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□ ORIGINAL ARTICLE □

Reactive Oxygen Species Scavenging Effects of Jeju Waters Containing Vanadium Components

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The aim of this study was to examine the reactive oxygen species (ROS) scavenging effects of Jeju waters containing vanadium components; S1 (8.0±0.9 μg/l), S2 (24.0±2.0 μg/l), and S3 (26.0±2.0 μg/l). Human Chang liver cells were incubated for 10 passages in media containing deionized distilled water (DDW group) and Jeju waters (S1, S2, and S3 groups). DDW and Jeju waters did not show cytotoxicity and scavenging effect of 1,1-diphenyl-2-picrylhydrazyl radical. Electron spin resonance spectrometer data showed that cells exposed to Jeju waters showed significantly scavenged superoxide anion induced by the xanthine/xanthine oxidase system and hydroxyl radicals induced by Fenton reaction (H_2O_2+$FeSO_4$) compared to cells exposed to DDW. Furthermore, the S3 group significantly scavenged intracellular ROS compared to the DDW group, as measured by spectrofluorometry, flow cytometry, and confocal microscopy after staining with 2',7'-dichlorodihydrofluorescein diacetate. These results suggest that vanadium components-containing Jeju waters exhibited antioxidant effect by scavenging ROS. (***Cancer Prev Res*** **15, 111-117, 2010)**

Key Words: Reactive oxygen species, Vanadium, Antioxidant effect

INTRODUCTION

Supplementation with micronutrients can delay or even cease disease progression.[1,2] Among these micronutrients, vanadium, a dietary micronutrient, has shown promising ability to inhibit murine leukemia, Ehrlich tumor, murine mammary adenocarcinoma, human lung cancer, human breast cancer, and human gastrointestinal tract cancer.[3~6] Vegetables such as mushrooms, dill seed, black pepper, parsley and foods such as cereals, fruits, and shellfish are common sources of vanadium.[7~9]

Vanadium is believed to regulate certain intracellular signaling pathways and capable of exerting unique beneficial effects at the cellular or sub-cellular levels at very low doses compared to toxic at higher doses.[10,11] It has been demonstrated that this trace element presents interesting biological and pharmacological properties, including the insulin-mimetic action, antihyperlipidemia, antihypertension, antiobesity, enhancement of oxygen affinity of hemoglobin and myoglobin and diuretic action,[12] all of which should be further explored for application in biomedical sciences.[13] Sodium orthovanadate showed antioxidant effect in streptozotocin-induced diabetic rats via induction of glutathione peroxidase, catalase, superoxide dismutase and glutathione content.[14] Recently Ashiq *et al*. reported that the vanadium-hydrazide complex showed superoxide and nitric oxide scavenging activities.[15]

The objective of this study was to investigate the ROS scavenging effect of Jeju waters containing vanadium components.

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MATERIALS AND METHODS

1. Reagents

Jeju waters containing vanadium components; S1 (8.0±0.9 μg/l), S2 (24.0±2.0 μg/l), and S3 (26.0±2.0 μg/l) were provided by the Jeju special self-governing province development corporation (Jeju, Korea). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) were purchased from the Sigma chemical company (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

2. Cell culture

Human Chang liver cells were obtained from the American type culture collection (Rockville, MD, USA), and cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2 in air, and cultured in DDW or Jeju waters with RPMI 1640, containing 0.1 mM non-essential amino acids, 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml).

3. Cell viability assay

The effects of DDW and Jeju waters on cell viability were determined by the MTT assay, based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenase in viable cells.[16] DDW or Jeju waters cultured cells (10 passages) were seeded in a 96 well plate at a concentration of 1×10^5 cells/ml, and incubated for an additional 24 h at 37°C. Fifty microliter of MTT stock solution (2 mg/ml) was then added to each well of a total reaction volume of 200 μl. After incubating for 4 h, the plate was centrifuged at 800×g for 5 min and the supernatants were then aspirated. Formazan crystals in each well were dissolved in 150 μl dimethylsulfoxide and the A_{540} was read by a scanning multi-well spectrophotometer.

4. DPPH radical scavenging activity

DPPH radical scavenging activity was measured using the method described by Nanjo *et al.*[17] 60 μl of DDW or Jeju waters was added to 60 μl of DPPH (60 μM) in methanol solution. After mixing vigorously for 10 sec, the solution was then transferred into a 100 μl teflon capillary tube fitted into the cavity of JES-FA electron spin resonance (ESR) spectrometer (JEOL, Tokyo, Japan). The parameters of the ESR spectrometer were set at a magnetic field of 336 mT, power of 5.00 mW, frequency of 9.4380 GHz, modulation amplitude of 0.8 mT, gain of 500, scan time of 0.5 min, scan width of 10 mT, time constant of 0.03 sec and a temperature of 25°C.

5. Detection of superoxide anion

Superoxide was produced by reaction of the xanthine/xanthine oxidase system and reacted with spin trap DMPO. The DMPO-・OOH adducts were detected using an ESR spectrometer (JEOL, Tokyo, Japan). ESR signaling was recorded at 5 min after mixing with 20 μl of xanthine oxidase (0.25 U/ml), 20 μl of xanthine (5 mM), 20 μl of DMPO (1.5 M), 20 μl of DDW or Jeju waters.[18] The parameters of the ESR spectrometer were set at a magnetic field of 336 mT, power of 5.00 mW, frequency of 9.4380 GHz, modulation amplitude of 0.2 mT, gain of 500, scan time of 0.5 min, scan width of 10 mT, time constant of 0.03 sec and a temperature of 25°C.[19,20]

6. Detection of hydroxyl radicals

Hydroxyl radicals were generated by the Fenton reaction ($H_2O_2+FeSO_4$) and then reacted with a nitrone spin trap, DMPO.[21] The resultant DMPO-OH adducts was detected using an ESR spectrometer. ESR signaling was recorded at 2.5 min after mixing with 20 μl of 0.3 M DMPO, 20 μl of 10 mM $FeSO_4$, 20 μl of 10 mM H_2O_2, 20 μl of DDW or Jeju waters. The parameters of the ESR spectrometer were set at a magnetic field of 336 mT, power of 1.00 mW, frequency of 9.4380 GHz, modulation amplitude of 0.2 mT, gain of 200, scan time of 0.5 min, scan width of 10 mT, time constant of 0.03 sec and a temperature of 25°C.[19,20]

7. Intracellular reactive oxygen species (ROS) measurement

The DCF-DA method was used to detect the levels of intracellular ROS.[22] DDW or Jeju waters cultured cells (10 passages) were seeded in a 96-well plate at 1.5×10^5 cells/well. Sixteen hours after plating, 600 μM of H_2O_2 was added to the plate. The cells were incubated at 37°C. After the addition of 25 μM of DCF-DA solution for 10 min, the fluorescence of 2',7'-dichlorofluorescein was detected using a Perkin Elmer LS-5B spectrofluorometer and a flow cytometer (Becton Dickinson, Mountain View, CA, USA), respectively. Image analysis for intracellular ROS generation was achieved by

seeding cells on a cover-slip loaded six well plate at 2×10^5 cells/well. Twenty four hours later, 100 μM DCF-DA was added to each well and the cells were incubated for an additional 30 min at 37°C. After washing with phosphate buffered saline, the stained cells were mounted onto a microscope slide in mounting medium (DAKO, Carpinteria, CA, USA). Images were captured using the laser scanning microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) by a confocal microscope.[22)]

8. Statistical analysis

All measurements were made in triplicate (n=3), and all

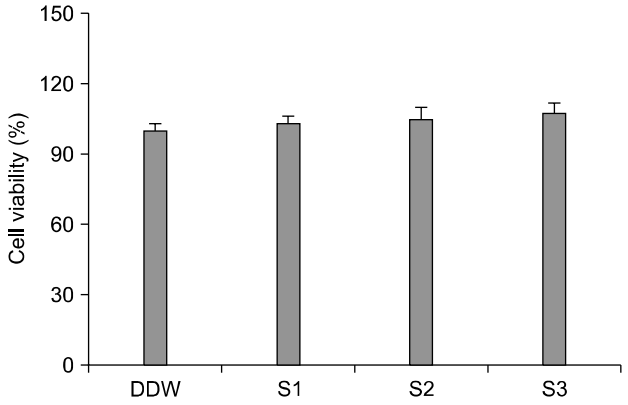


Fig. 1. Cytotoxic effect of Jeju waters on human Chang liver cells. DDW or Jeju waters cultured cells (10 passages) were seeded to a 96 well plate at a concentration of 1×10^5 cells/ml, and incubated for an additional 24 h at 37°C. Cell viability was determined by MTT assay. The data represent three experiments and are expressed as mean±SE.

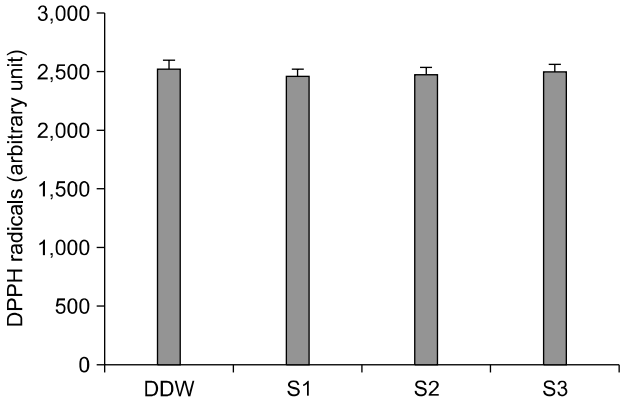
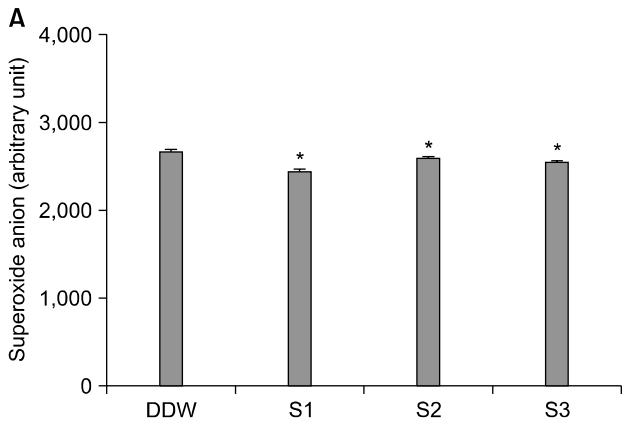


Fig. 2. Scavenging effect of Jeju waters against DPPH radicals. The amount of DPPH radical was determined using ESR spectrometer. The data represent three experiments and are expressed as mean±SE.



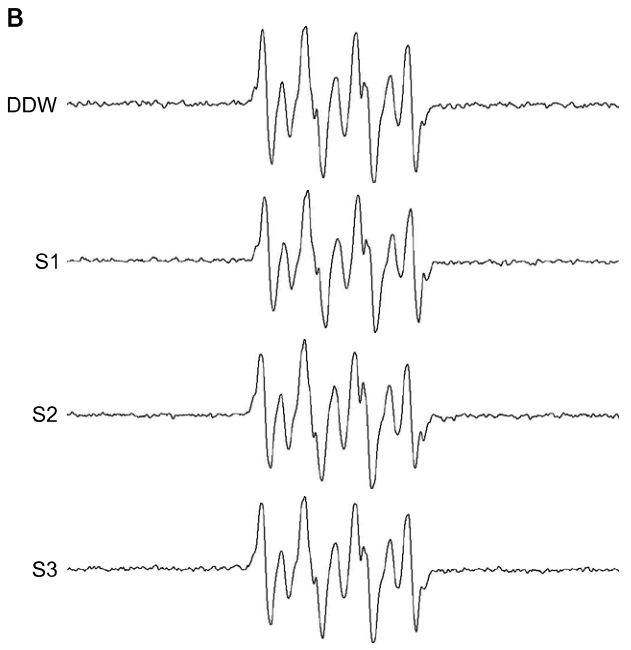


Fig. 3. Scavenging effect of Jeju waters against superoxide anion. Superoxide anion generated by xanthine and xanthine oxidase was reacted with DMPO, and the resultant DMPO-·OOH adducts were detected by ESR spectrometry. Results showed (A) histogram (mean±SE) and (B) representative raw data. The data represent three experiments and are expressed as mean±SE. *Significantly different from DDW group ($p<0.05$).

values are represented as mean±standard error (SE). Data were analyzed with analysis of variance (ANOVA) using the Tukey test. $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

1. Cytotoxic effect of Jeju waters containing vanadium components

To validate the cytotoxic effect of Jeju waters on Chang liver cells, cell viability after incubation of 10 passages with DDW, S1, S2, and S3 was measured by MTT assay. As shown in Fig. 1, Chang liver cells exposed to S1, S2, and S3 did not show toxic effect; cell viability of S1, S2, and S3 groups showed 103%, 105%, and 107%, respectively.

2. DPPH radical scavenging activity of Jeju waters

In our system, Jeju waters did not show DPPH radical scavenging activity (Fig. 2); DPPH radicals amount (arbitrary unit) of S1, S2, and S3 groups showed 2462, 2473 and 2500 respectively compared to 2528 of the DDW group. A DPPH radical-formation system offers a convenient and accurate method for titrating the oxidizable groups of compounds. Any molecule can donate an electron or hydrogen to a reaction system, which will bleach DPPH radical. Reaction of DPPH with hydrogen yields 2-(4-hydroxyphenyl)-2-phenyl-1-picryl hydrazine as a major product, and 2-(4-nitrophenyl)-2-phenyl-1-picrylhydrazine via a series of secondary processes. The concentration of DPPH at the end of a reaction depends on the concentration and structure of the compound being scavenged.[23] However, the DPPH assay was somewhat inappropriate for the evaluation of Jeju waters, because it measures the compounds' electron-donating activity. Vanadium component in Jeju waters appears to lack oxidizable groups, such as phenolic compounds, which are stabilized by the resonance delocalization. Therefore, Jeju waters are not easily prone to entering into hydrogen donation reactions with oxidizing agents. Due to the weak electron-donating ability of Jeju waters, they are poor radical quenchers in a DPPH assay.

3. ROS scavenging activity of Jeju waters in cell free system

Superoxide anion was produced by reaction of the

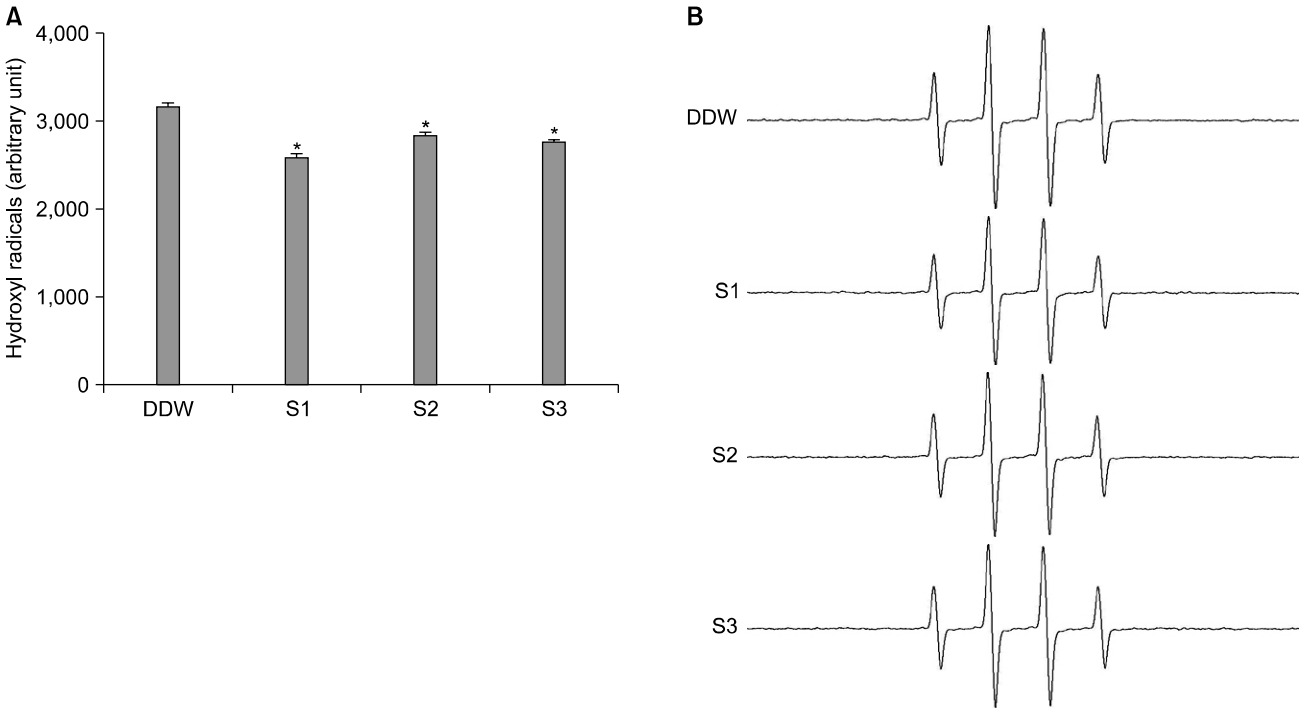


Fig. 4. Scavenging effect of Jeju waters against hydroxyl radicals. Hydroxyl radicals generated by the Fenton reaction (H_2O_2+ $FeSO_4$) were reacted with DMPO, and the resultant DMPO-OH adducts were detected by ESR spectrometry. Results are expressed (A) histogram (mean±SE) and (B) representative raw data. The data represent three experiments and are expressed as mean± SE. *Significantly different from DDW group ($p<0.05$).

xanthine/xanthine oxidase system and reacted with spin trap DMPO. The DMPO- • OOH adducts were detected using ESR spectrometer. In our system, Jeju waters demonstrated significant superoxide scavenging activity (Fig. 3). The quantity of superoxide anion (arbitrary unit) in S1, S2, and S3 groups was 2438, 2589 and 2549 respectively as compared to 2665 in DDW group. Hydroxyl radicals were generated by the Fenton reaction (H_2O_2+$FeSO_4$), and then reacted with a nitrone spin trap, DMPO. The resultant DMPO-OH adducts was detected using an ESR spectrometer. In our system, Jeju waters significantly exhibited hydroxyl radical scavenging activity (Fig. 4). The quantity of hydroxyl radical (arbitrary unit) in S1, S2, and S3 groups were 2587, 2833 and 2759 respectively compared to 3163 in the DDW group. The effects of vanadium

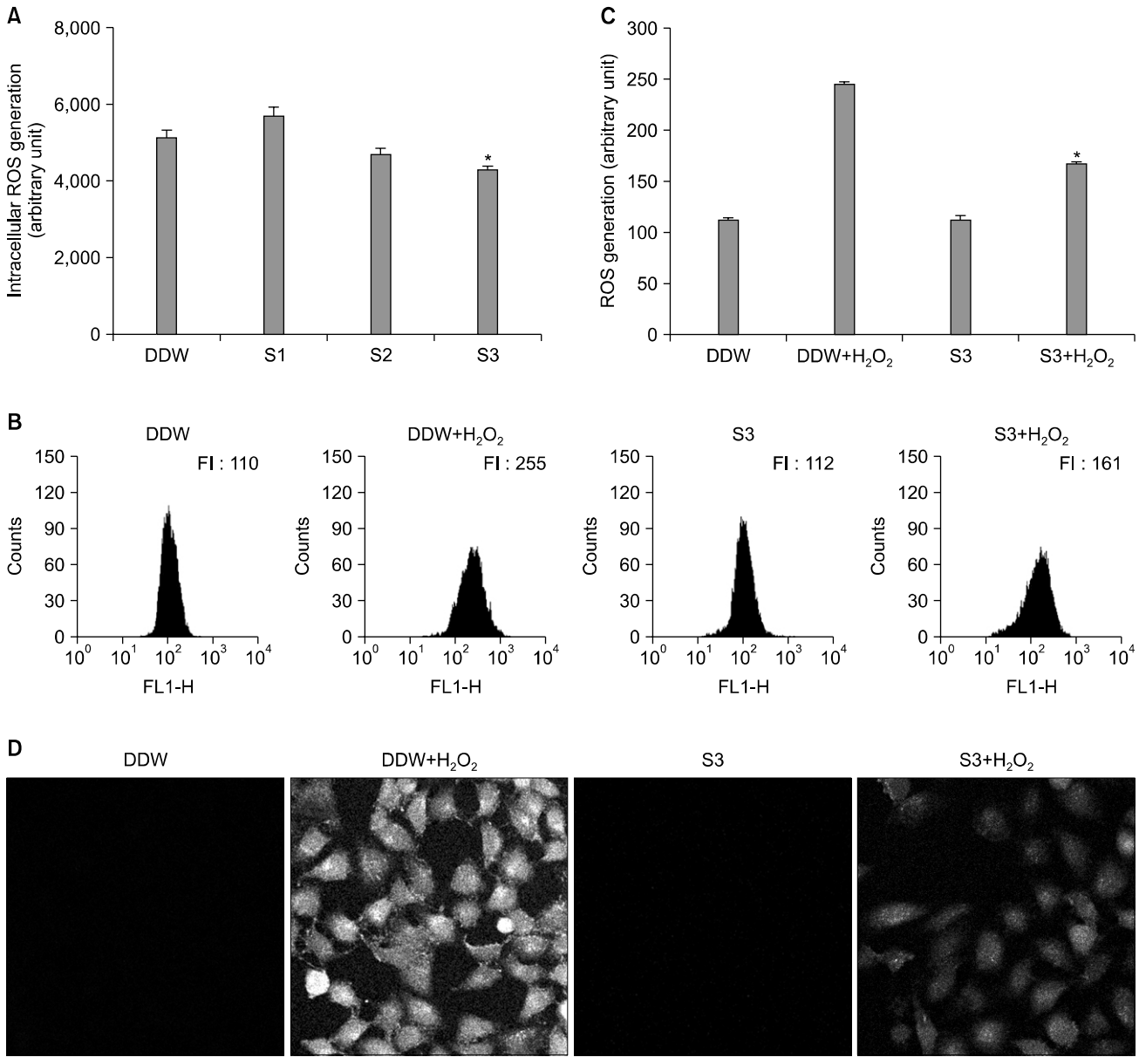


Fig. 5. Scavenging effect of Jeju waters against intracellular ROS. (A) DDW or Jeju waters cultured cells (10 passages) were treated with H_2O_2. After an additional 30 min, the DCF-DA was added and intracellular ROS generated were detected by spectrofluorometry. *Significantly different from DDW group ($p<0.05$). Intracellular ROS generated were detected by flow cytometry after the DCF-DA treatment. Results are expressed (B) representative raw data and (C) histogram (mean±SE). *Significantly different from DDW cells ($p<0.05$). (D) The representative confocal images illustrate increase in the red fluorescence intensity of DCF produced by ROS in DDW and H_2O_2 treated cells compared to S3 and H_2O_2 treated cells.

on the balance of oxidant-antioxidant are limited and remained controversial.[24~29] Vanadium is a catalytic metal and, as reported in some studies, it is probable that vanadium induces oxidative damage, lipid peroxidation and changes in the hematological, reproductive and respiratory systems.[24~26,30,31] However, it has been suggested that vanadium exerted antioxidant effects in other studies,[27~29] which is consistent with our findings.

4. Intracellular ROS scavenging activity of Jeju waters

The DCF-DA method was used to detect the levels of intracellular ROS.[22] This assessment can be achieved by inducing antioxidant enzymes such as catalase and superoxide dismutase. Typical antioxidants, such as polyphenols, show antioxidant effects *in vivo*, and are not only dependent on direct scavenging of ROS. Polyphenols may also offer indirect protection by activating endogenous defense systems.[32] This is the reason why the DCF-DA method is more appropriate to determine antioxidant activity associate with weak direct radical scavenging activity. In our system, the S3 group significantly showed intracellular ROS radical scavenging activity (Fig. 5A) on a spectrofluorometer. The quantity of intracellular ROS radical (arbitrary unit) in S1, S2, and S3 groups were 5683, 4679 and 4272 respectively compared to 5106 in the DDW group. Moreover, the fluorescence intensity of DCF-DA staining was measured by flow cytometry and confocal microscopy. The level of ROS detected using a flow cytometer revealed a fluorescence intensity of 161 for ROS stained by DCF-DA fluorescence dye in S3 and H_2O_2 treated cells compared to that of 255 in the DDW and H_2O_2 treated cells (Fig. 5B, C). Moreover, confocal microscopy showed that S3 reduced red fluorescence intensity with H_2O_2 treatment compared to that in the DDW and H_2O_2 treated cells (Fig. 5D), indicating a reduction in ROS generation. It has been reported that vanadyl ions (oxidation state of vanadium) inhibited excess nitric oxide production from the macrophages in the streptozotocin-induced diabetic model. Vanadium exhibited protective effect against genotoxicity and carcinogenesis in rat colon by down-regulating inducible nitric oxide synthase.[33] Considering vanadium is related with suppression of nitric oxide and carcinogenesis, we hypothesized that Jeju waters containing vanadium may be possess antioxidant activity.

CONCLUSION

Jeju waters containing vanadium components; S1 (8.0±0.9 μg/l), S2 (24.0±2.0 μg/l), and S3 (26.0±2.0 μg/l) significantly scavenged superoxide anion induced by xanthine/xanthine oxidase system, and hydroxyl radicals induced by the Fenton reaction ($H_2O_2+FeSO_4$) compared to DDW group. Furthermore, cells treated with S3 significantly scavenged intracellular ROS compared to cells treated with DDW, as measured by spectrofluorometry, flow cytometry, and confocal microscopy after staining with 2',7'-dichlorodihydrofluorescein diacetate. These results suggest that Jeju waters containing vanadium components possessed antioxidant effect via ROS scavenging. However, the effects of vanadium on the oxidant-antioxidant balance remain controversial. Further studies are required to explain the mechanisms by which Jeju waters exert antioxidant effects on cells, perticularly in terms of antioxidant enzymes.

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