

Jeju Ground Water Containing Vanadium Enhances Antioxidant Systems in Human Liver Cells

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Abstract Vanadium compounds have shown promise in the treatment of diabetes and in cancer prevention. The aim of this study is to investigate the effects of Jeju ground water, containing the vanadium compounds S1 ($8.0 \pm 0.9 \mu\text{g/l}$) and S3 ($26.0 \pm 2.0 \mu\text{g/l}$), and of vanadyl sulfate (VOSO_4 , $26 \mu\text{g/l}$) on antioxidant systems in human Chang liver cells. Cells were incubated for ten passages in media containing deionized distilled water, Jeju ground water (S1, S3), or VOSO_4 . S1 and S3 increased the gene and protein expression and the enzymatic activities of antioxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase, and heme oxygenase. VOSO_4 was likewise found to improve mRNA and protein expression as well as the activities of these enzymes. Taken together, these results suggest that the antioxidant properties of Jeju ground water, containing vanadium compounds, and of vanadyl sulfate were

due to stimulatory effects on antioxidant enzyme activities and antioxidant enzyme expression.

Keywords Jeju ground water · Vanadium · Antioxidant enzymes · Human Chang liver cells · Reactive oxygen species

Introduction

Supplementation with micronutrients has been shown to delay or even inhibit the progression of disease-related processes [1]. The micronutrient vanadium is obtained from dietary sources, most commonly vegetables, such as mushrooms, dill seed, black pepper, and parsley, and foods, such as cereals, fruits, and shellfish [2, 3]. Vanadium is believed to be important both in normal cell function and in development, in addition to showing promising effects in the inhibition of murine leukemia, fluid and solid Ehrlich ascites tumor, murine mammary adenocarcinoma, and human carcinomas of the lung, breast, and gastrointestinal tract [4, 5]. Vanadium is thought to regulate certain intracellular signaling pathways, and very low doses confer several unique beneficial effects at cellular or subcellular levels, whereas higher doses are toxic [6, 7]. The interesting biological and pharmacological properties of vanadium include insulin-mimetic action; anti-hyperlipidemia, anti-hypertension, and anti-obesity effects; enhancement of the oxygen affinity of hemoglobin and myoglobin; and diuretic action [8]. All of these properties can be exploited in biomedical applications. For example, a protective effect on the pancreas of streptozotocin-induced diabetic rats was demonstrated for vanadyl sulfate (VOSO_4), an oxidative form of vanadium [9]; insulin-mimetic vanadium (IV) and zinc (II) complex was found to possess anti-diabetic properties [10]; and the

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first human clinical trials for type 2 diabetes (phase I and phase II) were complemented recently with bis(ethylmalto)oxovanadium (IV), showing promising results of vanadium treatment of type 2 diabetes [11, 12].

The Mt. Fuji (Japan) ground water contains a very high concentration of vanadium [13], and the mineral water of this region is on the market as Vanadium Water as an agent to cope with diabetes [14]. Jeju Island, the largest island in Korea, is a volcanic island located about 140 km south of the Korean peninsula. The extent of the water resources on Jeju Island appears large, but the island has suffered from a water shortage mainly because of the lack of large perennial rivers and streams. The geology of Jeju Island is composed predominantly of permeable basalts into which rain and stream waters easily percolate to accumulate and move slowly in accessible. In generally, basalts contain the highest concentrations of vanadium. Therefore, Jeju ground water contains the vanadium due to the dissolution of vanadium from basalt and exists as oxidized state of vanadium (IV or V). Recent report has suggested that natural vanadium-containing Jeju ground water stimulates glucose uptake through the activation of AMP-activated protein kinase in L6 myotubes [15]. We also demonstrated that Jeju ground water, containing vanadium compounds, possesses *in vitro* and *in vivo* antioxidant activity [16–18], attenuates adipogenesis in 3T3-L1 preadipocytes [19], and exhibits immune activation properties on the peripheral immunocytes of mice irradiated with low dose gamma rays [20].

Enzymes comprising the main antioxidant systems include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and heme oxygenase-1 (HO-1). SOD catalyzes the conversion of superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide plus dioxygen [21] and is part of a defense system conferring protection against harmful processes in which $O_2^{\cdot-}$ appears to play an important role, including in inflammation, carcinogenesis, and aging [22, 23]. CAT is an ubiquitous enzyme found in nearly all living organisms exposed to oxygen; it catalyzes the decomposition of hydrogen peroxide, a harmful by-product of many normal metabolic process, to water and oxygen [24]. The selenoenzyme GPx is an antioxidant that catalyzes the reduction of hydroperoxides, including hydrogen peroxides, using reduced glutathione [25]. The stress-responsive enzyme HO-1 is induced by various oxidative agents; it plays a fundamental protective role against oxidative process by cleaving pro-oxidant heme into equimolar amounts of carbon monoxide, biliverdin/bilirubin, and free iron, all of which have significant biological properties, such as anti-oxidant, anti-inflammatory, and anti-apoptotic activities [26, 27].

In the present study, we examined the effects of vanadium on these antioxidant enzymes by measuring their mRNA and protein expression and activities in response to Jeju ground water and vanadyl sulfate.

Materials and Methods

Reagents

Jeju ground water containing the vanadium components, S1 (vanadium, $8.0 \pm 0.9 \mu\text{g/l}$, pH 7.8) and S3 (vanadium, $26.0 \pm 2.0 \mu\text{g/l}$, pH 8.4), Na^+ $5.06 \pm 1.0 \text{ mg/l}$, Ca^{2+} $3.4 \pm 0.5 \text{ mg/l}$, Mg^{2+} $3.0 \pm 1.0 \text{ mg/l}$, and K^+ $3.0 \pm 0.5 \text{ mg/l}$, was provided by the Jeju special self-governing province development corporation (Jeju, South Korea). Vanadyl sulfate hydrate ($\text{VOSO}_4 \cdot x\text{H}_2\text{O}$) was purchased from Sigma (St. Louis, MO, USA). CAT, GPx, and HO-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Copper/zinc superoxide dismutase (Cu/Zn SOD) antibody was purchased from Stressgen Corporation (Victoria, BC, Canada). All other chemicals and reagents used were of analytical grade.

Cell Culture

Human Chang liver cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2 in air. Cells were cultured with RPMI 1640 containing distilled deionized water (DDW), Jeju ground water (S1, S3), or VOSO_4 supplemented with 0.1 mM non-essential amino acids, 10% heat-inactivated fetal calf serum, streptomycin (100 $\mu\text{g/ml}$), and penicillin (100 units/ml).

SOD Activity

SOD activity was measured using a colorimetric assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. The kit utilizes WST-1, which produces a water-soluble formazan dye upon reduction with superoxide anion, and the product that was detected at 450 nm. SOD activity was calculated on the basis of the percent inhibition of superoxide anion.

CAT Activity

A CAT assay kit (Abcam, Cambridge, MA, USA) was used to measure CAT activity according to the manufacturer's protocol. In this assay, CAT reacts with H_2O_2 to produce water and oxygen. Unconverted H_2O_2 reacts with the OxiRed probe to produce a product that is detected at 570 nm. CAT activity is expressed in mU/ml.

GPx Activity

The FR 17 assay kit (Oxford Biomedical Research, MI, USA) was used to measure GPx activity, following the

manufacturer's protocol. The enzyme reaction is assessed by adding the substrate tert-butyl hydroperoxide. The rate of decrease in the absorbance at the 340 nm is directly proportional to GPx activity, which is expressed in mU/ml.

Quantification of HO-1 Concentration

HO-1 production was quantified using a Human HO-1 ELISA kit (Assay Designs Inc., Ann Arbor, MI, USA) following the manufacturer's protocol. Cell lysates were added to the coated plates and incubated for 1 h at room temperature with anti-human HO-1 antibody. After repeated washings, the plates were incubated with anti-rabbit IgG-horseradish peroxidase conjugate for 30 min and then treated with tetramethylbenzidine, substrate for peroxidase. The reaction was stopped after 15 min, and the optical density at 450 nm was read using a microplate reader. HO-1 concentration is expressed in ng/ml.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells using Trizol (GibcoBRL, Grand Island, NY, USA). Real-time quantitative polymerase chain reaction (PCR) was performed in 96-well optical plates with an iQTM5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primer pairs (Bionics, Seoul, South Korea) of the antioxidant enzymes are shown in Table 1. The PCR mixture contained 10 µl of 2×SYBR qPCR SuperMix Universal kit (Invitrogen, CA, USA), 10 µM each of the forward and reverse primers, and 1 µl of diluted template cDNA (10 ng). Fluorescence was measured at the end of each cycle to determine the amount of PCR products. The point at which the SYBR fluorescent signal reached statistically significant above background was defined as the cycle threshold (CT), the optimal value of which was chosen automatically. Transcript quantities represented the expression levels of target genes and were determined relative to those of reference genes. Relative expression levels were calculated using the following equation, based on the gene expression CT difference method

[28]: relative expression level = $\frac{E_{\text{HKG}}^{\text{CT}_{\text{HKG}}}}{E_{\text{GOI}}^{\text{CT}_{\text{GOI}}}}$, where E_{HKG} and E_{GOI} are the PCR efficiencies, and CT_{HKG} and CT_{GOI} are the threshold cycles for the reference housekeeping gene and a given interest gene, respectively.

Western Blotting Analysis

Cells were harvested, washed twice with PBS, lysed on ice for 30 min in 100 µl of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40], and then centrifuged at 13,000×g for 15 min. The supernatants were collected from the lysates and the protein concentrations were determined. Aliquots of the lysates (40 µg of protein) were boiled for 5 min and electrophoresed in a 10% SDS-polyacrylamide gel. The proteins in the gels were transferred onto nitrocellulose membranes, which were then incubated with the primary antibody and then with the secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK) and then exposed to X-ray film.

Statistical Analysis

All measurements were made in triplicate ($n=3$), and all values are the means±standard error (SE). Data were analyzed with analysis of variance using the Tukey test.

Results

Antioxidant Systems Are Enhanced by Jeju Ground Water Containing Vanadium Components

To investigate whether the antioxidant effects of S1 and S3 are mediated by antioxidant enzymes, the activities and the mRNA and protein levels of SOD, CAT, GPx, and HO-1 were measured. As shown in Fig. 1a, S1 and S3 increased SOD activity, inhibiting superoxide anion by 29% and 38%

Table 1 Primers and conditions used in the real-time PCR

Gene	Primer sequence	Product size (bp)	Anneal (°C)
GAPDH	F: 5'-AAGGTCGGAGTCAACGGATTT-3' R: 5'-GCAGTGAGG GTCTCTCTCCT-3'	1,054	60
Cu/Zn SOD	F: 5'-AAGATGACTTGGGCAAAGGT-3' R: 5'-AATCCCAATCACACCACAAG-3'	189	60
CAT	F : 5'-TCTGGAGAAGTGCGGAGATT-3' R : 5'-AGTCAGGGTGGACCTCAGTG-3'	190	55
GPx	F: 5'-TATAGAAGCCCTGCTGTCCA-3' R: 5'-CAAGCCCAGATACCAGGAA-3'	123	53
HO-1	F: 5'-CAGGCAGAGAATGCTGAGTTC-3' R: 5'-GATGTTGAGCAGGAACGCAGT-3'	555	55

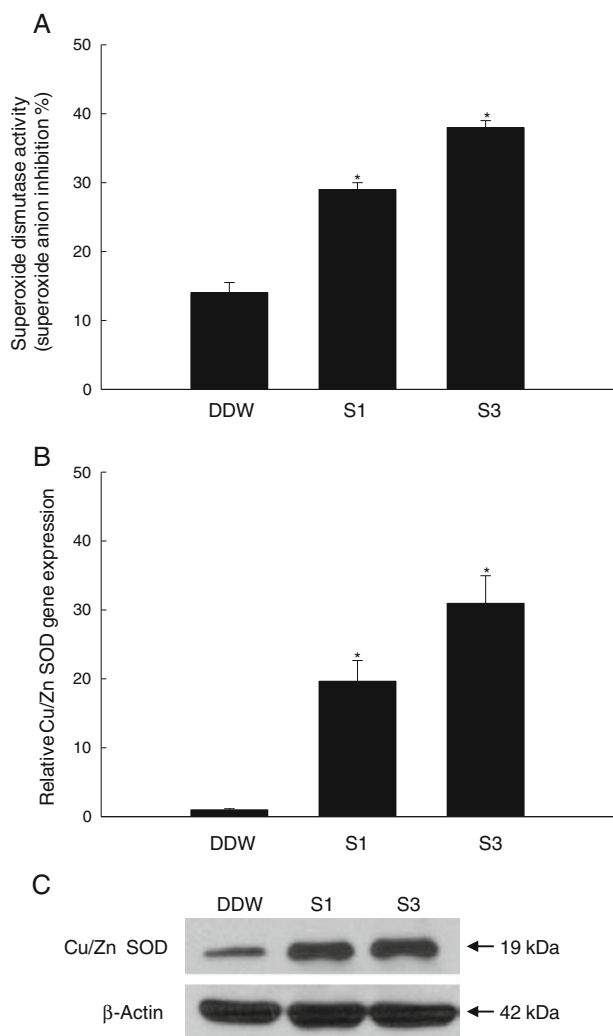


Fig. 1 Effects of S1 and S3 on SOD. **a** SOD activity was measured using a colorimetric assay kit. All values are represented as mean±SE. Asterisk indicates significant difference from the DDW group ($p<0.05$). **b** Expression of the Cu/Zn SOD gene was measured by real-time PCR. Each value represents the mean±SE. The values were normalized using GAPDH. Asterisk indicates significant difference from the DDW group ($p<0.05$). **c** Cell lysates were electrophoresed, and Cu/Zn SOD protein was then detected using Cu/Zn SOD specific antibody

compared with 14% in DDW treatment. Real-time PCR showed that S1 and S3 enhanced Cu/Zn SOD mRNA levels (Fig. 1b), and the results were consistent with the SOD expression level (Fig. 1c). S1 and S3 also augmented the activities of CAT, 111 and 130 mU/ml, respectively, compared with 39 mU/ml in DDW (Fig. 2a). The results of real-time PCR and Western blotting confirmed that CAT mRNA and protein, respectively, were enhanced by both S1 and S3 (Fig. 2b, c). Similarly, GPx activity was increased to 71 and 103 mU/ml by S1 and S3, respectively, compared with 49 mU/ml in DDW (Fig. 3a). GPx mRNA and protein levels were also increased (Fig. 3b, c). Finally, HO-1 concentration was increased to 30 and 36 ng/ml by S1 and S3,

respectively, compared with 20 ng/ml in DDW (Fig. 4a). HO-1 mRNA and protein expressions were increased by S1 and S3 (Fig. 4b, c). Taken together, these results suggest that S1 and S3 have positive effects not only on antioxidant activity but also on the mRNA and protein level of the enzymes that given these activities.

Induction of Antioxidant Systems by VOSO₄

To investigate whether the antioxidant effects of VOSO₄ were mediated by the same antioxidant enzymes examined in the previous section, the activities, mRNA levels, and protein expressions of SOD, CAT, GPx, and HO-1 were assessed in VOSO₄-treated cells. Cell viability assays confirmed that VOSO₄ at 26 μg/l was not toxic to human Chang liver cells (data not shown). As shown in Fig. 5a, superoxide anion level was inhibited by 32% in VOSO₄-treated cells, whereas it was inhibited by 19% in DDW-treated cells, showing that VOSO₄ increased SOD activity. Real-time PCR confirmed that VOSO₄ increased Cu/Zn SOD mRNA levels (Fig. 5b), and the results were consistent with the increase in the Cu/Zn SOD protein levels as measured by Western blot analysis (Fig. 5c). Similarly, VOSO₄ increased the activities of CAT and GPx, with values of 75 and 94 mU/ml compared with 19 and 57 mU/ml in DDW, respectively (Fig. 6a). VOSO₄ also resulted in higher mRNA (Fig. 6b) and protein expressions (Fig. 6c) of CAT and GPx. Similar increases were obtained for HO-1 (Fig. 7a–c). Taken together, these results suggest that VOSO₄ increases not only activities but also the mRNA and protein expression of antioxidant enzymes.

Discussion

Reactive oxygen species (ROS), and particularly superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$), are widely held to be a pathogenic factor in several human diseases, such as diabetes, atherosclerosis, cardiovascular disease, cancer, neurodegenerative disorders, and in the aging process [29]. The cellular production and removal of these species must be critically balanced, which is the function of several strategically employed cellular antioxidant systems that scavenge or neutralize these molecules [30]. Recently, the potential role of organic metal compounds as oxidants or antioxidants in biological systems has been the subject of increasing research. SOD, CAT, and GPx are vital antioxidant enzymes that protect cells against oxidative damage. SOD catalyzes the dismutation of the superoxide anion into molecular oxygen and H_2O_2 [31]. In humans, there are three forms of SOD: cytosolic Cu/Zn SOD (a ~16 kDa homodimer containing a copper/zinc reactive site), mitochondrial Mn SOD (a ~21 to 25 kDa homotetramer

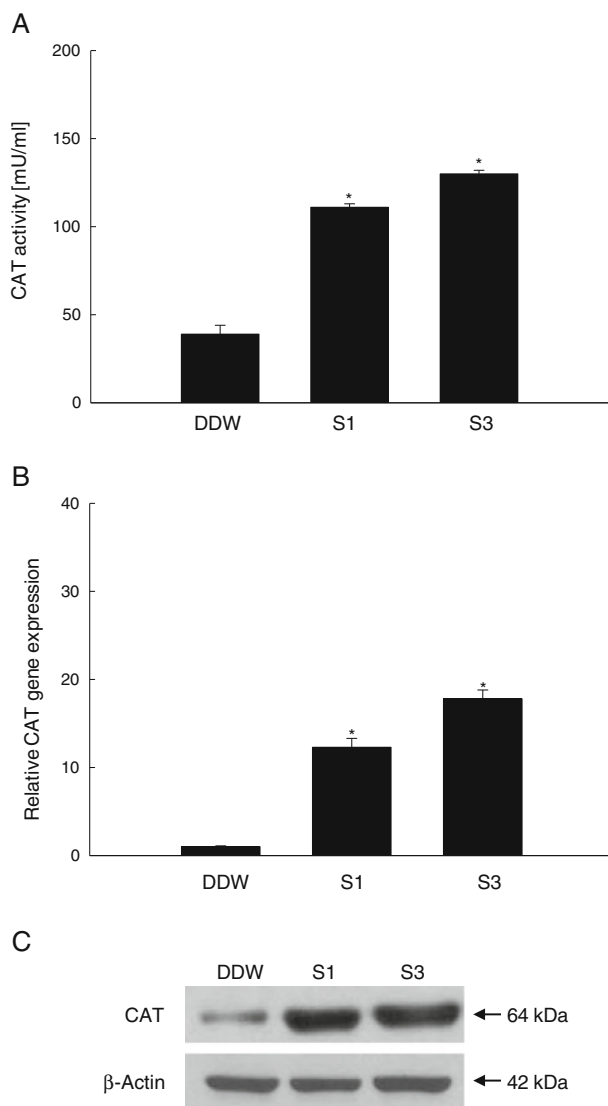


Fig. 2 Effects of S1 and S3 on CAT. **a** CAT activity was measured using an assay kit. All values are represented as mean±SE. *Asterisk* indicates significant difference from the DDW group ($p<0.05$). **b** CAT gene expression was measured by real-time PCR. Each value represents the mean±SE. The values were normalized using GAPDH. *Asterisk* indicates significant difference from the DDW group ($p<0.05$). **c** Cell lysates were electrophoresed, and CAT protein was detected using CAT specific antibody

containing a manganese reactive site), and extracellular SOD (a ~26 kDa homotetramer containing a copper/zinc reactive site) [32]. A deficiency of SOD, regardless of isoform and location, results in relatively higher levels of ROS, an altered cellular redox state, and, in turn, persistent oxidative stress [33]. CAT, a cytosolic homotetramer with subunit of ~60 kDa, catalyzes the reduction of H_2O_2 (generated by the dismutation of superoxide anion or by the reaction between ascorbate and Fe^{3+}) to water and molecular oxygen [34]. The Fenton reaction (using Fe^{2+}) can also eliminate H_2O_2 , by catalyzing its conversion to the hydroxyl radical, the

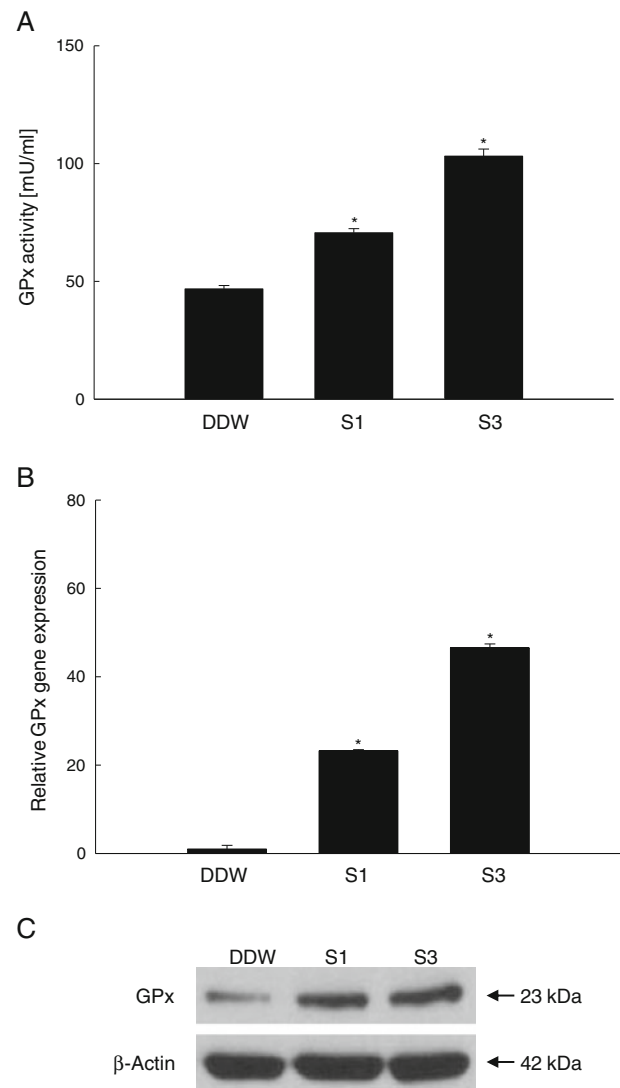


Fig. 3 Effects of S1 and S3 on GPx. **a** GPx activity was determined using an assay kit. All values are represented as mean±SE. *Asterisk* indicates significant difference from the DDW group ($p<0.05$). **b** GPx gene expression was measured by real-time PCR. Each value represents the mean±SE. The values were normalized using GAPDH. *Asterisk* indicates significant difference from the DDW group ($p<0.05$). **c** Cell lysates were electrophoresed, and GPx protein was then detected using GPx specific antibody

most reactive of known ROS. It was observed that CAT deficiency in mice renders kidneys more prone to oxidative stress [35], while humans deficient in CAT are predisposed to cumulative oxidant damage leading to type 1 and type 2 diabetes mellitus [36]. GPx, a selenium containing enzyme, detoxifies H_2O_2 to H_2O through the oxidation of reduced glutathione [37]. GPx overexpression is associated with enhanced protection against oxidative stress [38]. HO-1, another enzyme with potent antioxidant properties, degrades pro-oxidant heme into iron, carbon monoxide, and biliverdin [39]. Biliverdin is then directly converted into the strong anti-oxidant bilirubin by biliverdin reductase. The

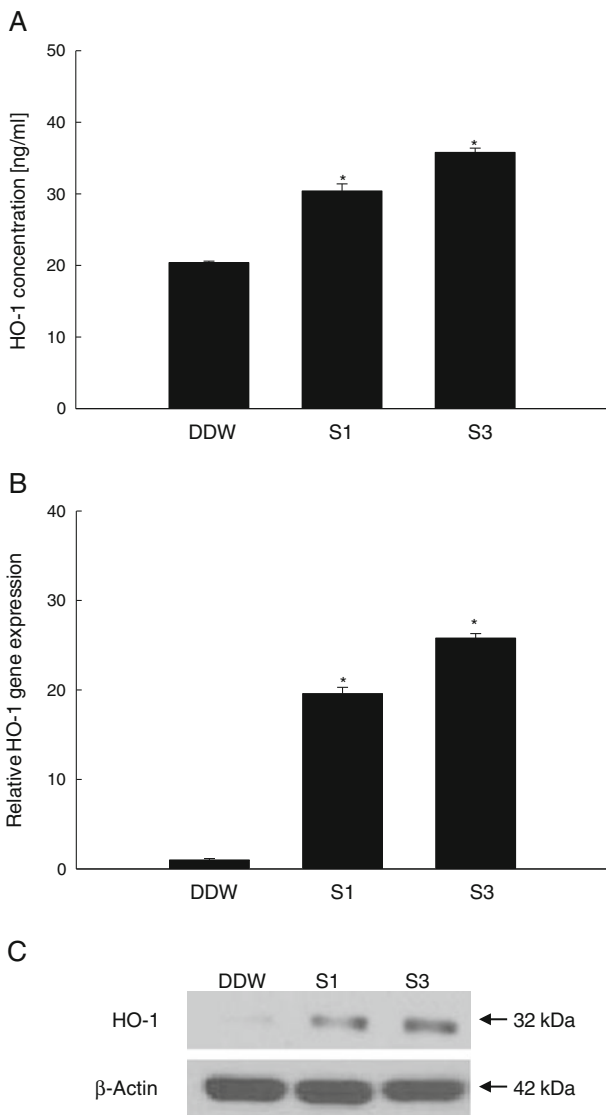


Fig. 4 Effects of S1 and S3 on HO-1. **a** HO-1 concentration was quantitated using HO-1 ELISA kit. All values are represented as mean±SE. Asterisk indicates significant difference from the DDW group ($p<0.05$). **b** HO-1 gene expression was measured by real-time PCR. Each value represents the mean±SE. The values were normalized using GAPDH. Asterisk indicates significant difference from the DDW group ($p<0.05$). **c** Cell lysates were electrophoresed, and HO-1 protein was then detected using HO-1 specific antibody

upregulation of HO-1 expression is a key event in the cellular maintenance of the adaptive survival response to diverse noxious stimuli. The strongly inducible HO-1 isoform has been shown to protect against oxidative stress, inflammation, and apoptosis [39, 40]. Selective overexpression of cytoprotective HO-1 in the endothelial cell line HMEC-1 was shown to prevent hyperglycemia-mediated O_2^- formation, thereby blocking ROS-induced DNA damage and caspase activation [41]. The effects of vanadium on the oxidant-antioxidant balance have been limited and controversial [42, 43]. In previous studies, vanadium

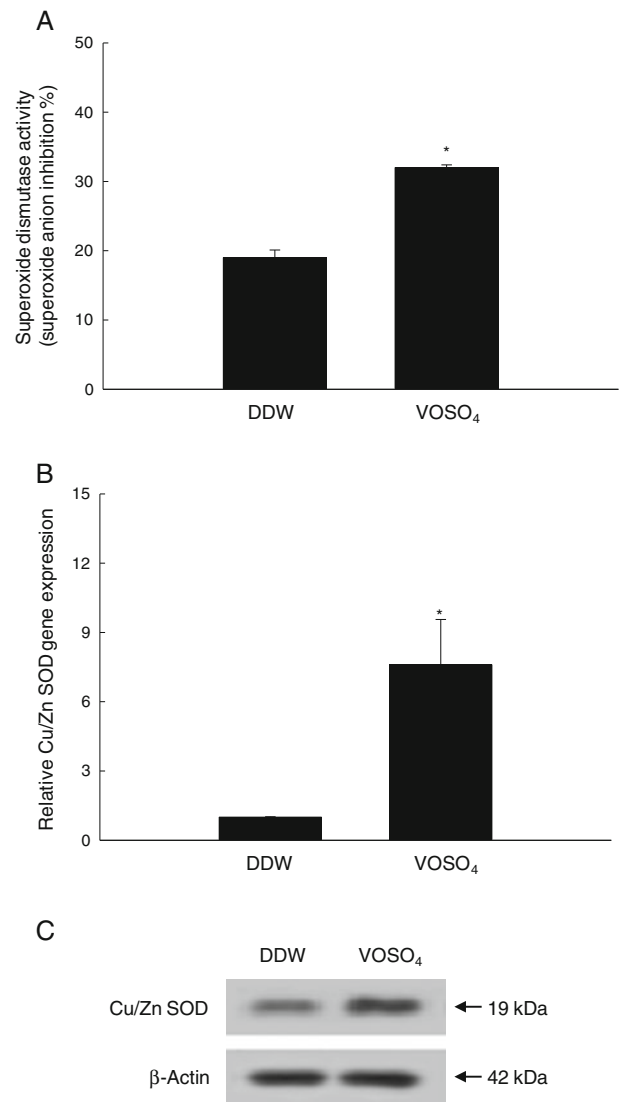


Fig. 5 Effect of VOSO₄ on SOD. **a** SOD activity was measured using a colorimetric assay kit. All values are represented as mean±SE. Asterisk indicates significant difference from the DDW group ($p<0.05$). **b** Cu/Zn SOD gene expression was measured by real-time PCR. Each value represents the mean±SE. The values were normalized using GAPDH. Asterisk indicates significant difference from the DDW group ($p<0.05$). **c** Cell lysates were electrophoresed, and Cu/Zn SOD protein was then detected using Cu/Zn SOD specific antibody

compounds were shown to cause oxidative stress [44]. In osteoblast and osteosarcoma cell lines, vanadate-induced cell toxicity, ROS formation, and lipid peroxidation increased with increasing concentration of vanadate [45]. In vitro incubation of vanadium (IV) with 2'-deoxyguanosine and DNA, in the presence of H₂O₂, resulted in enhanced 8-hydroxy-2'-deoxyguanosine formation and substantial DNA strand breaks [46]. Recent evidence has pointed to the indirect promotion of ROS production by vanadate, probably through mitochondrial interactions [44]. However, other studies suggested that vanadium exerts antioxidant

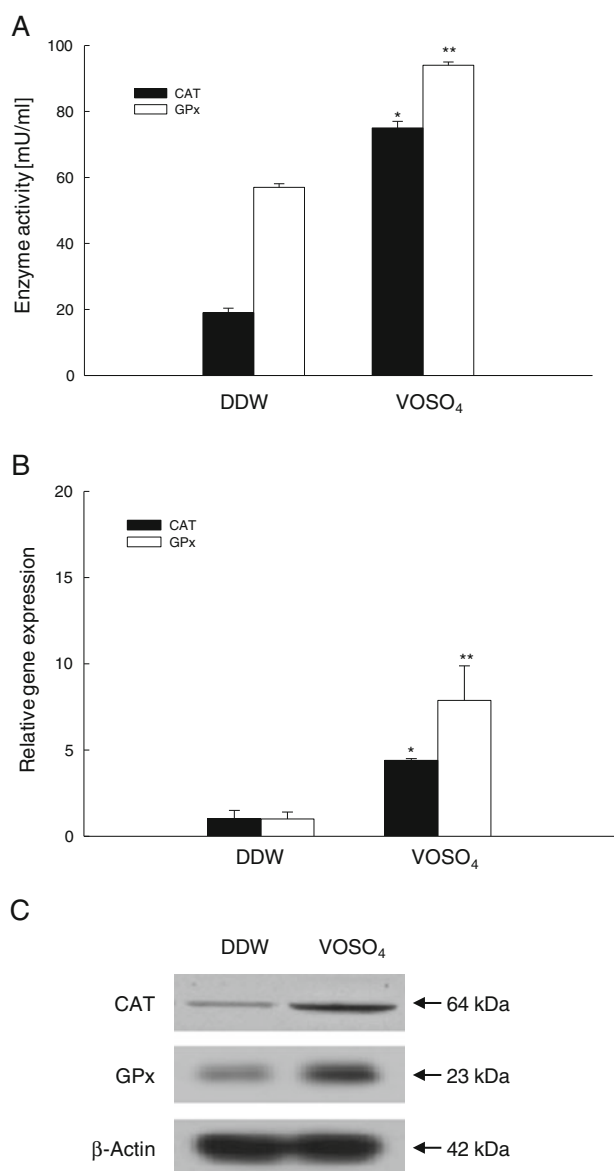


Fig. 6 Effect of VOSO₄ on CAT and GPx activities. **a** CAT and GPx activities were measured using the respective assay kit. All values are represented as mean±SE. *Single* and *double asterisks* indicate that CAT and GPx activities, respectively, are significantly different from the DDW ($p<0.05$). **b** CAT and GPx gene expression were measured by real-time PCR. Each value represents the mean±SE. The values were normalized using GAPDH. *Single* and *double asterisks* indicate that CAT and GPx gene expressions, respectively, are significantly different from the DDW ($p<0.05$). **c** Cell lysates were electrophoresed, and CAT and GPx proteins were then detected using CAT and GPx specific antibodies

effects [43], which is consistent with our findings. Vanadyl ions were previously shown to inhibit excess nitric oxide production by macrophages in the streptozotocin-induced diabetes model. In rat colon, vanadium exhibited protective effect against genotoxicity and carcinogenesis by the down-regulation of inducible nitric oxide synthase [47]. Vanadate

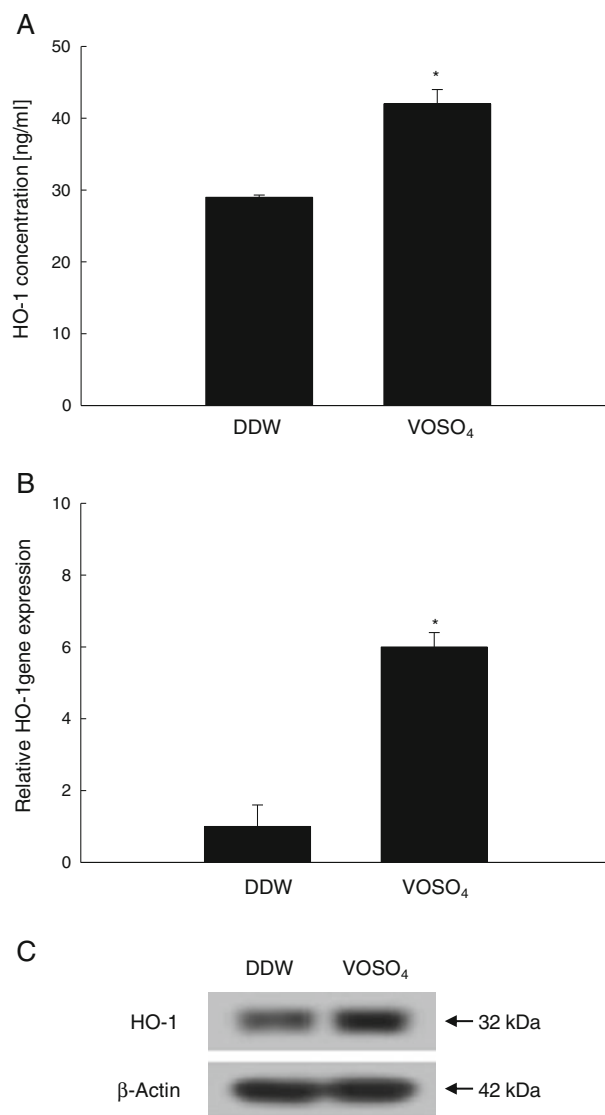


Fig. 7 Effect of VOSO₄ on HO-1. **a** HO-1 concentration was quantitated using HO-1 ELISA kit. All values are represented as mean±SE. *Asterisk* indicates significant difference from the DDW group ($p<0.05$). **b** HO-1 gene expression was measured by real-time PCR. Each value represents the mean±SE. The values were normalized using GAPDH. *Asterisk* indicates significant difference from the DDW group ($p<0.05$). **c** Cell lysates were electrophoresed, and the HO-1 protein was then detected using HO-1 specific antibody

treatment restored the decreased activity of antioxidant enzymes and the altered levels of plasma lipid peroxide, glycoproteins, and erythrocyte membrane phospholipids in diabetic rats [48].

In our study, S1, S3, and VOSO₄ significantly enhanced the activities as well as mRNA and protein level of SOD, CAT, GPx, and HO-1. Further studies are needed to investigate the effects of S1, S3, and VOSO₄ on antioxidant systems in vivo and to determine the underlying mechanisms involved in vanadium-mediated cellular response.

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