

RESEARCH ARTICLE

Magnesium Sulfate Induced Toxicity *in Vitro* in AGS Gastric Adenocarcinoma Cells and *in Vivo* in Mouse Gastric MucosaXulong Zhang¹, Agula Bo², Baofeng Chi², Yuan Xia², Xiong Su², Juan Sun^{2*}**Abstract**

Magnesium sulfate is widely used as a food additive and as an orally administered medication. The aim of this study was to evaluate the possible cytotoxicity of magnesium sulfate on AGS human gastric adenocarcinoma cells and gastric mucosa in mice. A trypan blue exclusion assay was used to determine the reduction in viability of AGS cells exposed to magnesium sulfate, and then effects on cell proliferation were quantified. The role of magnesium sulfate-mediated pro-inflammatory cytokine production in AGS cells was also investigated. mRNA expression for IL-1 β , IL-6, IL-8, and TNF- α was determined by RT-PCR, and secretion of these cytokines was measured by ELISA. Immunohistochemical evaluation of IL-1 β , IL-6, and TNF- α expression was conducted in mouse gastric mucosa. Addition of 3 to 50 mM magnesium sulfate to AGS cells inhibited both cell proliferation and cell viability in a dose-dependent manner. Magnesium sulfate had little effect on production of IL-1 β or IL-6 but significantly inhibited production of IL-8. The animal model demonstrated that magnesium sulfate induced production of IL-1 β , IL-6, and TNF- α . These preliminary data suggest that magnesium sulfate had a direct effect on the stomach and initiates cytotoxicity in moderate concentrations and time periods by inhibiting viability and proliferation of AGS cells and by regulating expression and/or release of pro-inflammatory cytokines.

Keywords: AGS cells - gastric - magnesium sulfate - proliferation - pro-inflammatory cytokine - toxicity - viability

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Introduction

Magnesium sulfate (MgSO₄) occurs naturally and is known as the mineral epsomite or Epsom salt. It is chemically prepared by neutralizing magnesium oxide, hydroxide, or carbonate with sulfuric acid and evaporating the resultant solution to form crystals (Code of Federal Regulations, 2001). As a medication, MgSO₄ is most commonly used during pregnancy as either an anticonvulsant (Bigdeliet et al., 2013). MgSO₄ also affects heart (Zhou et al., 2013), lung (Powell et al., 2012) and brain (Bachnas et al., 2013) function. MgSO₄ is currently the drug of choice for the treatment of eclampsia.

A shortened postpartum course of MgSO₄ (loading dose plus two 5g doses 4h apart) was as effective as the standard Pritchard regimen (loading dose plus 5g every 4h for 24h) in the management of eclampsia (Chama et al., 2013). Application of antenatal MgSO₄ in preterm delivery increases cord blood brain-derived neurotrophic factor levels that could play a potential role in fetal neuroprotection (Borja-Del-Rosario et al., 2013). To keep neonatal serum magnesium concentrations within a range that is effective for neuroprotection and safe for the neonates, the correct dose must be selected (Pryde and Mittendorf, 2011).

Given that any substance at high enough doses becomes toxic, the "best dose" is the lowest dose that

achieves efficacy while minimizing toxicity among susceptible fetuses (Samsonia and Kandelaki, 2013). A study by Katsumata and colleagues studied female rats treated with MgSO₄ at doses of 250, 500, or 1000mg/kg administered subcutaneously three times daily on day 15 through day 20 of gestation. The first-generation offspring of the rats in the 1000mg/kg group demonstrated low body weight, delays in development such as the eruption of lower incisors or opening of the eyes, and a reversible change in the ribs (wavy rib) (Katsumata et al., 1998). Other studies have also demonstrated the developmental toxicity of magnesium sulfate. High-dose MgSO₄ exposure induced apoptotic cell death in the developing neonatal mouse brain (Dribben et al., 2009). MgSO₄ also caused oxidative stress through generation of reactive oxygen species resulting in DNA damage in testicular cells and reproductive abnormalities (Rasool et al., 2014).

Because MgSO₄ can be orally administered at pharmacologic doses (Eberhardt et al., 1991) and is used as a food additive (Code of Federal Regulations, 2001), the gastric lining is a vulnerable site for adverse effects of MgSO₄. In this study, a human gastric adenocarcinoma epithelial cell line (AGS) was used to evaluate the impact of MgSO₄ on cell viability and proliferation and the regulation of inflammatory cytokines. The effects of MgSO₄ on gastric tissue in mice *in vivo* were also examined.

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Materials and Methods

Test material

Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, Wako Pure Chemical Industries, Ltd, Osaka, Japan) was used in this study. The test material was a minimum of 99.5% pure. The test material was freely soluble in water. The pH of a 50g/L solution at 25°C was 5.0-8.0. This test material was used throughout this study.

AGS cell culture

AGS human gastric adenocarcinoma cells (Dainippon Pharmaceutical Co, Ltd, Osaka, Japan) were maintained in Ham's F12 with L-glutamine nutrient mixture (Gibco, Invitrogen Corporation) in the presence of 10% fetal bovine serum (FBS) (Gibco) and supplemented with penicillin 100IU/mL (Gibco) and streptomycin 100 μg /mL (Gibco). The cells were incubated at 37°C in 5% CO_2 .

Cell viability assay

A trypan blue exclusion assay was used to determine the viability of AGS cells exposed to MgSO_4 . AGS cells were seeded in 24-well culture plates at a density of 5×10^3 cells/well. The culture plate was incubated at 37°C in a humidified atmosphere of 5% CO_2 with MgSO_4 0 to 50mM. Cell viability was determined using a hemocytometer by trypan blue exclusion after 24 and 48h.

Cell proliferation assay

AGS cells were seeded in 96-well culture plates at a density of 4×10^3 cells/well. The culture plate was incubated at 37°C in a humidified atmosphere of 5% CO_2 with MgSO_4 0 to 50mM. Cell proliferation was determined at 24, 48, and 72h using a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases (Quick Cell Proliferation Assay, Medical & Biological Laboratories Co, Ltd, Nagoya, Japan). The test was conducted according to the manufacturer's instructions.

Cytokine evaluation

AGS Cells were seeded in 6-well culture plates at a density of 5×10^5 cells/well in 2mL F12 medium. The culture plate was incubated at 37°C in a humidified atmosphere of 5% CO_2 with MgSO_4 0 to 50mM to measure the levels of interleukin-1 β (IL-1 β), IL-6, IL-8, and tumor necrosis factor- α (TNF- α) and their messenger RNAs (mRNAs).

Measurement of IL-1 β , IL-6, IL-8, and TNF- α : Levels of IL-1 β , IL-6, IL-8, and TNF- α were measured by a specific enzyme-linked immunosorbent assay (ELISA; Immunotech Beckman-Coulter Company, Marseille, France). A one-step immunologic sandwich-type assay was used for IL-6 and TNF- α and a two-step immunologic sandwich-type assay was used for IL-1 β and IL-8. The bound enzymatic activity was detected by the addition of a chromogenic substrate that was detected at an absorbance of 450 nm for IL-1 β and IL-8 and at 405 nm for IL-6 and TNF- α . The absorbance intensity was correlated with the cytokine concentration in the samples and standards. The sample results are calculated by interpolation from a

standard curve. The lower limits of detection were 3 pg/mL for IL-6, 8 pg/mL for IL-8, 1.5 pg/mL for IL-1 β , and 5pg/mL for TNF- α . Each experiment was repeated three times and the results from all experiments were combined.

Isolation of mRNA from cultured AGS cells and reverse transcription: Total RNA was extracted from AGS cells after the collection of the culture supernatants using phenol and guanidine thiocyanate (Isogen, Nippon Gene, Tokyo, Japan) and chloroform according to the manufacturer's instructions. Aliquots (2.5 μg) of total RNA were incubated at 70°C for 5 min, chilled on ice, and subjected to reverse transcription. The final reaction volume was 10 μl . The reaction solution contained Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Ltd, Strathclyde, UK) and the following additional components: 5X First-Strand Buffer, dithiothreitol 0.1 mol, and dNTP 2.5 mmol. The random primer hexadeoxyribonucleotide mixture (6-mer) pd(N)6 (TaKaRa, Shiga, Japan) was incubated at 22°C for 10 min, then at 37°C for 60 min, heated to 80°C for 5min, and then stored at -20°C until used.

Conventional reverse transcriptase polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) 1 μl was added to a 29 μl reaction mixture containing 3 μl of 10X PCR buffer, 1 μl of 4 nmol of each primer, 0.1 μl of 5U/ μl of Taq deoxyribonucleic acid (DNA) polymerase, and deionized water. Oligonucleotide primers were selected as in previous studies (Yumoto et al., 1999; Tanahashi et al., 2000). Glyceraldehyde 3-phosphate dehydrogenase was used as a control.

PCR was performed with an automatic thermal cycler (Program Temperature Control System PC-701, ASTEC, Fukuoka, Japan). The amplification cycle consisted of initial denaturation of target DNA at 94°C for 2min and then cycled 36 times, denaturation at 94°C for 45s, annealing at 60°C for 45s, and completion of an extension step at 72°C for 1 min. The final cycle included completion of an extension step for 5min at 72°C to ensure full extension of the product. Aliquots (10 μl) of each PCR product were subjected to gel electrophoresis in Agarose S 1.5% with ethidium bromide for detection (Wako Pure Chemical Industries, Osaka, Japan). Photographs were taken under ultraviolet illumination to detect amplified DNA.

Animal studies

Ten 5-week-old female BALB/c mice were housed in groups of two in metal cages. The room was maintained at an ambient temperature $25 \pm 2^\circ\text{C}$, and a relative humidity of $55 \pm 5\%$. Food (CE-2 pelleted diet) and water were available ad libitum.

After acclimation for 1 week, the mice underwent intragastric dosing once daily. The experimental group (n=5) received 0.05 mL of 50mM MgSO_4 and the control group (n=5) received a corresponding amount of distilled water. After 10 days of treatment, all mice were sacrificed, and the stomachs were excised.

Immunohistochemistry

The frozen blocks of mouse gastric tissue were sectioned (6- μ m thick) and fixed in ice-cold acetone for 20 min. Representative slides were used for immunocytochemical staining. The sections were fixed in paraformaldehyde 4% for 10 min and in acetone for 10 min. Then the slides were treated with 3% H₂O₂ (Wako) in methanol for 5 min and blocked with 10% normal rabbit serum (Vector Laboratories, Inc., Burlingame, CA, USA.) for 10 min. The following primary polyclonal antibodies were used: goat anti-mouse TNF- α , IL-1 β , and IL-6 (R & D Systems, Inc., Minneapolis, MN, USA). The tissue sections were incubated for 60min at room temperature with 75% humidity with the different primary antibodies diluted according to the manufacturer's instructions. A biotinylated rabbit anti-goat immunoglobulin (Vector Laboratories, Inc.) was applied for 30min at room temperature as the secondary antibody. After three 5-minute rinses with PBS, the reaction was amplified with an ABC Kit (Vector Laboratories, Inc.) for 30min and visualized using diaminobenzidine (Vector Laboratories, Inc.) for about 5min. Sections were counterstained with Mayer hematoxylin, dehydrated (Muto pure chemicals LTD), and mounted on glass slides (Fisher scientific). For the negative controls, the specific primary antibodies were replaced by purified goat control immunoglobulin G (Vector Laboratories, Inc.) and subjected to the same techniques under the same conditions.

Data analysis

Student's t test and ANOVA with a post-hoc test were used when appropriate to analyze the study data. Statistical significance was assessed at the 5% level ($p < 0.05$ was considered statistically significant).

Results

MgSO₄ decreased viability of AGS cells

MgSO₄ treatment decreased viability of AGS cells in a concentration-dependent manner and showed a significant decrease in viability at 50mM (Figure 1). AGS cell viability was least affected by 3.1mM MgSO₄ at 24 and 48h of incubation (not statistically significant, NS) and was most affected (68%) by 50mM MgSO₄ at a concentration of 50mM at 48h of incubation. Compared to control conditions, treating cells with the highest concentration of MgSO₄ (50mM) significantly decreased their viability ($p < 0.01$ at 48h and $p < 0.05$ at 24h of incubation) (Figure 1).

For 24h of incubation, inhibition by the second-lowest concentration of MgSO₄ was even higher than that by the lowest concentration, but the difference was not statistically significant ($p = 0.09$) because there was a larger variance. Compared with control cells, the treated cells had the following percent viability: at 24h of incubation, 44% viability at the highest concentration of MgSO₄ (50mM, $p < 0.05$) and 78% at the lowest concentration (3.1mM, NS); at 48 h of incubation, 32% viability at the highest concentration of MgSO₄ (50mM, $p < 0.01$) and 86% at the lowest concentration (3.1mM, NS) (Figure 1).

A colorimetric assay based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular

mitochondrial dehydrogenases (Quick Cell Proliferation Assay) was used to assess the effect of MgSO₄ on the proliferation of AGS cells (Figure 2). The lowest concentration of MgSO₄ was decreased to 1.6mM, because the trypan blue exclusion assay showed a large decrease in cell viability at the lowest concentration of MgSO₄ (3.1mM). Since the percentage of viable AGS cells at 48 h was similar to that seen at 24h, an additional observation at 72 h of incubation was performed. The various concentrations of MgSO₄ (1.6-50mM) studied had little effect on AGS cell proliferation after 24h of incubation (Figure 2). At 48h of incubation with 1.6-25mM MgSO₄, the AGS cell proliferation rate was similar to that of control cells. At 72h of incubation with 1.6-12.5mM MgSO₄, the proliferation of treated cells was not significantly different from controls. At the highest MgSO₄ concentration (50mM), the maximal inhibition of proliferation was observed at 48h of incubation. MgSO₄ 50mM completely inhibited cell proliferation after incubation for 72h.

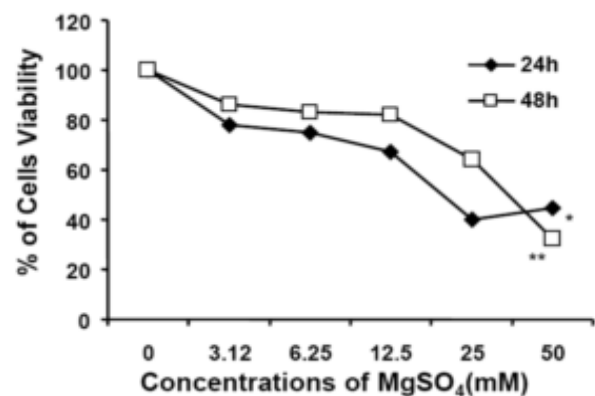


Figure 1. The Percentage of viable AGS Human Gastric Adenocarcinoma Cells after Exposure to MgSO₄ During 24 and 48h of Incubation. AGS cells were seeded in 24-well culture plates at a density of 5×10^3 cells/well. The culture plate was incubated at 37°C in a humidified atmosphere of 5% CO₂ with MgSO₄ at 0 to 50mM. Cell viability was determined using a hemocytometer by trypan blue exclusion after 24 and 48h

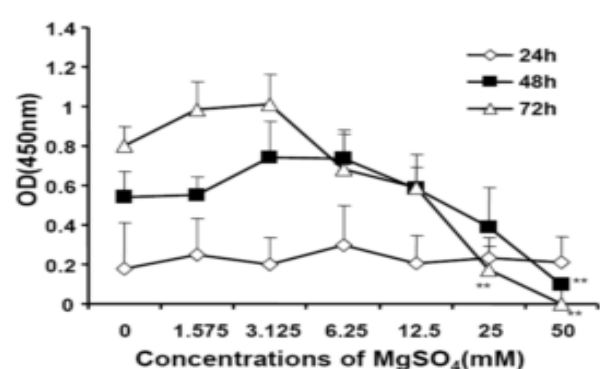


Figure 2. Dose-response effects of MgSO₄ Compounds on the Proliferation of AGS Cells During 24, 48, and 72h of Incubation. AGS cells were seeded in 96-well culture plates at a density of 4×10^3 cells/well. The culture plate was incubated at 37°C in a humidified atmosphere of 5% CO₂ with MgSO₄ 0 to 50mM. Cell proliferation was determined at 24, 48, and 72h using a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases

MgSO₄ influenced cytokines secretion of AGS cells in vitro

Cytokines (IL-1β, IL-6, IL-8, and TNF-α) secreted by MgSO₄-treated and untreated AGS cells were then measured using an ELISA. Figure 3 showed the changes in cytokine protein secretion profiles of AGS cells exposed to MgSO₄ for 24h. An increase in TNF-α secretion occurred at the highest MgSO₄ concentration used (50mM). The AGS cell suspensions exposed for 24h to MgSO₄ ranging in concentration from 6.25 to 25mM released twice as much TNF-α as control cells, but these effects were not statistically significant. AGS cells exposed to 50mM MgSO₄ released 4 times more TNF-α than control cells. AGS cells exposed to either MgSO₄ or vehicle released little IL-6. Fluctuations in the levels of IL-1β secreted were minimal and occurred regardless of exposure to MgSO₄. The levels of IL-8 were not significantly affected by MgSO₄ (6.2-50mM).

Cytokine mRNA expression was also assessed by

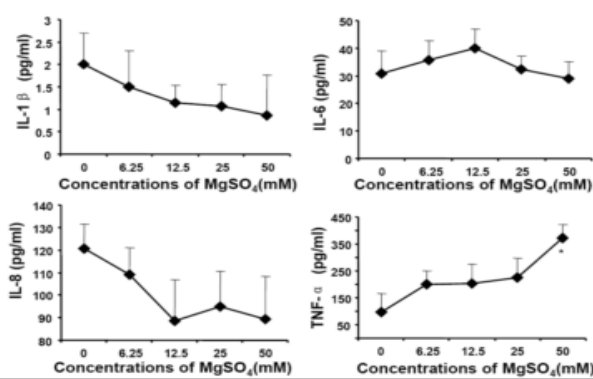


Figure 3. Cytokine Protein Secretion Profiles of MgSO₄-stimulated AGS Cell. Levels of interleukin-1β (IL-1β), IL-6, IL-8, and tumor necrosis factor-α (TNF-α) were measured by a specific enzyme-linked immunosorbent assay. A one-step immunologic sandwich-type assay was used for IL-6 and TNF-α and a two-step immunologic sandwich-type assay was used for IL-1β and IL-8. The bound enzymatic activity was detected by the addition of a chromogenic substrate that was detected at an absorbance of 450nm for IL-1β and IL-8 and at 405nm for IL-6 and TNF-α

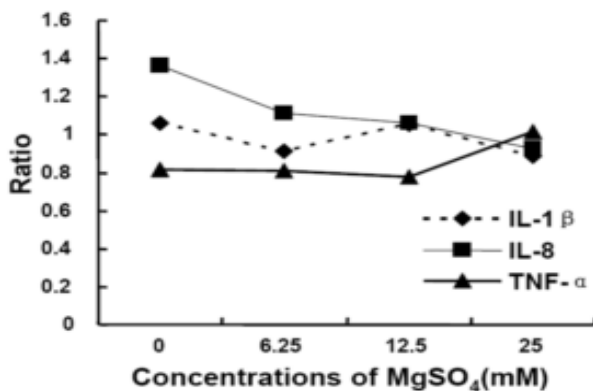


Figure 4. Expression of mRNAs for the Cytokines Interleukin-1β (IL-1β), IL-6, IL-8, and Tumor Necrosis Factor-α (TNF-α) in AGS Cells Treated with MgSO₄. Total RNA was extracted from AGS cells after the collection of the culture supernatants. Aliquots (2.5μg) of total RNA were subjected to reverse transcription with Moloney murine leukemia virus reverse transcriptase

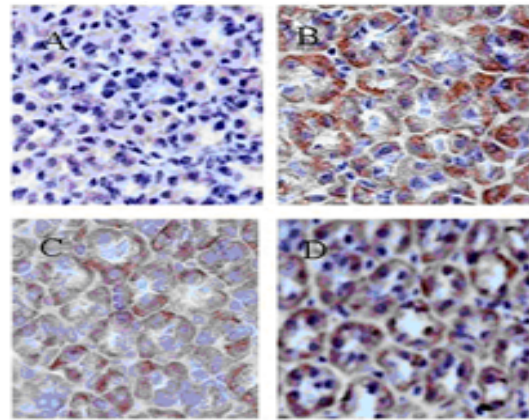


Figure 5. Mouse Gastric Tissue Treated with MgSO₄ and Stained with different Antibodies by Means of an ABC Kit. The tissue sections were incubated with the different primary antibodies [goat anti-mouse IL-1β (B), IL-6 (C) and TNF-α (D)] diluted according to the manufacturer’s instructions. A biotinylated rabbit anti-goat immunoglobulin G was applied as the secondary antibody. The reaction was amplified using an ABC Kit. In the negative controls (A), the specific primary antibodies were replaced by purified goat immunoglobulin G

RT-PCR (Figure 4). TNF-α mRNA was expressed constitutively by cultured AGS cells, and upregulation of gene expression occurred in response to 50mM MgSO₄ exposure. The observed pattern of IL-1β gene secretion was similar to that of the protein secretion determined by ELISA, and the levels of mRNA were similar to those of control cells. IL-6 expression was not detected from AGS cells, although a measurable amount of secreted IL-6 was detected in the previous experiment. The levels of IL-8 mRNA in AGS cells treated with MgSO₄ were slightly lower than in control cells.

MgSO₄ influenced cytokines secretion in vivo

Immunohistochemical analysis was performed on gastric tissue from mice treated with oral MgSO₄ (Figure 5). Intense TNF-α staining was found in mouse gastric tissue consistent with the findings from RT-PCR and ELISA. Staining for IL-6 and IL-1β was also noted. Almost no staining occurred in control tissues treated with G immunoglobulin G. Immunohistochemistry for IL-8 was not performed due to unavailability of mouse anti-IL-8 antibodies. No staining occurred in the gastric tissue of control mice (n=5; data not shown).

Discussion

Previous cell proliferation assays showed no significant difference in chondrocyte viability after treatment with MgSO₄ (Baker et al., 2011). The current study suggests that proliferation still occurs at the lower concentrations of MgSO₄ (3.12-25mM). These data suggest that high oral doses could adversely affect the gastric mucosa. A prior study showed that adding MgSO₄ (0.1%) to the culture medium somewhat increased AGS cell proliferation (Eng, 1981). This finding is consistent with the results of the current study. These data indicate a potentially complex concentration-response relationship for MgSO₄ in AGS cells. Cell proliferation may be enhanced at

lower concentrations (1.6-6.2mM), but is decreased at higher concentrations (25-50mM). However, MgSO₄ can be toxic or even lethal when administered at high doses (Mittendorf et al., 2006). A decrease in AGS cell proliferation was observed at MgSO₄ concentration greater than 6.2mM. The inhibition of cell proliferation was time- and concentration-dependent at MgSO₄ concentrations ranging from 3.125 to 50mM.

Administering MgSO₄ to pre-eclamptic placentas significantly attenuated the increased secretion of IL-1 β into the maternal circulation (Amash et al., 2012). In this study, regardless of cell exposure to MgSO₄, low levels of secreted IL-1 β mRNA and protein were detected.

IL-6 is associated with various cancers although MgSO₄ showed no effect on IL-6 levels in AGS cells, *in vivo* levels of IL-6 were significantly increased in mouse gastric tissue. It has been established that IL-6 plays an active role in various immunological responses (Sun et al., 1995; Sun et al., 1995; Sun et al., 1995; Sun et al., 1996). It has also been shown that the perfused pre-eclamptic placenta secretes increased levels of IL-6 into the fetal and the maternal circulations compared to normal placenta. Exposure of pre-eclamptic placentas to MgSO₄ decreased IL-6 levels in the maternal circulation when compared with the control group. In the fetal circulation, when compared with the control group, the addition of MgSO₄ resulted in a non-significant tendency toward decreased IL-6 levels (Amash et al., 2010). These contradictions may result from differences in experimental design, or species and tissue differences, or differences in the MgSO₄ preparations and route of administration.

IL-8 exerts an important effect on the regulation of inflammatory responses and is regulated by some compounds (Liu, 2014). No studies on the effect of MgSO₄ on IL-8 were found in an extensive literature search. Our *in vitro* data showed, however, that MgSO₄ inhibited release of this cytokine, but the results were not statistically significant.

Repeated measurements illustrated that there was no significant difference in TNF- α when trauma patients with systemic inflammatory response syndromes were randomly assigned into groups receiving MgSO₄ or placebo treatment (Mirrahimi et al., 2012). Another study indicated that an increased level of TNF- α in preterm infants resulted from magnesium deficiency and may be ameliorated by magnesium supplementation (Caddell et al., 1999). In our *in vitro* experiment, an increase in cytokine levels was observed only for TNF- α . This finding was confirmed by our *in vivo* data. Exposure to MgSO₄ was found to upregulate the level of TNF- α in gastric cells during both *in vitro* and *in vivo* experiments.

Many researchers have studied the effect of MgSO₄ on inflammatory cytokines. MgSO₄ exposure increased intracellular magnesium levels, reducing the production of IL-1 β , IL-8, and TNF- α following stimulation with various factors (Suzuki-Kakisaka et al., 2013). A assessment of the effect of MgSO₄ on cytokine production in humans showed reduced maternal TNF- α and IL-6 production following MgSO₄ treatment (Sugimoto et al., 2012). The cell culture results in the current study showed that exposure to MgSO₄ exerted less of an effect

on inflammatory cytokines than sodium acetate (Sun et al., 2005; Sun et al., 2006). Exposure to MgSO₄ produced various effects on pro-inflammatory cytokines in human cells and increased cytokine levels in a mouse gastric tissue.

MgSO₄ (6.25-50mM) affected the secretion of the cytokines, IL-1 β , IL-6, IL-8, and TNF- α . In AGS cells, the secretion of IL-1 β and IL-8 decreased and that of TNF- α increased with increasing concentrations of MgSO₄. IL-6 secretion was not correlated with MgSO₄ concentration. The transcription levels of IL-8 and TNF- α were consistent with the levels of protein expression in AGS cells treated with MgSO₄, but there was no significant change in IL-1 β transcription with MgSO₄ treatment.

The effects of magnesium sulfate on inflammatory cytokines were variable. TNF- α was increased in both AGS cell and mouse gastric mucosa. IL-8 expression (mRNA and protein) was decreased in AGS cells but not in mouse gastric mucosa. In contrast to the *in vivo* studies, IL-6 was not detected in AGS cells. Expression of IL-1 β showed (protein and mRNA) in response to MgSO₄ was also different between AGS cells and mouse gastric mucosa. The reasons for discrepancies in cytokine activity may be differences between species and the biological variability in cytokine responses to MgSO₄. It is likely that a combination of several different factors contributes to the timing and magnitude of inflammatory cytokine response. Recently, a study indicate that interactions in cytokine genes may contribute to cancer risk. *In vivo* cytokine regulated by a number of different factors and cytokine activity is frequently unpredictable. Many population-based studies examined the association between cytokine and cancer risk also proved it. Therefore, further research is needed to improve the understanding of these associations.

In conclusion, AGS cell viability and proliferation are inhibited by MgSO₄ at concentrations of 25-50mM. This study represents an essential step in understanding the effect of direct *in vitro* and *in vivo* exposure of magnesium sulfate on inflammatory cytokines. Additional studies are needed to identify other mechanisms of MgSO₄ action that may affect cytokines.

Acknowledgements

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