results

*Effect of increasing concentrations and incubation periods of EcV and Asc on viability of the cultured cells.* Results presented in Fig. 1A indicated that fibroblast cultures grown in normal MEM and incubated with increasing EcV concentrations of

0.10, 0.25 and 0.50  $\mu\text{g/ml}$  MEM for 4, 12 and 24 h did not cause significant loss of cell viability compared to venom-free controls. As an example percentage cell viabilities equaled  $98.3 \pm 4.12$ ,  $101.6 \pm 4.34$  and  $99.4 \pm 4.16\%$  in cultures incubated with 0.10, 0.25 and 0.50  $\mu\text{g/ml}$  for 4 h, respectively, against 100% assigned to venom-free controls. Moreover, very similar values were obtained for cultures incubated with the same venom concentrations for 12 and 24 h. Other Fig. 1A data however, revealed that incubation of cultures with 1.0, 1.5, 2.5 and 4.0  $\mu\text{g}$  EcV/ml MEM for 4, 12 and 24 h resulted in very significant and progressive losses of cell viability proportional to venom concentration and very similar in magnitude regardless of the incubation period. As an example percentage cell viabilities equaled  $60.3 \pm 2.41$ ,  $37.8 \pm 1.48$ ,  $19.3 \pm 0.77$  and  $6.14 \pm 0.23\%$  in cultures incubated with 1.0, 1.5, 2.5 and 4.0  $\mu\text{g}$  EcV/ml, respectively, against 100% assigned for venom-free controls ( $P < 0.001$  for all comparisons). In light of Fig. 1A results, cultures were incubated with 0.5  $\mu\text{g}$  EcV/ml MEM for 4 h prior to investigation of the oxidative status of cells.

In contrast, Fig. 1B data show that incubation of fibroblast cultures with increasing Asc concentrations equivalent to 200, 300, 400 and 500  $\mu\text{M}$  (chosen to approximately represent double, triple, quadruple and quintuple human plasma levels), did not result in any significant loss of cell viability when compared to control cultures cultivated in routine MEM approximately containing 100  $\mu\text{M}$  Asc. In addition, cell viabilities were very similar in magnitude regardless of whether the incubation was performed for 4, 12 or 24 h.

*Effect of increasing EcV concentrations and incubation time on PCC of fibroblast cultures.* As illustrated in Fig. 2, incubation of venom-free control cultures with increasing EcV concentrations (0.10-1.00  $\mu\text{g/ml}$  MEM) for 4, 12 and 24 h resulted in very significant progressive increases in PCC that were dose-dependent. After incubation for 4 h, values equaled

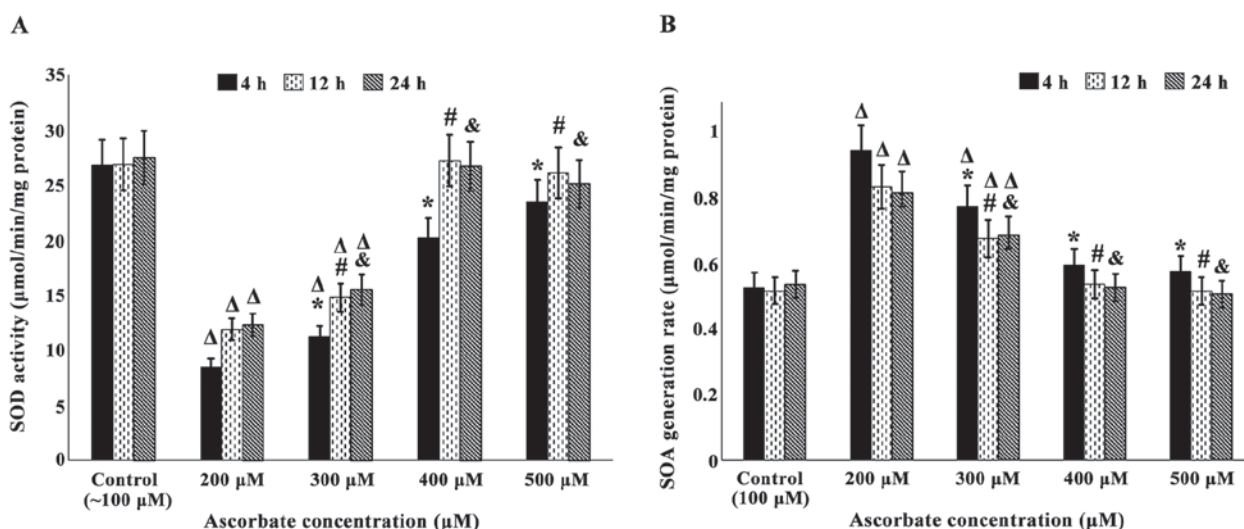


Figure 3. (A) Effect of incubation of EcV treated fibroblast cultures (0.5  $\mu\text{g/ml}$  MEM for 4 h) with ascorbate (200-500  $\mu\text{M}$ ) for 4, 12 and 24 h on SOD activity. Confluent passage 5 cultures were used. Control cultures (venom-free) were grown in routine MEM. Values shown are means  $\pm$  SD of triplicate determinations of 10 cultures.  $^{*}, \#$   $P < 0.001$  when comparing SOD activities in EcV-treated cultures supplemented with 300, 400 and 500  $\mu\text{M}$  ascorbate for 4 h ( $^{*}$ ), 12 h ( $^{\#}$ ) and 24 h ( $\&$ ), against those supplemented with 200  $\mu\text{M}$  ascorbate.  $^{\Delta} P < 0.001$  when SOD activities in EcV-treated cultures supplemented with 200 and 300  $\mu\text{M}$  ascorbate were compared to either those supplemented with 400 and 500  $\mu\text{M}$  ascorbate, or with venom-free controls at each incubation period. (B) Effect of incubation of EcV-treated cultures (0.5  $\mu\text{g/ml}$  MEM for 4 h) with ascorbate (200-500  $\mu\text{M}$ ) for 4, 12 and 24 h on SOA generation rates. Confluent passage 5 cultures were used. Controls (venom-free) were grown in routine MEM. Values shown are means  $\pm$  SD of triplicate determinations of 10 cultures.  $^{*}, \#$   $P < 0.001$  when comparing SOA generation rates in EcV-treated cultures supplemented with 300, 400 and 500  $\mu\text{M}$  ascorbate for 4 h ( $^{*}$ ), 12 h ( $^{\#}$ ) and 24 h ( $\&$ ), against those supplemented with 200  $\mu\text{M}$  ascorbate.  $^{\Delta} P < 0.001$  when SOA generation rates in EcV-treated cultures supplemented with 200 and 300  $\mu\text{M}$  ascorbate were compared to either those supplemented with 400 and 500  $\mu\text{M}$  ascorbate, or with venom-free controls at each incubation period. EcV, *Echis coloratus* venom; MEM, Eagle's Minimum Essential Medium; SOD, superoxide dismutase; SOA, superoxide anions.

3.65 $\pm$ 0.29, 4.91 $\pm$ 0.39, 6.01 $\pm$ 0.48 and 6.05 $\pm$ 0.48 nmol/mg tissue at 0.10, 0.25, 0.50 and 1.00  $\mu\text{g}$  EcV/ml MEM, respectively, against 2.31 $\pm$ 0.18 nmol/mg tissue recorded for venom-free controls ( $P < 0.001$  for all comparisons). As also evident from Fig. 2, such PCC values were very similar in magnitude regardless of whether the incubation period with EcV was performed for 4, 12 or 24 h. Furthermore, PCC values in cultures incubated with 0.50 and 1.00  $\mu\text{g}$  EcV/ml MEM reached maximal levels and were very similar in value regardless of the incubation period.

In light of the above results and those related to the effect of increasing EcV concentrations and incubation time on cell viability (presented in Fig. 1A), cultures were incubated with 0.50  $\mu\text{g}$  EcV/ml MEM for 4 h prior to investigation of the antioxidant/oxidant status of cells. Under such conditions envenomed cells are metabolically viable and proliferate normally but are being subjected to OS.

**Effect of increasing Asc concentrations and incubation time of EcV-treated cultures on SOD and SOA as markers of OS.** As evident from Fig. 3A data, incubation of EcV-treated cultures with increasing Asc concentrations for 4 h resulted in very significant progressive SOD activity increases that were dose dependent (11.2 $\pm$ 1.01, 20.2 $\pm$ 1.71 and 23.5 $\pm$ 1.97  $\mu\text{mol/min/mg}$  protein at 300, 400 and 500  $\mu\text{M}$  Asc, respectively, against 8.51 $\pm$ 0.74  $\mu\text{mol/min/mg}$  protein recorded in EcV-treated cultures incubated with 200  $\mu\text{M}$  Asc;  $P < 0.001$  for all comparisons). However, such increased values were very significantly lower compared to that obtained in venom-free controls (26.8 $\pm$ 2.28  $\mu\text{mol/min/mg}$  protein;  $P < 0.001$  for 200-400  $\mu\text{M}$  Asc and  $P < 0.05$  for 500  $\mu\text{M}$  Asc). In addition, Fig. 3A data

revealed that very significant higher enzyme activity increases were obtained when EcV-treated cultures were incubated with the same Asc concentrations for 12 h (14.8 $\pm$ 1.26, 27.2 $\pm$ 2.32 and 26.1 $\pm$ 2.31  $\mu\text{mol/min/mg}$  protein at 300, 400 and 500  $\mu\text{M}$  Asc, respectively, against 11.9 $\pm$ 0.99  $\mu\text{mol/min/mg}$  protein recorded in cultures incubated with 200  $\mu\text{M}$  Asc;  $P < 0.001$  for all comparisons). Furthermore, although SOD activities in EcV-treated cultures incubated with 200 and 300  $\mu\text{M}$  Asc were still significantly lower than that obtained for venom-free controls (11.9 $\pm$ 0.99 and 14.8 $\pm$ 1.26, respectively, against 26.9 $\pm$ 2.36  $\mu\text{mol/min/mg}$  protein;  $P < 0.001$  for both comparisons), the enzyme activities in venom-treated cultures incubated with 400 and 500  $\mu\text{M}$  Asc were of very similar magnitude and not statistically different when compared to that of venom-free controls (27.2 $\pm$ 2.32 and 26.1 $\pm$ 2.31, respectively, against 26.9 $\pm$ 2.36  $\mu\text{mol/min/mg}$  protein). Fig. 3A data also indicated a very similar pattern and magnitude of SOD activity increases when EcV-treated cultures were incubated with the same Asc concentrations for 24 h compared to those incubated for 12 h (15.5 $\pm$ 1.36, 26.7 $\pm$ 2.22 and 25.1 $\pm$ 2.13  $\mu\text{mol/min/mg}$  protein at 300, 400 and 500  $\mu\text{M}$  Asc, respectively, against 12.3 $\pm$ 1.02  $\mu\text{mol/min/mg}$  protein obtained for venom-treated cultures incubated with 200  $\mu\text{M}$  Asc;  $P < 0.001$  for all comparisons). Moreover, the above enzyme activities at 400 and 500  $\mu\text{M}$  Asc were of very similar magnitude with that obtained in venom-free cultures (27.5 $\pm$ 2.39  $\mu\text{mol/min/mg}$  protein).

Concurrently, Fig. 3B demonstrates that incubation of EcV-treated cultures with increasing Asc concentrations for 4 h resulted in very significant dose-dependent gradual reductions in SOA generation. Rates equaled 0.78 $\pm$ 0.064, 0.6 $\pm$ 0.049 and 0.58 $\pm$ 0.047  $\mu\text{mol/min/mg}$  protein at 300, 400 and 500  $\mu\text{M}$

Table I. Effect of incubation of fibroblast cultures with EcV, Asc and EcV plus Asc on antioxidant enzyme activities.

Sample groups/ incubation time (n=10)	Enzymatic parameters				
	GPx	CAT	SOD	GR	GST
Group I					
Control cultures	1.88±0.15	3.75±0.31	25.2±2.21	2.70±0.25	92.8±7.88
Group II					
Cultures + EcV (4 h)	1.01±0.10 <sup>a</sup>	2.08±0.19 <sup>a</sup>	12.8±1.14 <sup>a</sup>	1.48±0.13 <sup>a</sup>	44.6±3.81 <sup>a</sup>
Group III					
Cultures + Asc (12 h)	1.97±0.17	3.80±0.30	23.5±2.12	2.68±0.24	95.0±7.94
Group IV					
Cultures + EcV (4 h) + Asc (12 h)	1.95±0.16	3.62±0.29	23.7±2.22	2.76±0.27	84.9±6.21 <sup>b</sup>

The values for GPx, CAT, SOD and GR are presented in  $\mu\text{mol}/\text{min}/\text{mg}$  protein and the values for GST are presented in  $\text{nmol}/\text{min}/\text{mg}$  protein. Concentrations of EcV and Asc are indicated in the text. Confluent passage 5 cultures were used. The values shown are means  $\pm$  SD for triplicate determinations of the 10 cultures. <sup>a</sup> $P<0.001$  when values obtained in EcV-incubated cultures were compared to those in venom-free controls. <sup>b</sup> $P<0.05$  when GST activity obtained for cultures incubated with EcV + Asc was compared to that in venom-free control. EcV, *Echis coloratus* venom; Asc, ascorbate; GPx, glutathione peroxidase; CAT, catalase; SOD, superoxide dismutase; GR, glutathione reductase; GST, glutathione S-transferase.

Asc, respectively, against  $0.95\pm0.079$   $\mu\text{mol}/\text{min}/\text{mg}$  protein recorded in EcV-treated cultures incubated with  $200$   $\mu\text{M}$  Asc ( $P<0.001$  for all comparisons). However, such lower rates were still significantly higher than that recorded for SOA generation in venom-free controls ( $0.53\pm0.045$   $\mu\text{mol}/\text{min}/\text{mg}$  protein;  $P<0.001$  for  $200$  and  $300$   $\mu\text{M}$  Asc,  $P<0.01$  for  $400$   $\mu\text{M}$  Asc and  $P<0.05$  for  $500$   $\mu\text{M}$  Asc). Data also showed that lower magnitude SOA rate reductions were obtained when EcV-treated cultures were incubated with the same Asc concentrations for  $12$  h ( $0.68\pm0.057$ ,  $0.54\pm0.044$  and  $0.52\pm0.042$   $\mu\text{mol}/\text{min}/\text{mg}$  protein at  $300$ ,  $400$  and  $500$   $\mu\text{M}$  Asc respectively against  $0.84\pm0.067$   $\mu\text{mol}/\text{min}/\text{mg}$  protein recorded in cultures incubated with  $200$   $\mu\text{M}$  Asc;  $P<0.001$  for all comparisons). Although the rates in envenomed cultures incubated with  $200$  and  $300$   $\mu\text{M}$  Asc were still very significantly higher than that obtained for venom-free controls ( $0.84\pm0.067$  and  $0.68\pm0.057$   $\mu\text{mol}/\text{min}/\text{mg}$  protein, respectively, against  $0.52\pm0.041$   $\mu\text{mol}/\text{min}/\text{mg}$  protein;  $P<0.001$  for both comparisons), those in cultures incubated with  $400$  and  $500$   $\mu\text{M}$  Asc were of very similar magnitude and not significantly different from the rate in venom-free controls ( $0.54\pm0.044$  and  $0.52\pm0.042$   $\mu\text{mol}/\text{min}/\text{mg}$  protein, respectively, against  $0.52\pm0.041$   $\mu\text{mol}/\text{min}/\text{mg}$  protein). Additionally Fig. 3B data indicate a very similar pattern as well as magnitude of SOA generation rate reductions when EcV-treated cultures were incubated with the same Asc concentrations for  $24$  h rather than  $12$  h ( $0.69\pm0.056$ ,  $0.53\pm0.042$  and  $0.51\pm0.041$   $\mu\text{mol}/\text{min}/\text{mg}$  protein at  $300$ ,  $400$  and  $500$   $\mu\text{M}$  Asc, respectively, against  $0.82\pm0.065$   $\mu\text{mol}/\text{min}/\text{mg}$  protein recorded in cultures incubated with  $200$   $\mu\text{M}$  Asc;  $P<0.001$  for all comparisons). Moreover, the above-mentioned rates at  $400$  and  $500$   $\mu\text{M}$  Asc were of very similar magnitude to that obtained in venom-free controls ( $0.54\pm0.043$   $\mu\text{mol}/\text{min}/\text{mg}$  protein).

Thus, Fig. 3 results indicate that a  $400$  or  $500$   $\mu\text{M}$  Asc concentration and an incubation period of either  $12$  or  $24$  h were required to achieve maximal restoration of SOD

activities and SOA generation rates in envenomed cultures to values similar to those recorded in venom-free controls. Hence, in all subsequent experiments investigating the effect of Asc on the activities and levels of antioxidants and pro-oxidants, EcV-treated cultures were incubated with  $400$   $\mu\text{M}$  Asc for  $12$  h.

*Effect of incubation of cultures with EcV, Asc and EcV plus Asc on antioxidant enzyme activities.* Table I data clearly indicate that incubation of control fibroblast cultures with EcV ( $0.5$   $\mu\text{g}/\text{ml}$  MEM for  $4$  h) resulted in highly significant reductions in the activities of all investigated antioxidant enzymes. All enzymes underwent activity percentage reductions of similar magnitude when compared to activities recorded in venom-free control cultures ( $P<0.001$  for all comparisons). Percentage reductions equaled  $47.3\pm4.18$ ,  $44.6\pm4.09$ ,  $49.1\pm4.34$ ,  $44.2\pm4.18$  and  $52.3\pm4.46\%$  of control activities for GPx, CAT, SOD, GR and GST, respectively. In contrast, incubation of control cultures in serum-free MEM supplemented with  $400$   $\mu\text{M}$  for  $12$  h did not cause any significant changes in the activities of any of the studied enzymes. Also, Table I data show that incubation of the EcV-treated cultures with serum-free MEM containing  $400$   $\mu\text{M}$  Asc for  $12$  h resulted in the restoration of GPx, CAT, SOD and GR activities to values very similar and not significantly different from those recorded for venom-free controls. However, GST activity was partially restored to levels significantly lower compared to those documented for venom-free controls ( $84.9\pm6.12$   $\text{nmol}/\text{min}/\text{mg}$  protein against  $92.8\pm7.88$   $\text{nmol}/\text{min}/\text{mg}$  protein;  $P<0.05$ ).

*Effect of incubation of cultures with EcV, Asc and EcV plus Asc on oxidant generation.* Table II data demonstrate that SOA,  $\text{H}_2\text{O}_2$  and LPO generation rates in EcV-incubated cultures ( $0.5$   $\mu\text{g}/\text{ml}$  for  $4$  h) underwent very significant increases compared to those obtained for venom-free controls ( $0.92\pm0.09$  against  $0.58\pm0.06$   $\mu\text{mol}/\text{min}/\text{mg}$  protein for SOA,

Table II. Effect of incubation of fibroblast cultures with EcV, Asc and EcV plus Asc on oxidant generation rates.

Samples groups/ incubation time (n=10)	Oxidant generation rates		
	SOA	H <sub>2</sub> O <sub>2</sub>	LPO
Group I Control cultures	0.58±0.06	1.69±0.17	35.1±3.34
Group II Cultures + EcV (4 h)	0.92±0.09 <sup>a</sup>	2.59±0.25 <sup>a</sup>	51.2±4.56 <sup>a</sup>
Group III Control + Asc (12 h)	0.56±0.06	1.67±0.15	35.8±.26
Group IV Cultures + EcV (4 h) + Asc (12 h)	0.53±0.06	1.64±0.15	34.7±3.29

The values for SOA are presented in  $\mu\text{mole}/\text{min}/\text{mg}$  protein and the values for H<sub>2</sub>O<sub>2</sub> and LPO are presented in  $\text{pmole}/\text{min}/\text{mg}$  protein. Concentrations of EcV and Asc are indicated in the text. Confluent passage 5 cultures were used. The values shown are means  $\pm$  SD for triplicate determinations of the 10 cultures. <sup>a</sup>P<0.001 when values obtained in EcV-incubated cultures were compared to those in venom-free controls. EcV, *Echis coloratus* crude venom; Asc, ascorbate; SOA, superoxide anions; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; LPO, lipid peroxides.

Table III. Effect of incubation of fibroblast cultures with EcV, Asc and EcV plus Asc on GSH and GSSG levels.

Samples groups/ incubation time (n=12)			
	GSH	GSSG	GSH/GSSG
Group I Control cultures	48.5±3.95	0.79±0.068	60.7±4.97
Group II Cultures + EcV (4 h)	32.1±2.24 <sup>a</sup>	1.11±0.092 <sup>a</sup>	28.1±2.15 <sup>a</sup>
Group III Control + Asc (12 h)	46.7±3.74	0.82±0.071	57.3±4.57
Group IV Cultures + EcV (4 h) + Asc (12 h)	46.9±3.67	0.76±0.052	59.2±4.90

The values for GSH, GSSG and GSH/GSSG are presented in  $\text{nmol}/\text{mg}$  protein. Concentrations of EcV and Asc are indicated in the text. Confluent passage 5 cultures were used. Controls cultures were venom-free and grown in routine MEM. The values shown are means  $\pm$  SD for triplicates of the 10 cultures. <sup>a</sup>P<0.001 when values obtained for EcV-incubated cultures were compared to those in venom-free controls. EcV, *Echis coloratus* crude venom; Asc, ascorbate; GSH, reduced glutathione; GSSG, oxidized glutathione; MEM, Eagle's Minimum Essential Medium.

2.59±0.25 against 1.69±0.17  $\text{pmol}/\text{min}/\text{mg}$  protein for H<sub>2</sub>O<sub>2</sub> and 51.2±4.56 against 35.1±3.34  $\text{pmol}/\text{min}/\text{mg}$  protein for LPO; P<0.001 for all comparisons). Such increases amounted to 37.4±3.69, 52.2±5.44 and 44.7±4.56% of control levels for SOA, H<sub>2</sub>O<sub>2</sub> and LPO, respectively. In contrast, incubation of venom-free control cultures with serum-free MEM containing 400  $\mu\text{M}$  Asc for 12 h did not significantly alter the generation rates of any of the studied oxidants. Table II data also show that incubation of the EcV-treated cultures with serum-free MEM containing 400  $\mu\text{M}$  Asc for 12 h, caused very significant decline in all oxidant generation rates to values very similar and not statistically different from those recorded for venom-free cultures (0.53±0.06  $\mu\text{mol}/\text{min}/\text{mg}$  protein for SOA and 1.64±0.15 and 34.7±3.29  $\text{pmol}/\text{min}/\text{mg}$  protein for H<sub>2</sub>O<sub>2</sub> and LPO, respectively).

*Effect of incubation of cultures with EcV, Asc and EcV plus Asc on GSH and GSSG levels.* Results presented in Table III indicated that cellular GSH levels in EcV-treated cultures significantly declined by 34.4±0.29%, and those of GSSG significantly increased by 40.1±0.33% of levels recorded in venom-free control cultures. Levels equaled 32.1±2.24 and 1.11±0.092  $\text{nmol}/\text{mg}$  protein against 48.5±3.95 and 0.79±0.068  $\text{nmol}/\text{mg}$  protein for GSH and GSSG, respectively (P<0.001 for both comparisons). Consequently the GSH/GSSG ratio was significantly decreased in the EcV-treated cultures compared to controls (28.9±2.15 against 60.7±4.97). However, these levels were restored upon incubation of the envenomed cultures with serum-free MEM containing 400  $\mu\text{M}$  Asc for 12 h reaching very similar values to those recorded in venom-free controls (46.9±3.67 against 48.5±3.95  $\text{nmol}/\text{mg}$  protein for

GSH and  $0.76 \pm 0.052$  against  $0.79 \pm 0.068$  nmol/mg protein for GSSG). This resulted in restoration of the GSH/GSSG ratio to a value similar to that obtained for venom-free controls ( $59.2 \pm 4.90$  against  $60.7 \pm 4.97$ ).

*Effect of incubation of cultures with EcV, Asc and EcV plus Asc on relative gene expression of antioxidant enzymes.* As evident from Fig. 4, fibroblast *hsGPx*, *hsGR*, *hsGST*, *hsCAT* and *hsSOD* gene expression levels were very significantly downregulated by  $52.2 \pm 4.18$ ,  $45.9 \pm 3.62$ ,  $59.8 \pm 4.84$ ,  $52.4 \pm 4.35$  and  $53.0 \pm 4.38\%$  of control levels, respectively ( $P < 0.001$  upon comparison of the fold-change in the gene expression levels of all enzymes in EcV-treated cells relative to venom-free controls). However, gene expression levels of all enzymes except GST were restored to values similar and not significantly different from those of control cultures when the venom-treated cultures were incubated with Asc ( $400 \mu\text{M}$  for 12 h). For GST, the fold-changes in envenomed cultures were moderately but significantly lower than those recorded for controls ( $0.99 \pm 0.086$  against  $1.10 \pm 0.090$ ;  $P < 0.01$ ).

## Discussion

Human fibroblast cultures have been previously extensively used by us for the study of metabolic changes related to different pathologic conditions including incubation of cells with snake venom proteins (41-43). The *in vitro* maintained human tissue model system provided in the present study is an appropriate experimental tool for the investigation of the effect of different concentrations and incubation periods of EcV, Asc and EcV plus Asc on the antioxidant/oxidant status of envenomed fibroblasts. To this end, it was essential to choose an EcV concentration and incubation period that would minimize kinetic errors without affecting the proliferative and metabolic viability of the cells. Thus, any observed changes in the oxidative status of cells can be attributed to the activity of the venom. Fig. 1A data suggest that incubating cultures with EcV concentrations up to  $0.5 \mu\text{g/ml}$  MEM for 4, 12 and 24 h maintained control cellular viability. However, the use of 1.0, 1.5, 2.5 and  $4.0 \mu\text{g/ml}$  for the same incubation periods caused progressive loss of viability in a dose-dependent fashion regardless of the incubation time. Results also showed that incubation of cultures with EcV concentrations at 0.10, 0.25, 0.50 and  $1.00 \mu\text{g/ml}$  MEM caused significant progressive increases in the PCC of cells which peaked at 0.50 and  $1.00 \mu\text{g}$  of the venom (Fig. 2). Furthermore, the magnitude of such increases were very similar regardless of whether the incubation was performed for 4, 12 or 24 h. In light of the above results, it was decided that in all subsequent experiments, cell cultures will be incubated with EcV ( $0.50 \mu\text{g/ml}$  MEM for 4 h) prior to harvesting for investigation. This ensured that although cells at such venom concentrations were metabolically and proliferatively viable, they were being subjected to OS.

It was also important to choose an Asc concentration and incubation time that would not affect the proliferative and metabolic viability of cells. To this end, Fig. 1B data show that incubation of cultures with Asc ( $200$ - $500 \mu\text{M}$ ) for 4, 12 and 24 h did not cause any significant changes in cell viability regardless of the incubation period. However, incubation of

EcV-treated oxidatively-stressed cultures with the same Asc increasing concentrations and incubation times caused progressive statistically very significant increases in SOD activity that was chosen as a marker antioxidant (Fig. 3A). Such increases were dose-dependent and reached very similar peak values in envenomed cultures incubated with  $400$  and  $500 \mu\text{M}$  Asc regardless of whether the incubation was performed for 12 or 24 h. Furthermore, incubation of the EcV-treated cultures with the vitamin at the same concentrations and incubation periods caused progressive decreases in the levels of SOA chosen as a marker oxidant (Fig. 3B). Such decreases were also shown to reach similar lowest levels when the oxidatively stressed cells were incubated with  $400$  and  $500 \mu\text{M}$  Asc for 12 and 24 h. Hence, in all subsequent experiments that investigated the activities and levels of a variety of antioxidants and oxidants, EcV-treated cultures were incubated with  $400 \mu\text{M}$  Asc for 12 h thus minimizing kinetic errors.

Throughout the present study passage 5 cultures were used since we previously showed that fibroblasts beyond passages 10 and 15 enter an early phase of senescence causing many metabolic changes including lowered rates of growth and replication, protein synthesis and changes in the activities of many key and antioxidant enzymes as well as alterations in cellular morphology (27,36,44,45). Other optimal culture conditions were also provided to ensure maximal rates of fibroblast growth, multiplication and metabolism. These included the use of sufficient MEM volumes, addition of Hepes buffer to both culture and trypsinisation media and streptomycin and penicillin to prevent contamination.

As illustrated in Table I, incubation of cultures with EcV resulted in highly significant decreases of similar magnitude in the activities of GPx, GR, GST, SOD and CAT compared to those documented for control cultures. These findings are in broad agreement with previous studies which reported that *Echis pyramidum*, *Echis ocellatus* and *Naja Haje* envenomation of rats and mice caused significant decreases of hepatic and renal GPx, CAT and SOD activities (22-25). In the present study all enzyme activities were expressed in terms of cellular protein, however the ratios of protein/DNA for the 10 cultures were similar in value regardless of EcV incubation (mean =  $14.6 \pm 1.35 \mu\text{g protein}/\mu\text{g DNA}$ ). Furthermore, incubation of cultures with  $0.5 \mu\text{g/ml}$  EcV for 4 h did not significantly change the protein yield ( $671 \pm 41.1 \mu\text{g}/75 \text{ cm}^2$  flask of cells) indicating no proteolytic activity of the venom at the above concentration and incubation time. Although proteolytic activity has been reported for some venoms, none was detected by us for *Echis coloratus* purified fractions (41). The absence of proteolytic activity, however, could have been a result of protease inhibitors contributed by the fetal calf serum component of MEM. Furthermore, the possibility of cell membrane rupture that could have resulted from the venom's phospholipase A2 activity is ruled out since no antioxidant enzyme activity was detected in EcV or MEM prior to or post-incubation of cells with the venom. In contrast, incubation of cultures with EcV ( $>8 \mu\text{g/ml}$ ) resulted in rounding and lysis of cells. Our previous study (41) also showed that incubation of fibroblast sonicates with EcV ( $0.5 \mu\text{g/ml}$  MEM for 3 h) did not cause any significant changes in the activities of key cytosolic and mitochondrial enzymes. This finding coupled with the fact all presently investigated antioxidant

enzyme activities underwent reductions of similar magnitude (44-52% of control activities), suggest that EcV executes its effect at the cellular level rather than directly at the protein enzyme molecules. Several of our previous studies reported similar findings where TCA cycle enzyme activities reduced by 50-60% (44), and phosphofructokinase and citrate synthase activities by 60-62% (41) of control values upon incubation of fibroblast cultures with *Walterinnesia aegyptia* and EcVs, respectively. Furthermore, these effects were venom dose-dependent and exhibited saturation kinetics. In the present study Figs. 1A and 2 data show that loss of cell viability and the increased levels of PCC were also proportional to EcV concentrations and reached maximal values at 0.50-1.00  $\mu$ g EcV/ml MEM. These findings further indicate that venom proteins execute their effect at the cellular level possibly via cellular or mitochondrial receptors, the mechanism of which needs elucidation.

Concurrent with the above antioxidant enzyme activity reductions, EcV-treated cultures exhibited very significant increases in the generation rates of  $H_2O_2$ , LPO and SOA (Table II) which were similar in magnitude and ranged from 37-52% of control levels. In addition, although GSH levels were significantly decreased, GSSG levels underwent significant increases leading to a drastically lowered GSH/GSSG ratio equivalent to about 51% of the value recorded for control cultures (Table III). GSH is an important antioxidant for the maintenance of homeostasis and redox balance as well as the prevention of lipid peroxidation (46). The significant decrease presently noted in GR activity of EcV-treated cells (Table I), could have resulted in the lowered GSH levels. Alternatively, the GSH decline could have been a result of GSH reacting directly with excessively generated  $H_2O_2$  leading to increased GSSG formation. These findings indicated that the venom-treated cells were subjected to OS which is in broad agreement with results reported by other workers (21-25). However, such studies only investigated a few parameters of the antioxidant/oxidant status of envenomed animals and were not related to EcV. In comparison, the present study examined the effect of EcV on a comprehensive list of antioxidants and their corresponding oxidants using human tissue. Furthermore, results showed that the antioxidant capacity decreases and those of the corresponding oxidant generation increases were of similar magnitude. Other results unique to the present study demonstrated that the expression levels of all investigated antioxidant genes in EcV-treated cultures underwent significant downregulations of similar magnitude ranging from 46-60% of the levels recorded in control cultures (Fig. 4). Such downregulation was also in a similar range of the corresponding reductions seen in antioxidant enzyme activities. These results further suggest that EcV executes its effect at the cellular and compartmental levels. The lowered gene expression levels in the EcV-treated cells must have caused the subsequent reduction in antioxidant enzyme activities, and were probably a result of DNA damage incurred by the increased ROS generation leading to downregulation of transcription and translation processes. To this end, SOA have been reported to activate key cellular hallmark events including DNA damage and mitochondrial alterations (14) thus triggering apoptosis. Moreover, SOD loss has been shown to induce phosphorylation of a DNA damage marker ( $\gamma$ -H2AX), and upregulation of p21, a target

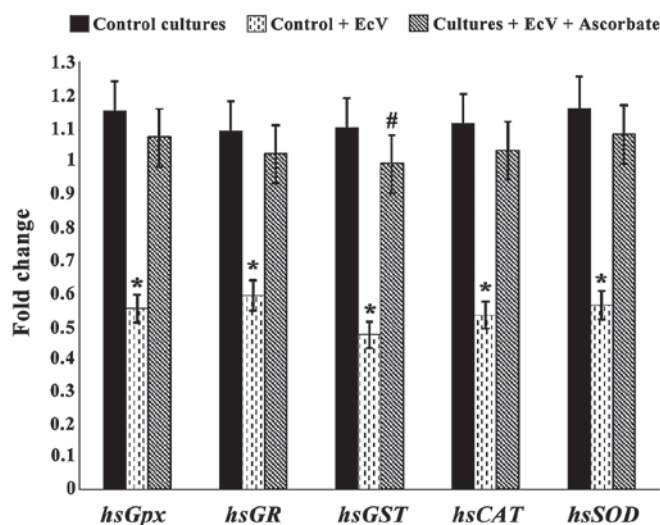


Figure 4. Relative gene expression of *hsGpx*, *hsGR*, *hsGST*, *hsCAT* and *hsSOD* in control, EcV-treated and EcV-treated plus ascorbate fibroblasts. Confluent passage 5 cultures were used. Control (venom-free) cultures were grown in routine MEM. Concentrations of EcV and ascorbate are indicated in the text. Fold-change values are means  $\pm$  SD for triplicates of the 10 cultures. \* $P < 0.001$  when comparing fold-change in the gene expression levels of each enzyme in EcV-treated fibroblasts relative to venom-free controls. # $P < 0.01$  upon fold-change comparison in the gene expression level of GST in EcV plus ascorbate-treated fibroblasts relative to controls. EcV, *Echis coloratus* crude venom; *hsGpx*, *hsGR*, *hsGST*, *hsCAT* and *hsSOD*: *Homo sapiens* glutathione peroxidase, glutathione reductase, glutathione S-transferase, catalase and superoxide dismutase respectively; MEM, Eagle's Minimum Essential Medium.

gene of p53 in fibroblasts (47). The demonstrated increased  $H_2O_2$  generation could have also interacted with SOA thus producing the more reactive hydroxyl radicals (48) known to react with purines and pyrimidines causing DNA damage and lowered antioxidant gene expression.

The antioxidant property of vitamin C stems from its reducing and electron donating ability. It donates two electrons from a double bond between the second and third carbon atoms of its molecule. The donated electrons are received by compounds with unpaired electrons like ROS, which thus get non-enzymatically neutralized (49). When vitamin C loses one electron it becomes oxidized to Asc which is relatively stable and fairly unreactive making it a potent free radical scavenger (50). Asc is not synthesized by human cells including fibroblasts (50), and is taken up by  $NA^+$ -dependent protein transporters hSVCT1 and hSVCT2 which are products of separate genes (51). Although Asc plasma concentration is 60-100  $\mu$ M, its intracellular levels are several orders of magnitude higher indicating that it is normally concentrated and accumulated in cellular compartments by the transporter proteins (52). Results of the present study (Tables I-III) demonstrated very significant restoration of the activities and levels of all the investigated antioxidants and oxidants to values very similar to those recorded in control cultures when the EcV-treated cultures were incubated with 400  $\mu$ M Asc for 12 h, suggesting that Asc ameliorates the venom-induced OS. Similar findings are scarce and have been reported in only one study where administration of Asc (50 mg/kg body weight) to *Bitis arietans* envenomed rats increased GPx, SOD and CAT activities, and reduced liver peroxidation levels (32,33). Unique

to the present study is that the noted downregulation of the investigated antioxidant gene expression levels in the envenomed cultures were restored to fold-change levels similar to those recorded for venom-free controls when the former were incubated with 400  $\mu$ M Asc for 12 h (Fig. 4). The percentage upregulation of the antioxidant gene expression levels incurred by Asc approximately equaled 93, 73, 110, 94 and 93% of control levels for GPx, GR, GST, CAT and SOD, respectively, and correlated well with the recorded corresponding increases in the enzyme activities which equaled 91, 75, 86, 87 and 90% respectively.

In light of the present study findings, it is concluded that incubation of EcV-treated cultures with high Asc concentrations (400 or 500  $\mu$ M) acted to scavenge ROS thus preventing OS and helped to alleviate DNA damage and the downregulation of antioxidant gene expression levels. Asc could have also acted to alleviate ROS-related DNA damage possibly causing downregulation of the expression levels of genes responsible for the synthesis of the hsSVCT1 and hsSVCT2 transporter proteins.

### Acknowledgements

The present study was financially supported by King Saud University, Vice Deanship of Research Chairs.

### References

- Mallow D, Ludwig D and Nilson G (eds): True Vipers: Natural History and Toxinology of Old World Vipers. Kreiger Publication Company, Malabar, FL, 2003.
- Serrano SM, Shannon JD, Wang D, Camargo AC and Fox JW: A multifaceted analysis of viperid snake venoms by two-dimensional gel electrophoresis: an approach to understanding venom proteomics. *Proteomics* 5: 501-510, 2005.
- Mackessy SP (ed): Handbook of Venoms and Toxins of Reptiles. CRC press, Boca Raton, FL, 2009.
- Kini RM: Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes. *Toxicon* 42: 827-840, 2003.
- Al-Jammaz I: Physiological effects of LD50 of *Echis coloratus* crude venom on rat at different time intervals. *J King Saud Univ Sci* 15: 121-129, 2003.
- Al-Asmari AK, Abbas Manthiri RM, Osman NMA, Al-Otaibi AF and Al-Asmari SA: Beneficial role of quercetin on *Echis coloratus* snake venom induced hepato-renal toxicity in rats. *J Biol Sci* 16: 112-119, 2016.
- Annobil SH: Complications of *Echis colorata* snake bites in the Asir region of Saudi Arabia. *Ann Trop Paediatr* 13: 39-44, 1993.
- Boviatsis EJ, Kouyialis AT, Papatheodorou G, Gavra M, Korfiatis S and Sakas DE: Multiple hemorrhagic brain infarcts after viper envenomation. *Am J Trop Med Hyg* 68: 253-257, 2003.
- Fernandez S, Hodgson W, Chaisakul J, Kornhauser R, Konstantakopoulos N, Smith AI and Kuruppu S: In vitro toxic effects of puff adder (*Bitis arietans*) venom, and their neutralization by antivenom. *Toxins (Basel)* 6: 1586-1597, 2014.
- Murphy MP: How mitochondria produce reactive oxygen species. *Biochem J* 417: 1-13, 2009.
- Sena LA and Chandel NS: Physiological roles of mitochondrial reactive oxygen species. *Mol Cell* 48: 158-167, 2012.
- Pickering AM, Vojtovich L, Tower J and A Davies KJ: Oxidative stress adaptation with acute, chronic, and repeated stress. *Free Radic Biol Med* 55: 109-118, 2013.
- Cai Z and Yan LJ: Protein oxidative modifications: beneficial roles in disease and health. *J Biochem Pharmacol Res* 1: 15-26, 2013.
- Aboul-Soud MA, Al-Othman AM, El-Desoky GE, Al-Othman ZA, Yusuf K, Ahmad J and Al-Khedhairi AA: Hepatoprotective effects of vitamin E/selenium against malathion-induced injuries on the antioxidant status and apoptosis-related gene expression in rats. *J Toxicol Sci* 36: 285-296, 2011.
- Nordberg J and Arnér ES: Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 31: 1287-1312, 2001.
- Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M and Telser J: Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39: 44-84, 2007.
- Al Asmari AK, Khan HA, Manthiri RA, Al Yahya KM and Al Otaibi KE: Effects of *Echis pyramidum* snake venom on hepatic and renal antioxidant enzymes and lipid peroxidation in rats. *J Biochem Mol Toxicol* 28: 407-412, 2014.
- Yamasaki SC, Villarreal JS, Barone JM, Zambotti-Villela L and Silveira PF: Aminopeptidase activities, oxidative stress and renal function in *Crotalus durissus terrificus* envenomation in mice. *Toxicon* 52: 445-454, 2008.
- Valenta J, Stach Z and Svítek M: Acute pancreatitis after viperid snake cerastes cerastes envenoming: a case report. *Prague Med Rep* 111: 69-75, 2010.
- Sagheb MM, Sharifian M, Moini M and Salehi O: Acute renal failure and acute necrotizing pancreatitis after *Echis carinatus* sochureki bite, report of a rare complication from southern Iran. *Prague Med Rep* 112: 67-71, 2011.
- Doussset E, Carrega L, Steinberg JG, Clot-Faybessé O, Jouirou B, Sauze N, Devaux C, Autier Y, Jammes Y, Martin-Eauclaire MF and Guieu R: Evidence that free radical generation occurs during scorpion envenomation. *Comp Biochem Physiol C Toxicol Pharmacol* 140: 221-226, 2005.
- Al Asmari A, Al Moutaery K, Manthari RA and Khan HA: Time-course of lipid peroxidation in different organs of mice treated with *Echis pyramidum* snake venom. *J Biochem Mol Toxicol* 20: 93-95, 2006.
- Asmari AK, Khan HA, Banah FA, Buraidi AA and Manthiri RA: Serum biomarkers for acute hepatotoxicity of *Echis pyramidum* snake venom in rats. *Int J Clin Exp Med* 8: 1376-1380, 2015.
- Onyeama HP, Ebong PE and Eteng MU: Evaluation of the effects of *Calliandra portoricensis* extracts on oxidative stress enzymes in Wistar rats challenged with venom of *Echis ocellatus*. *J Appl Pharm Sci* 2: 199-202, 2012.
- Tohamy AA, Mohamed AF, Abdul Moneim AE and Diab MSM: Biological effects of *Naja haje* crude venom on hepatic and renal tissues of mice. *J King Saud Univ Sci* 26: 205-212, 2014.
- Halliwell B: Vitamin C: antioxidant or pro-oxidant in vivo? *Free Radic Res* 25: 439-454, 1996.
- Ghneim HK and Al-Sheikh YA: The effect of aging and increasing ascorbate concentrations on respiratory chain activity in cultured human fibroblasts. *Cell Biochem Funct* 28: 283-292, 2010.
- Banerjee P, Bhattacharyya SS, Bhattacharjee N, Pathak S, Boujedaini N, Belon P and Khuda-Bukhsh AR: Ascorbic acid combats arsenic-induced oxidative stress in mice liver. *Ecotoxicol Environ Saf* 72: 639-649, 2009.
- Retsky KL and Frei B: Vitamin C prevents metal ion-dependent initiation and propagation of lipid peroxidation in human low-density lipoprotein. *Biochim Biophys Acta* 1257: 279-287, 1995.
- Chen K, Suh J, Carr AC, Morrow JD, Zeind J and Frei B: Vitamin C suppresses oxidative lipid damage in vivo, even in the presence of iron overload. *Am J Physiol Endocrinol Metab* 279: E1406-E1412, 2000.
- ElShama SS, EL-Meghawry A, El-Kenawy AE and Osman HE: Vitamin C daily supplements and its ameliorative effects. In: Vitamin C. Guiné R (ed). Nova Science Publishers, Inc., Hauppauge, NY, pp47-64, 2013.
- Klenner FR: Observations on the dose of administration of ascorbic acid when employed beyond the range of a vitamin in human pathology. *J Appl Nutr* 23: 61-68, 1971.
- Khan W, Osman NA, Alahmari AM, Amaan A and Al-Asmari A: Vitamin C protects against viper venom induced hepatotoxicity and oxidative damage in rat liver. *MOJ Toxicol* 2: 00026, 2016.
- Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-63, 1983.
- Ghneim HK and Alshehly MM: Biochemical markers of oxidative stress in Saudi women with recurrent miscarriage. *J Korean Med Sci* 31: 98-105, 2016.
- Al-Sheikh YA and Ghneim HK: 'The effect of micronutrients on superoxide dismutase in senescent fibroblasts'. *Cell Biochem Funct* 29: 384-393, 2011.
- Habig WH, Pabst MJ and Jakoby WB: Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 249: 7130-7139, 1974.
- Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.

39. Reznick AZ and Packer L: Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol* 233: 357-363, 1994.
40. Ghneim HK, Al-Sheikh YA, Alshebly MM and Aboul-Soud MA: Superoxide dismutase activity and gene expression levels in Saudi women with recurrent miscarriage. *Mol Med Rep* 13: 2606-2612, 2016.
41. Al-Saleh SS, Ghneim HK, Haddad HY and Khan SU: Separation and purification of *Echis coloratus* venom and some biological and biochemical effects of the proteins. *Cell Biochem Funct* 20: 153-162, 2002.
42. Al-Saleh S, Ghneim H and Khan S: The effect of crude and purified *Cerastes vipera* venom protein fractions on respiratory chain function in cultured human fibroblasts. *Cell Physiol Biochem* 13: 315-320, 2003.
43. Ghneim HK, Al-Sheikh YA and Aboul-Soud MA: The effect of *Walterinnesia aegyptia* venom proteins on TCA cycle activity and mitochondrial NAD(+)-redox state in cultured human fibroblasts. *Biomed Res Int* 2015: 738147, 2015.
44. Ghneim HK and Al-Sheikh YA: Effect of selenium supplementation on glutathione peroxidase and catalase activities in senescent cultured human fibroblasts. *Ann Nutr Metab* 59: 127-138, 2011.
45. Ghneim HK: Enzymatic variations related to glucose and glycogen catabolism in serially subcultured human fibroblasts. *Cell Physiol Biochem* 4: 44-56, 1994.
46. Couto N, Malys N, Gaskell SJ and Barber J: Partition and turnover of glutathione reductase from *Saccharomyces cerevisiae*: a proteomic approach. *J Proteome Res* 12: 2885-2894, 2013.
47. Lei XG, Zhu JH, McClung JP, Aregullin M and Roneker CA: Mice deficient in Cu,Zn-superoxide dismutase are resistant to acetaminophen toxicity. *Biochem J* 399: 455-461, 2006.
48. Kehrer JP: The Haber-Weiss reaction and mechanisms of toxicity. *Toxicology* 149: 43-50, 2000.
49. Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, Chen S, Corpe C, Dutta A, Dutta SK and Levine M: Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J Am Coll Nutr* 22: 18-35, 2003.
50. Welch RW, Bergsten P, Butler JD and Levine M: Ascorbic acid accumulation and transport in human fibroblasts. *Biochem J* 294: 505-510, 1993.
51. Savini I, Rossi A, Pierro C, Avigliano L and Catani MV: SVCT1 and SVCT2: key proteins for vitamin C uptake. *Amino Acids* 34: 347-355, 2008.
52. Linster CL and Van Schaftingen E: Vitamin C. Biosynthesis, recycling and degradation in mammals. *FEBS J* 274: 1-22, 2007.