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Research article

Magnesium-enriched deep-sea water inhibits NLRP3 inflammasome activation and dampens inflammation

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ABSTRACT

The NLRP3 inflammasome is an essential component of the innate immune system, but excessive activation can lead to inflammatory diseases. Ion fluxes across the plasma membrane or from intracellular stores are known to regulate NLRP3 inflammasome activation. Deep-sea water (DSW) contains high concentrations of many mineral ions, which could potentially influence NLRP3 inflammasome activation. However, the impact of DSW on NLRP3 inflammasome activation has not been investigated. Here, we demonstrated that DSW with water hardness levels up to 500 mg/L did not affect cell viability or the expression of NLRP3 inflammasome components in macrophages derived from THP-1 cells. However, the DSW significantly inhibited IL-1β secretion and caspase-1 activation in response to NLRP3 activators such as nigericin, ATP, or monosodium urate (MSU) crystals. Mechanically, it was discovered that the presence of 5 mM magnesium ions ($Mg2^+$), equivalent to the $Mg2^+$ concentration found in the DSW with a water hardness of 500 mg/L, inhibits NLRP3 inflammasome activation. This indicates that $Mg2^+$ contributes to the

Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; CAPS, cryopyrin-associated periodic syndrome; DSW, deep-sea water; ECL, enhanced chemiluminescence; IACUC, Institutional Animal Care and Use Committee; LDH A, lactate dehydrogenase A; $Mg2^+$, magnesium ions; MSU, monosodium urate; NLRP3 NOD-, LRR- and pyrin domain-containing protein 3; PBS, phosphate-buffered saline; PKR, double-stranded RNA-dependent protein kinase; PMA, phorbol myristate acetate; pro-IL-1β, pro-interleukin 1 beta; RPMI, Roswell Park Memorial Institute.

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mechanism by which DSW mitigates NLRP3 inflammasome activation. Moreover, DSW administration effectively lessens MSU-triggered peritonitis in mice, a commonly used model for examining the impacts of NLRP3 inflammasome activation. These results show that DSW enriched with Mg^{2+} could potentially be beneficial in modulating NLRP3 inflammasome-associated diseases.

1. Introduction

NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome is a complex of proteins found in the cytoplasm, activated by disturbances from infections or non-infectious injuries [1]. When activated, NLRP3 begins to form oligomers and attracts the protein apoptosis-associated speck-like protein containing a CARD (ASC), which in turn activates caspase-1. This enzyme is crucial for converting pro-interleukin 1 beta (pro-IL-1β) into IL-1β, an important proinflammatory cytokines [2]. Growing research shows that uncontrolled NLRP3 inflammasome activation is linked to the development of multiple inflammatory conditions, including gout, diabetes, atherosclerosis, and Muckle-Wells syndrome [3]. In the case of gout, NLRP3 inflammasome activation in macrophages is specifically caused by the presence of monosodium urate (MSU) crystals [4,5].

NLRP3 inflammasome activation involves a two-step process requiring both priming and activation signals [1]. First, a priming signal is necessary to prepare the macrophages for activation. This priming step is triggered by various factors which activate NF-κB. As a result, expression of NLRP3 and pro-IL-1β increases. Following priming, NLRP3, which is a pattern recognition receptor, can be activated by a wide range of bacterial pore-forming toxins (e.g., nigericin), endogenous danger molecules (e.g., ATP) [6], and crystal molecules (e.g., MSU) [7], which leads to assembly of the NLRP3 inflammasome. Despite the chemical and structural diversity of these activating stimuli, they all seem to trigger a common cellular signal that NLRP3 can sense. These signals include ion flux, mitochondrial dysfunction, lysosomal disruption, trans-Golgi disassembly and metabolic alterations [2]. Notably, emerging evidence suggests that changes in the intracellular concentrations of different ions, such as potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), manganese, zinc, iron, and chloride, may modulate NLRP3 activation by various activators [8]. The permeation of the plasma cell membrane to K^+ and Na^+ is a common response induced by many NLRP3 agonists [9]. A reduction in intracellular K^+ concentration is sufficient to activate the NLRP3 inflammasome, while an increase in intracellular Na^+ is not strictly necessary for inflammasome activation. Consequently, potassium efflux enhances NLRP3 inflammasome activation by promoting the interaction between NLRP3 and NEK7 [10]. Specifically, an increase in cytoplasmic Ca^{2+} levels plays a crucial role in NLRP3 inflammasome activation triggered by various stimuli [11,12]. Ca^{2+} can originate from different sources, including the endoplasmic reticulum, lysosomal lumen, and the extracellular environment [8]. Interestingly, extracellular Mg^{2+} antagonizes the role of Ca^{2+} in NLRP3 inflammasome activation by inhibiting Ca^{2+} influx [12,13].

Deep-sea water (DSW) is defined as ocean water from a depth of over 200 m [14]. Salient features of DSW include its high inorganic nutrient salt concentration, clarity, enriched trace elements, and stability [15]. Previous research indicates that using DSW as a treatment can be effective in addressing health issues commonly linked to lifestyle diseases, including diabetes, obesity, cardiovascular disease, cancer, and dermatological conditions [16]. DSW treatment could facilitate a decrease in the incidence of cardiovascular disease [15,17], inhibit the progression of atherosclerosis [18], improve glucose tolerance and regulate function of glucagon and insulin [19,20], inhibit adipocyte differentiation [21–23], inhibit the metastatic potential of malignant cells [24], and protect the mice from 2,4-dinitro-chlorobezene-induced atopic dermatitis-like skin lesions [25]. In addition, DSW promotes osteoporosis recovery through bone regeneration [26] and accelerates recovery from physical fatigue [27]. Although DSW is known to provide potential health benefits, more evidence is still needed to understand the mechanisms whereby DSW regulates physiological functions.

Using macrophage cell culture *in vitro* as well as a mouse peritonitis model *in vivo*, we propose that DSW treatment has beneficial effects by modulating NLRP3 inflammasome-associated diseases. We shows that DSW with a water hardness of 500 mg/L can inhibit NLRP3 inflammasome activation in response to various stimuli, and that Mg^{2+} plays an active role in the effects of DSW by inhibiting NLRP3 inflammasome activation. Finally, we show that treating mice with DSW, which is enriched in Mg^{2+}, lessens MSU-induced peritonitis—a model frequently used to explore the effects of NLRP3 inflammasome activation *in vivo*. These results enhance our knowledge of magnesium's influence on NLRP3 inflammasome activation and imply that DSW with high magnesium content may mitigate diseases associated with this inflammasome.

2. Materials and methods

2.1. Reagents

$MgCl_2$, nigericin, ATP, and phorbol myristate acetate (PMA) were procured from Sigma-Aldrich Chemical Company. MSU crystals were sourced from InvivoGen.

2.2. Cell culture

THP-1 human monocytic leukemia cells, obtained from the Biosource Collection and Research Center (Taiwan), were cultured in Roswell Park Memorial Institute (RPMI) medium as described previously [5]. Differentiation into macrophages was induced by

treating the cells with 200 nM PMA for 20 h, adhering to previously established protocols [28]. For experimental treatments, the DSW was diluted to predetermined hardness levels (100, 300, or 500 mg/L) using cell culture medium. Post-differentiation, macrophages were incubated for 24 h in media containing either the adjusted DSW or 5 mM $MgCl_2$. Subsequently, cells were refreshed with media maintaining identical DSW or $MgCl_2$ concentrations. Inflammasome activation was achieved by treating the cells with 10 μM nigericin for 45 min, 5 mM ATP for 1 h, or 200 μg/mL MSU crystals for 2 h.

2.3. Preparation of DSW

DSW enriched with Mg^{2+} was provided by the Stone and Resource Industry Research and Development Center, Taiwan. DSW was collected at the site far from the coastline, 1.7 km, and at a depth of 312 m of the Pacific Ocean near the Zhiben River in Taiwan and filtered with membranes to remove calcium and sulfuric acid. To enrich the crude DSW with Mg^{2+}, it was subjected to vacuum concentration until a hardness of 250,000 mg/L as $CaCO_3$ was achieved. The DSW used in this study was sterilized through filtration and then utilized. The hardness of DSW was determined based on the Mg^{2+} and Ca^{2+} concentrations as follows: hardness = Mg^{2+} (mg/L) x 4.116 + Ca^{2+} (mg/L) x 2.497. The mineral content of the DSW at a hardness of 250,000 mg/L was shown in Table 1. Based on the mineral content of DSW at a hardness of 250,000 mg/L (Table 1), we prepared artificially constructed DSW with a hardness of 250,000 mg/L by mixing 2,500 mM $MgCl_2$, 37.9 mM NaCl, 13.8 mM KCl, and 0.6 mM $CaCl_2$ in water.

2.4. Cell Counting Kit-8 assay

For viability assays, THP-1 cells were plated at a density of 7.2×10^4 cells per well in a 96-well format and differentiated into macrophages using PMA. Post-differentiation, cells were exposed to DSW at various hardness levels for 24 h. Cell viability was then determined by adding 10 μl of Cell Counting Kit-8 solution (Dojindo Molecular Technologies, Japan) to each well, followed by a 2-h incubation. Viability was quantified by measuring the absorbance at 450 nm, with results expressed as the absorbance difference between treated/control wells and blank wells.

2.5. Animal experiments

The mouse studies adhered to ethical guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of MacKay Medical College (approval number A1090020). C57BL/6 mice were sourced from the National Laboratory Animal Center in Taiwan. To investigate the impact of DSW on peritonitis, we diluted the DSW to a hardness of 250 mg/L using phosphate-buffered saline (PBS). Subcutaneously, mice received pretreatment with 2 mL of this diluted DSW near the dorsal skin at 0.5, 12, and 24 h before MSU treatment, following a previously described protocol [29]. PBS served as the control in this experiment. Peritonitis induction followed a modified version of our previous studies [30]. Three hours after administering 1 mg of MSU, we collected peritoneal lavage fluid for analysis. Peritoneal cells were stained with specific antibodies and analyzed for neutrophil presence ($CD45^+/Ly6G^+/CD11b^+$) using the CytoFLEX S flow cytometer by Beckman Coulter. Antibodies included CD11b-BV605 (Cat # 101257) and CD45-APC (Cat # 103112) from BioLegend, along with Ly6G-PE (Cat # 551461) from BD Biosciences. All experimental protocols were approved by the IACUC of MacKay Medical College and adhered to the ARRIVE guidelines 2.0 (https://arriveguidelines.org) and relevant guidelines. The mice treated with three subcutaneous treatments of 2 mL each of either DSW or PBS showed increased urination. Nonetheless, there was no difference between the two groups, and their appetite and activity levels were unaffected.

2.6. Immunoblot analysis

We conducted experiments following our established protocol with modifications [28]. Cells were lysed using RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl, and 1 % Igepal CA-630, supplemented with a protease inhibitor mix (2 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL pepstatin). The lysate was chilled on ice for 30 min. For assessing matured IL-1β and cleaved caspase-1, supernatants were treated with a dilution of trichloroacetic acid (10 % final concentration) and cooled at 4 °C for 10 min. Proteins from these samples were resolved by electrophoresis on 10 % SDS polyacrylamide gels, and transferred to PVDF membranes from Millipore. Blots were incubated with primary antibodies targeting ASC (Santa Cruz #sc-22514), NLRP3 (Adipogen #AG-20B-0014-C100), IL-1β (Santa Cruz #sc-32294), cleaved caspase-1 (Cell Signaling #3866), pro-caspase-1 (Cell Signaling #2225), and GAPDH (Santa Cruz #sc-32233), followed by HRP-linked secondary antibodies. Bands were visualized using Clarity™ Western ECL or Immobilon ECL Ultra Western HRP substrates (Millipore),

Table 1
Mineral content of DSW at a hardness of 250,000 mg/L used in this study.

Mineral	Concentration (mg/L)
Mg^{2+}	60,695
Na^+	870
K^+	540
Ca^{2+}	25

and band intensities were analyzed using ImageJ software.

2.7. IL-1β ELISA

We analyzed both mouse peritoneal lavage fluids and cell culture supernatants for the presence of mouse IL-1β and human IL-1β, respectively, using kits from eBioscience. The procedures followed were consistent with those described in previous studies [5].

2.8. Statistical analysis

All statistical analyses employed either the two-way ANOVA followed by Tukey's post hoc test for multiple comparisons or the Student's t-test for paired samples using SPSS software as outlined in previous studies [31]. A p-value below 0.05 was deemed to indicate statistical significance.

3. Results

3.1. DSW had a high abundance of many mineral ions

Ion flux, from external or internal sources, has a large effect on NLRP3 inflammasome activation [8]. DSW is rich in many mineral ions, which could potentially influence NLRP3 inflammasome activation. However, the impact of DSW on NLRP3 inflammasome activation has not been investigated until now. Hence, we performed a study to evaluate the impact of DSW on NLRP3 inflammasome activation. In this study, the DSW was rich in many ions, including magnesium, sodium, potassium, and calcium (Table 1). The hardness of DSW was determined based on the Mg^{2+} and Ca^{2+} concentrations as follows: hardness = Mg^{2+} (mg/L) x 4.116 + Ca^{2+} (mg/L) x 2.497. Thus, the DSW we obtained had a hardness of 250,000 mg/L as shown in Table 1.

3.2. DSW at a water hardness of 500 mg/L did not affect cell viability

Because DSW is a hypertonic solution that may cause cell death at long incubation times, we first assessed the effect of DSW on cell viability. The THP-1-derived macrophages in the culture medium were treated with or without DSW for 24 h, and the cell viability was evaluated by CCK-8 assay. As shown in Fig. 1, DSW with hardness levels up to 500 mg/L did not affect the cell viability (Fig. 1). Therefore, we excluded the possibility that DSW may cause cell death in our tested conditions.

3.3. Inhibition of NLRP3 inflammasome activation by DSW

We also studied if DSW affected the priming signal of the NLRP3 inflammasome in macrophages by measuring the expression levels of its components. After exposure to DSW for 12 or 24 h, there was no change in the expression levels of NLRP3, ASC, pro-caspase-1 (p45 kDa), and pro-IL-1β (p31 kDa) between macrophages treated with or without DSW (Fig. 2A), indicating that DSW did not influence the NLRP3 inflammasome priming signal. Therefore, we further explored how DSW impacts NLRP3 inflammasome activation in macrophages by measuring the secretion of IL-1β and the expression levels of cleaved caspase-1 (p20 kDa) and mature IL-1β (p17 kDa) in response to NLRP3 stimuli, including nigericin, ATP, or MSU. Upon NLRP3 inflammasome activation, IL-1β secretion was reduced in DSW-treated cells in a dose-dependent manner compared with untreated cells (Fig. 2B). Consistently, cleaved caspase-1 and mature IL-1β expression levels were reduced in cells treated with DSW compared to those in untreated cells (Fig. 2C). These results demonstrated that DSW hinders the activation signal of the NLRP3 inflammasome rather than the priming signal. Given that DSW inhibited NLRP3 inflammasome activation in response to three different NLRP3 stimuli, the results suggest that DSW inhibited a common pathway of NLRP3 inflammation activation. The original blots of Fig. 2 were placed in Supplementary Fig. 1.

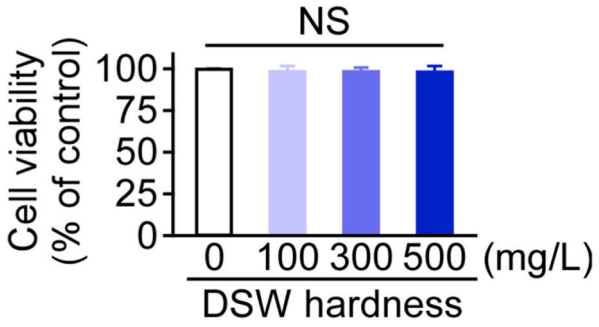

Fig. 1. Effect of DSW on cell viability of THP-1-derived macrophages. Cells were incubated for 24 h in cell culture medium containing diluted DSW at a hardness of 100, 300, and 500 mg/L, or medium alone (0 mg/L), as indicated. Cell viability was assessed using the medium without DSW as the baseline, set at 100 %. The data represent the mean and standard deviation (mean ± SD) of four independent experiments.

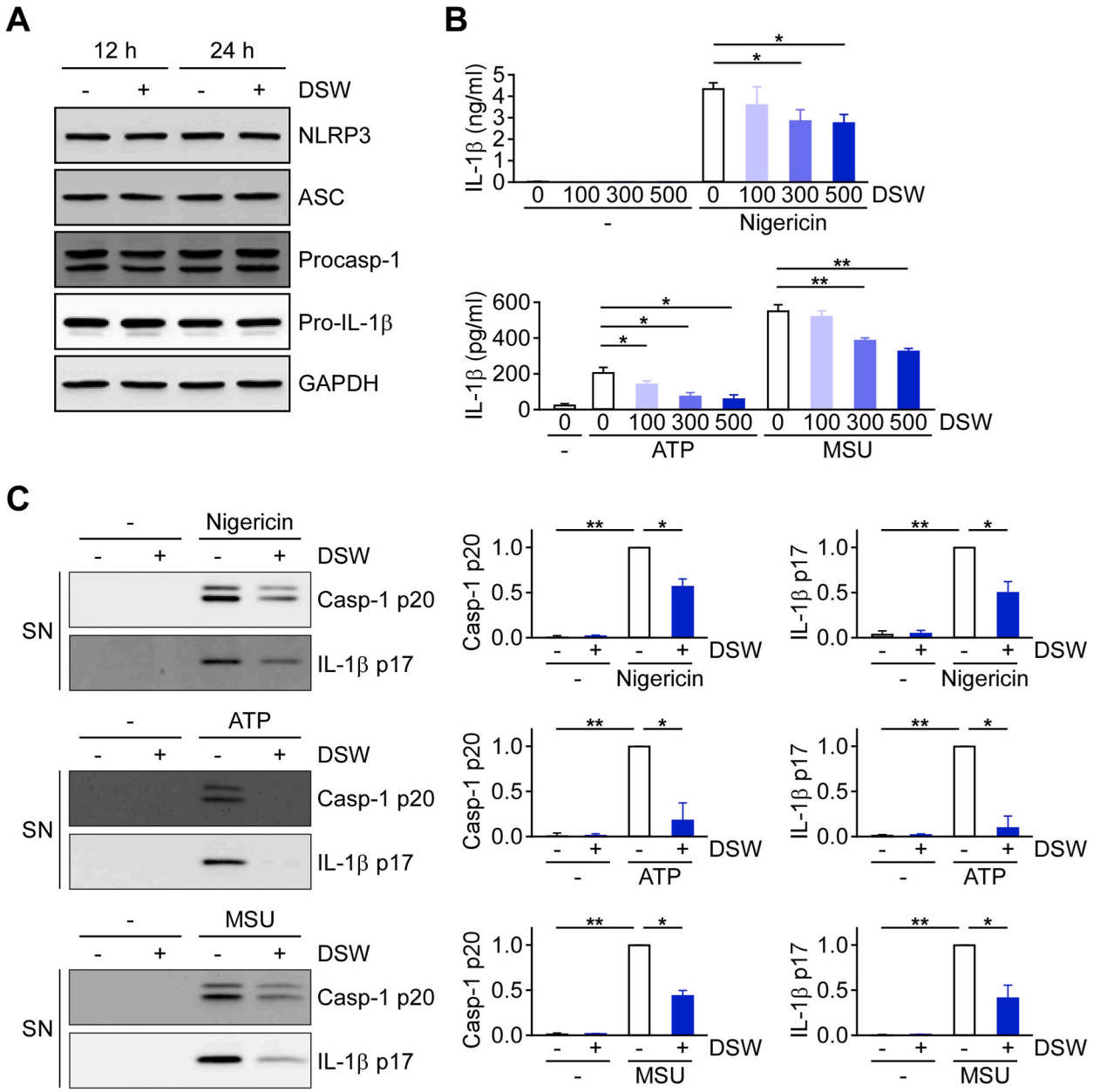

Fig. 2. Effect of DSW on NLRP3 inflammasome activation. A Immunoblot analysis of NLRP3 inflammasome molecules in cell lysates. THP-1-derived macrophages were incubated for 12 or 24 h in cell culture medium containing diluted DSW at a hardness of 500 mg/L, or in medium alone. GAPDH was used as the internal control. B IL-1β ELISA on the culture supernatant. THP-1-derived macrophages were incubated for 24 h in cell culture medium containing diluted DSW at a hardness of 100, 300, and 500 mg/L, or in medium alone, followed by stimulation with 10 μM nigericin for 45 min, 5 mM ATP for 1 h, or 200 μg/mL MSU for 2 h n = 4 biological replicates. The mean ± SD was shown. *$p < 0.05$ and **$p < 0.01$ by two-way ANOVA with Tukey's multiple comparisons test. C Immunoblot analysis of NLRP3 inflammasome molecules in culture supernatants. THP-1-derived macrophages were incubated for 24 h in cell culture medium containing diluted DSW at a hardness of 500 mg/L, or in medium alone, followed by stimulation with 10 μM nigericin for 45 min, 5 mM ATP for 1 h, or 200 μg/mL MSU for 2 h. The densitometric analysis of caspase-1 (p20) and IL-1β (p17) was shown in the right panel. n = 3 biological replicates. The mean ± SD was shown. *$p < 0.05$ and **$p < 0.01$ by two-way ANOVA with Tukey's multiple comparisons test.

3.4. *Mineral ions in DSW mediate inhibition of NLRP3-dependent IL-1β secretion*

Recent reports showed that extracellular Mg^{2+} antagonizes the role of Ca^{2+} in NLRP3 inflammasome activation by Ca^{2+} influx inhibition [12,13]. Additionally, K^+ efflux is recognized as the common trigger for NLRP3 inflammasome activation by bacterial toxins and particulate matter [9]. To explore the mechanisms underlying DSW-mediated inhibition of the NLRP3 inflammasome, we

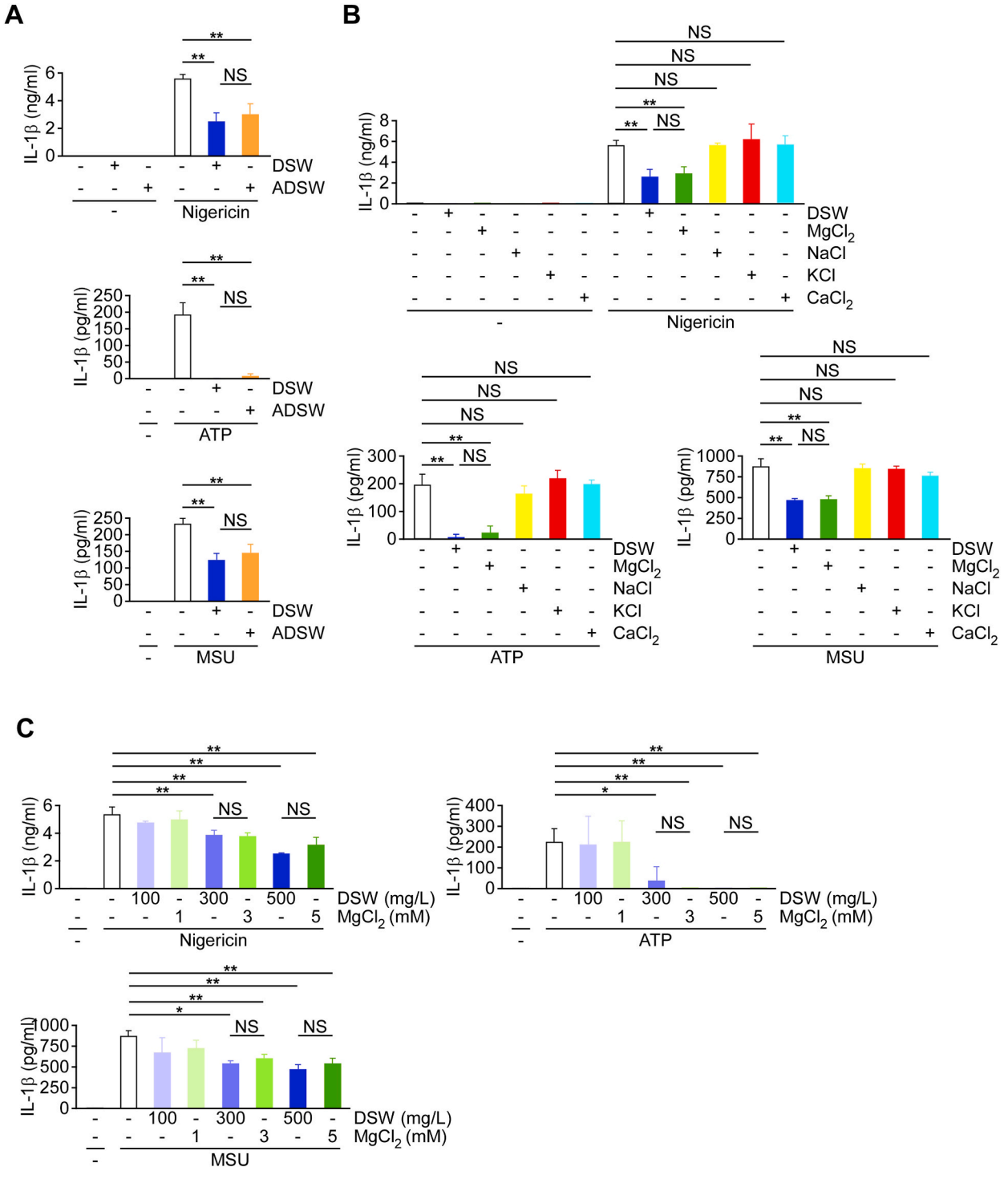

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Fig. 3. Effect of the mineral ions in DSW on NLRP3-dependent IL-1β secretion. A IL-1β ELISA on the culture supernatant. THP-1-derived macrophages were incubated for 24 h in cell culture medium with or without the diluted DSW or artificially constructed DSW at a hardness of 500 mg/L, followed by stimulation with 10 μM nigericin for 45 min, 5 mM ATP for 1 h, or 200 μg/mL MSU for 2 h n = 4 biological replicates. The mean ± SD was shown. *$p < 0.05$ and **$p < 0.01$ by two-way ANOVA with Tukey's multiple comparisons test. NS no significant difference ($p > 0.05$). B IL-1β ELISA on the culture supernatant. THP-1-derived macrophages were incubated for 24 h in cell culture medium containing either diluted DSW at a hardness of 500 mg/L, 5 mM $MgCl_2$, 75.7 μM NaCl, 27.6 μM KCl, 1.2 μM $CaCl_2$, or in medium alone, followed by stimulation with 10 μM nigericin for 45 min, 5 mM ATP for 1 h, or 200 μg/mL MSU for 2 h n = 4 biological replicates. The mean ± SD was shown. *$p < 0.05$ and **$p < 0.01$ by two-way ANOVA with Tukey's multiple comparisons test. NS no significant difference ($p > 0.05$). C IL-1β ELISA on the culture supernatant. THP-1-derived macrophages were incubated for 24 h in cell culture medium containing either diluted DSW, $MgCl_2$, or in medium alone as indicated, followed by stimulation with 10 μM nigericin for 45 min, 5 mM ATP for 1 h, or 200 μg/mL MSU for 2 h n = 4 biological replicates. The mean ± SD was shown. *$p < 0.05$ and **$p < 0.01$ by two-way ANOVA with Tukey's multiple comparisons test. NS no significant difference ($p > 0.05$).

examined the effect of the mineral ions in DSW on NLRP3 inflammasome inhibition using artificially constructed DSW composed of the same proportion of ionized water. In response to NLRP3 stimuli, both 500 mg/L DSW and 500 mg/L artificially constructed DSW reduced IL-1β secretion to similar levels (Fig. 3A), indicating that the mineral ions in DSW play a role in regulating the NLRP3 inflammasome. We further examined the effect of the full spectrum of DSW minerals that might be involved in regulating NLRP3 inflammasome activity. According to Table 1, DSW at a hardness of 250,000 mg/L contains 60,695 mg/L Mg^{2+}, 870 mg/L Na^+, 540 mg/L K^+, and 25 mg/L Ca^{2+} ions. When the cells were treated with DSW at a hardness of 500 mg/L, they were exposed to 121.39 mg/L (5 mM) Mg^{2+}, 1.74 mg/L (75.7 μM) Na^+, 1.08 mg/L (27.6 μM) K^+, and 0.05 mg/L (1.2 μM) Ca^{2+} ions. Therefore, we compared the effects of 500 mg/L DSW to those of 5 mM $MgCl_2$, 75.7 μM NaCl, 27.6 μM KCl, and 1.2 μM $CaCl_2$, which correspond to the concentrations of Mg^{2+}, Na^+, K^+, and Ca^{2+} ions in DSW with a hardness of 500 mg/L, on NLRP3 inflammasome activation. In response to NLRP3 stimuli, the levels of IL-1β secretion were reduced similarly in $MgCl_2$- or DSW-treated cells compared with untreated cells (Fig. 3B). Treatments with NaCl, KCl, and $CaCl_2$ did not affect IL-1β secretion compared to untreated cells. To determine whether the effect of DSW is primarily due to Mg^{2+}, we compared a range of DSW hardness levels at 100, 300, and 500 mg/L with Mg^{2+} solutions at equivalent concentrations of 1, 3, and 5 mM $MgCl_2$. Similarly to DSW, Mg^{2+} treatment had a dose-dependent effect in reducing IL-1β secretion (Fig. 3C). Notably, there was no difference in IL-1β secretion levels between cells treated with DSW and those treated with Mg^{2+} at equivalent concentrations. These results suggest that Mg^{2+} plays a major role in DSW-mediated inhibition of NLRP3-dependent IL-1β secretion.

3.5. Magnesium ions in DSW mediate inhibition of NLRP3 inflammasome

Next, we investigated whether Mg^{2+} affected the priming signal of the NLRP3 inflammasome in macrophages by measuring the

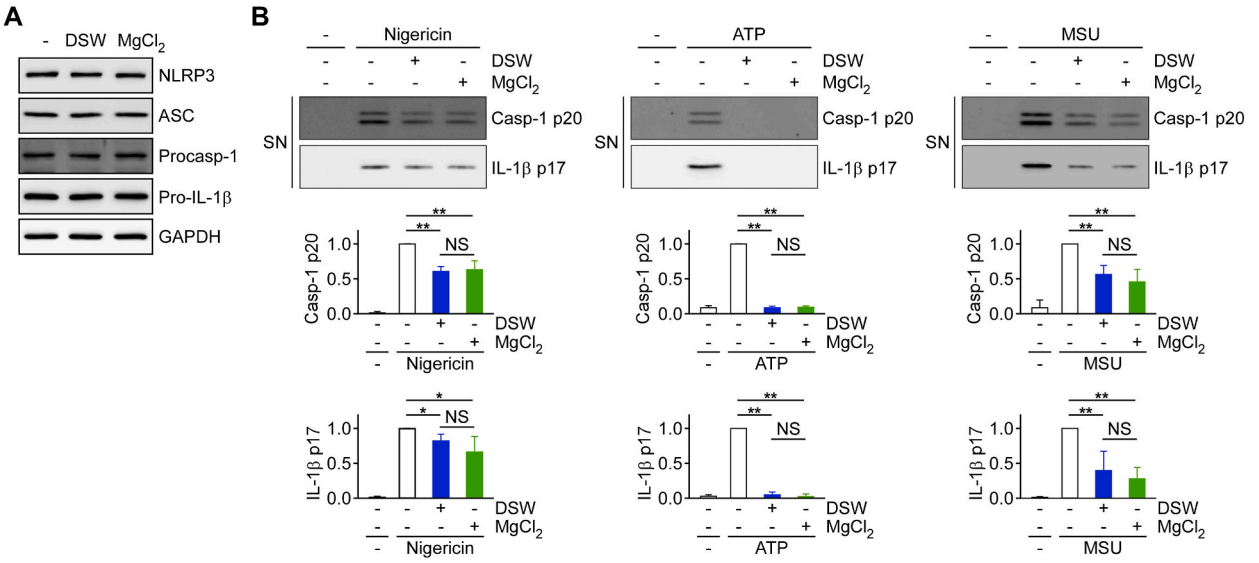

Fig. 4. Effect of Mg^{2+} on NLRP3 inflammasome activation. A Immunoblot analysis of NLRP3 inflammasome molecules in cell lysates. THP-1-derived macrophages were incubated for 24 h in cell culture medium containing either diluted DSW at a hardness of 500 mg/L, 5 mM $MgCl_2$, or in medium alone. GAPDH was used as the internal control. B Immunoblot analysis of NLRP3 inflammasome molecules in culture supernatants. THP-1-derived macrophages were incubated for 24 h in cell culture medium containing either diluted DSW at a hardness of 500 mg/L, 5 mM $MgCl_2$, or in medium alone, followed by stimulation with 10 μM nigericin for 45 min, 5 mM ATP for 1 h, or 200 μg/mL MSU for 2 h. The densitometric analysis of caspase-1 (p20) and IL-1β (p17) was shown in the right panel. n = 3 biological replicates. The mean ± SD was shown. *$p < 0.05$ and **$p < 0.01$ by two-way ANOVA with Tukey's multiple comparisons test. NS no significant difference ($p > 0.05$).

expression levels of its components. Like DSW, $MgCl_2$ treatment for 24 h did not change the expression levels of NLRP3 inflammasome molecules in macrophages (Fig. 4A). Importantly, the expression levels of cleaved caspase-1 and mature IL-1β were reduced in $MgCl_2$- or DSW-treated cells compared with untreated cells (Fig. 4B). These results demonstrated that Mg^{2+} plays a major role in DSW-mediated inhibition of NLRP3 inflammasome activation. The original blots of Fig. 4 were placed in Supplementary Fig. 2.

3.6. DSW protected mice from MSU-induced peritonitis

To evaluate the physiological significance of the inhibitory effect of DSW on NLRP3 inflammasome activation, we utilized a mouse model of MSU-mediated peritonitis, a well-established disease model for examining the effects of NLRP3 inflammasome activation (Fig. 5A) [5]. DSW was prepared at a hardness of 250 mg/L using PBS, with PBS serving as the control for our experiments. Considering the need to administer a substantial volume (2 mL) of fluid, subcutaneous injections were preferred over intravenous injections to control the administered fluid volume effectively and to ensure consistency across treatments, rather than opting for oral administration. This approach allowed us to investigate whether DSW treatment could mitigate NLRP3 inflammasome activation triggered by MSU, assessed both *in vitro* (Fig. 2C) and *in vivo*. Our findings indicated that MSU notably induced IL-1β secretion (Fig. 5B) and neutrophil influx (Fig. 5C) into the peritoneal cavity. However, these inflammatory responses were significantly inhibited by pretreatment with DSW, suggesting a protective role of DSW against NLRP3 inflammasome-mediated inflammation.

4. Discussion

Infection or sterile injury is sensed in the cytosol of cells by the NLRP3 protein, which assembles into the NLRP3 inflammasome complex [1]. This complex, in turn, activates caspase-1, which is responsible for processing and release of inflammatory cytokines like IL-1β and IL-18. These cytokines play a role in the development of persistent low-grade inflammation in the body, which is associated with inflammation-related diseases. Therefore, targeting the NLRP3 inflammasome has the potential to offer therapeutic benefits in managing conditions related to inflammation. In this regard, we studied the effect of DSW on NLRP3 inflammasome activation *in vitro* and *in vivo*. The results demonstrated that Mg^{2+} in DSW inhibits NLRP3 activation in macrophages *in vitro* due to stimulation with three different stimuli, and it inhibits MSU-induced inflammation in the peritoneal cavity in mice.

Recent studies have highlighted the role of the NLRP3 inflammasome role in the pathogenesis of various inflammation-associated diseases. NLRP3 has been proposed as a therapeutic target for conditions including cancer [3], metabolic diseases (like diabetes and obesity) [3], neurodegenerative disorders (such as Parkinson's disease, Alzheimer's disease, Huntington's disease, prion diseases, and amyotrophic lateral sclerosis) [32], rheumatic disease [33], cardiovascular diseases [34], non-alcoholic fatty liver disease and steatohepatitis [35], COVID-19 [36], multiple sclerosis [37], inflammatory bowel disease [38], chronic obstructive pulmonary disease [39], and gout [40]. Our findings reveal that DSW can inhibit NLRP3 inflammasome activation in macrophages in response to MSU

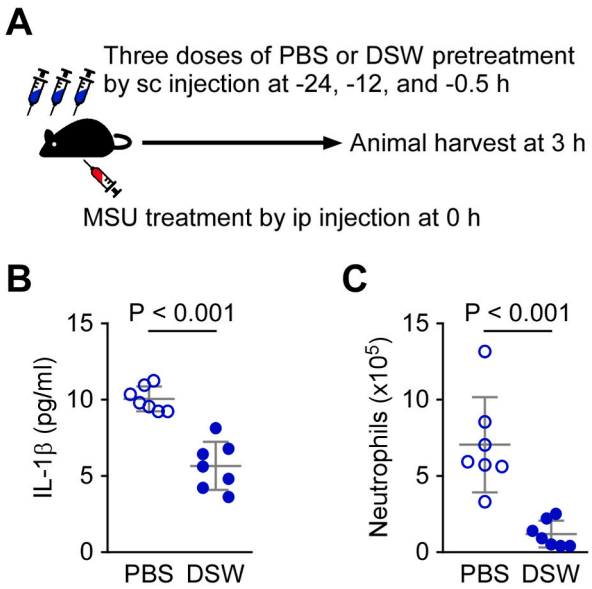

Fig. 5. Protective effect of DSW on MSU-induced peritonitis. (A) Schematic presentation of MSU-induced peritonitis in mice. Mice were treated with PBS containing diluted DSW at a hardness of 250 mg/L, or with PBS alone, at 0.5, 12, and 24 h prior to MSU treatment. Three hours post-MSU treatment, peritoneal lavage fluid was collected. (B) Determination by ELISA of IL-1β in the peritoneal lavage fluid. (C) Absolute number of CD45+/CD11b+/Ly6G + neutrophils in the peritoneal lavage fluid. Data are from three independent experiments with a total of seven mice in each group. Each symbol represents an individual mouse; small horizontal lines indicate the mean ± SD. All results were analyzed with the Student's t-test.

crystals *in vitro* and reduce MSU crystals-induced peritonitis in mice *in vivo*, underscoring the potential of DSW to alleviate gout symptoms. Notably, DSW inhibiting NLRP3 inflammasome activation extends to reactions induced by MSU crystals, the bacterial pore-forming toxin nigericin, and the endogenous danger molecule ATP. This broad-spectrum inhibition parallels the effects achieved with Mg^{2+}, which impedes Ca^{2+} influx—a known trigger of NLRP3 inflammasome activation [12,13]. Thus, our results indicate the significant therapeutic potential of DSW in addressing the severity of various NLRP3 inflammasome-associated inflammatory diseases triggered by diverse stimuli. Furthermore, DSW exhibits protective effects against diabetes and atherosclerosis [18–20], diseases where NLRP3 inflammasome activation plays a critical role in pathogenesis [3].

However, we also note that the inhibition efficacy of DSW varies with different NLRP3 activators. ATP-mediated activation is completely blocked by DSW, while nigericin and MSU activation are only partially inhibited. This difference may be due to the distinct mechanisms and pathways involved in NLRP3 inflammasome activation by ATP, nigericin, and MSU. ATP activates NLRP3 through the P2X7 purinergic receptor, causing a weak Ca^{2+} influx [41]. The calcium-sensing receptor then activates the NLRP3 inflammasome through phospholipase C, which catalyzes inositol-1,4,5-trisphosphate production, thereby inducing the release of Ca^{2+} from endoplasmic reticulum stores [12]. The increased cytoplasmic Ca^{2+} promotes the assembly of inflammasome components. Although nigericin and MSU, like ATP, depend on Ca^{2+} flux to activate the NLRP3 inflammasome [12], their specific pathways differ. Nigericin, a K^+/H^+ ionophore, activates NLRP3 by lowering intracellular K^+ through exchange with H^+ ions [9]. MSU causes lysosomal damage, releasing cathepsin B, which is crucial for MSU-induced NLRP3 activation [42] but not for ATP-induced activation [43]. These differences underscore the necessity of tailoring DSW application based on the specific NLRP3 activation pathway involved. By understanding the distinct mechanisms through which various stimuli activate the NLRP3 inflammasome, we could better develop DSW as a therapeutic option for different pathological conditions.

Given that clinical trials have shown that magnesium supplementation can ameliorate inflammatory disorders in older adults [44], and Mg^{2+}-enriched DSW improves insulin sensitivity metrics in prediabetic adults and could be used to reduce the incidence of diabetes [45], Mg^{2+}-enriched DSW could be formulated as oral supplements in liquid solutions to treat NLRP3 inflammasome-associated diseases. Clinical trials would determine the optimal dosage, ensuring patient compliance with convenient formulations. Monitoring would involve assessing biomarkers like IL-1β levels to gauge treatment efficacy and regularly checking serum Mg^{2+} levels to prevent toxicity, as hypermagnesemia can cause adverse effects such as neuromuscular and cardiovascular toxicity [46]. Clinical outcomes, including symptom severity and quality of life, would be closely monitored. Mg^{2+}-enriched DSW could become a valuable treatment for NLRP3 inflammasome-associated diseases, offering a novel therapeutic approach for inflammatory conditions. However, while our studies suggest that Mg^{2+} is the primary component of DSW responsible for inhibiting the NLRP3 inflammasome, the precise molecular mechanisms by which Mg^{2+} inhibits NLRP3 inflammasome activation remain unclear. Additionally, the long-term effects and safety of DSW administration warrant further investigation. Addressing these limitations in future research can provide a more comprehensive understanding of the potential benefits and risks of using Mg^{2+}-enriched DSW to modulate NLRP3 inflammasome-associated diseases.

Emerging evidence suggests that the DSW involves more than just inhibiting Ca^{2+} influx. DSW is shown to modulate glycolysis [47], a critical process for NLRP3 inflammasome activation [48,49]. Upon NLRP3 stimuli, macrophages increase hexokinase-dependent glycolysis, leading to a rise in mitochondrial reactive oxygen species production, essential for activating the NLRP3 inflammasome [48,50]. Furthermore, hexokinase influences lactate production, the end product of glycolysis, which then promotes phosphorylation of the double-stranded RNA-dependent protein kinase (PKR) [49]. Inhibiting PKR has been shown to reduce NLRP3 inflammasome activation [49,51]. Additionally, targeting lactate dehydrogenase A (LDH A), which is responsible for converting pyruvate to lactate, can block NLRP3 inflammasome activation [5]. Intriguingly, treatment with DSW at a water hardness of 500 mg/L, the same concentration used in our study, has been shown to decrease LDH A protein expression in C2C12 myotubes via activation of peroxisome proliferator-activated receptor gamma coactivator-1α [47]. This suggests that DSW may regulate NLRP3 inflammasome activation by modulating lactate metabolism, a hypothesis that warrants further investigation.

Compared to other sources of DSW, the Mg^{2+}-enriched DSW used in our study to inhibit NLRP3 inflammasome activation contains Mg^{2+} (60,695 mg/L), Na^+ (870 mg/L), K^+ (540 mg/L), and Ca^{2+} (25 mg/L) (Table 1). In contrast, the DSW used to regulate lipid metabolism is a balanced DSW containing Mg^{2+} (810.8 mg/kg), K^+ (268.7 mg/kg), Na^+ (264.8 mg/kg), and Ca^{2+} (270.0 mg/kg) [21]. Another formulation for improving abnormal lipid metabolism includes DSW with Mg^{2+} (96,000 mg/L), K^+ (10,000 mg/L), Na^+ (9,000 mg/L), and Ca^{2+} (40 mg/L) [52]. For regulating lactate metabolism, a balanced DSW contains Mg^{2+} (405 mg/L), K^+ (0.8 mg/L), Na^+ (106 mg/L), and Ca^{2+} (134 mg/L) [47]. Additionally, DSW used to modulate cardiovascular hemodynamics has a composition of Mg^{2+} (199.3 mg/L), K^+ (70.4 mg/L), Na^+ (73.6 mg/L), and Ca^{2+} (72.1 mg/L) [53]. Notably, the active component for inhibiting NLRP3 inflammasome activation, Mg2+, is the most concentrated and predominant component in these various DSW formulations. Our Mg^{2+}-enriched DSW, with a hardness of 500 mg/L, effectively inhibits the NLRP3 inflammasome in THP-1-derived macrophages. In comparison, the DSW used by Hwang et al. at a hardness of 1000 mg/L inhibits adipocyte differentiation in 3T3-L1 cells [21]. The DSW used by Ha et al. at a hardness of 2000 mg/L mediates lactate metabolism by regulating the gene expression levels of lactate dehydrogenases A and B, a monocarboxylate transporter, and a mitochondrial pyruvate carrier in C2C12 myoblast cells [47]. Our DSW, with a hardness of 500 mg/L, achieves effective inhibition of the NLRP3 inflammasome at a lower dose compared to the hardness of 1000 and 2000 mg/L used in other studies. Taken together, the active substance Mg^{2+} ion for inhibiting the NLRP3 inflammasome activation is the major component in DSW used across various studies. Therefore, DSW from different sources also has a high potential to inhibit NLRP3 inflammasome activation and modulate NLRP3 inflammasome-mediated inflammatory diseases.

Numerous NLRP3 inflammasome inhibitors have been identified, yet none have received FDA approval for clinical use to date. These inhibitors, which are at various stages of research and development, highlight the continuous effort to find effective treatments for conditions such as malignant diseases, oral mucositis, osteoarthritis, gout, familial cold autoinflammatory syndrome, and

cryopyrin-associated periodic syndrome (CAPS) [54]. Among these drugs, RRx-001, IFM-2427, Dapansutrile, VTX-2735, and ZYIL-1 have progressed to at least Phase II clinical trials [54]. RRx-001 stands out as the most advanced NLRP3 inhibitor in terms of human clinical trials. It is currently undergoing Phase III trials for treating small cell lung cancer (NCT05566041) and was granted fast-track status by the FDA in 2023 to potentially prevent or lessen severe oral mucositis in patients receiving chemotherapy and radiation for head and neck cancer [55]. Furthermore, in 2023, ZYIL-1 received orphan drug designation in the United States for the treatment of CAPS. Despite these advancements, the clinical application of these compounds is still in progress, with some undergoing clinical trials. Unlike RRx-001, which covalently binds to cysteine 409 of NLRP3 via its bromoacetyl group and thereby blocks the NLRP3-NEK7 interaction critical for the assembly and activation of the NLRP3 inflammasome [56], Mg2+ impedes Ca2+ influx, a known trigger of NLRP3 activation [2]. This suggests that Mg2+ acts at a stage upstream of the NLRP3-NEK7 interaction affected by RRx-001. According to Chen et al. [56], RRx-001 pretreatment almost completely inhibits IL-1β secretion in response to NLRP3 stimuli, including nigericin, ATP, and MSU, suggesting that it has a more substantial effect on NLRP3 inflammasome inhibition than DSW. In contrast to these potential clinical drugs, DSW presents a more accessible and cost-efficient alternative for treating diseases associated with the NLRP3 inflammasome. Given its availability and lower production costs, DSW stands out as a significant option and merits further investigation as a therapeutic option for these conditions.

In conclusion, our study expands current knowledge about the beneficial effects of Mg^{2+}-enriched DSW in modulating inflammatory responses. Our results show here that DSW with a water hardness of 500 mg/L can inhibit caspase-1 activation and IL-1β secretion in macrophages in response to various NLRP3 stimuli, and Mg^{2+} plays a major role in DSW inhibition of NLRP3 inflammasome activation. Finally, DSW treatment reduced NLRP3-dependent MSU-mediated peritonitis in mice. According to the present results, Mg^{2+}-enriched DSW provides an efficient treatment for experimental peritonitis, and Mg^{2+} ions could provide an additional basis for development of anti-inflammatory drugs suitable for treatment of NLRP3 inflammasome-associated diseases.

Data availability statement

Data will be made available on request.

Ethics approval

The study was conducted in compliance with the ethical approval of the Institutional Animal Care and Use Committee (IACUC) of MacKay Medical College (approval number: A1090020).

Consent to participate

Not applicable.

Consent for publication

All the authors have read the manuscript and agreed to submit the paper to the journal.

Author information

Not applicable.

CRediT authorship contribution statement

Hsueh-Hsiao Wang: Investigation. **Chi-Ruei Huang:** Investigation. **Hsin-Chung Lin:** Funding acquisition, Conceptualization. **Hsin-An Lin:** Writing – review & editing, Funding acquisition. **Yu-Jen Chen:** Funding acquisition, Conceptualization. **Kuen-Jou Tsai:** Methodology, Funding acquisition. **Chieh-Tien Shih:** Validation, Methodology. **Kuo-Yang Huang:** Supervision, Funding acquisition. **David M. Ojcius:** Writing – review & editing, Conceptualization. **Ming-Hang Tsai:** Writing – review & editing, Funding acquisition. **Kuang-Wen Tseng:** Validation. **Lih-Chyang Chen:** Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35136.

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