

## ORIGINAL ARTICLE

# Effect of hypertonic saline and macrophage migration inhibitory factor in restoration of T cell dysfunction

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**Purpose:** Trauma-induced suppression of cellular immune function likely contributes to sepsis, multiple organ dysfunction syndrome and death. T cell proliferation decreases after traumatic stress. The addition of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which depresses immune function after hemorrhage and trauma, to T-cells decreases T-cell proliferation; and hypertonic saline restores PGE<sub>2</sub>-induced T-cell suppression. Recently, it has become apparent that macrophage migration inhibitory factor (MIF) plays a central role in several immune responses, including T-cell proliferation. However, the role of MIF in mediating hypertonic saline (HTS) restoration of T cell dysfunction is unknown. Therefore, we hypothesize that T cell immune restoration by HTS occurs, at least in part, by a MIF-mediated mechanism. **Methods:** Jurkat cells were cultured in Roswell Park Memorial Institute media, at a final concentration of  $2.5 \times 10^6$  cell/mL. The effects of HTS on T-cell proliferation following PGE<sub>2</sub>-induced suppression were evaluated in Jurkat cells: HTS at 20 or 40 mmol/L above isotonicity was added. MIF levels were determined by enzyme-linked immunosorbent assay and western blot analysis. **Results:** PGE<sub>2</sub> caused a 15.0% inhibition of Jurkat cell proliferation, as compared to the control. MIF levels decreased in PGE<sub>2</sub>-suppressed cells, as compared to the control. MIF levels were higher in cells treated with HTS than PGE<sub>2</sub>-stimulated cells. **Conclusion:** The role of HTS in restoring Jurkat cells proliferation suppressed by PGE<sub>2</sub>, at least in part, should be mediated through a MIF pathway.

**Key Words:** Hypertonic solutions, Macrophage Migration-Inhibitory factors, Prostaglandins E, Injuries, T-lymphocytes

## INTRODUCTION

Immunological suppression is a well recognized consequence of trauma and hemorrhagic shock, and contributes to infectious complications, ultimately leading to sepsis and multiple organ dysfunction syndrome (MODS) [1]. There are several mechanisms of post-traumatic immune

impairment, including T cell dysfunction. T cell dysfunction after traumatic stress is characterized by a decrease in T cell proliferation. The addition of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which depresses immune function after hemorrhage and trauma, to T cells decreases T-cell proliferation, while hypertonic saline (HTS) restores PGE<sub>2</sub>-induced T-cell suppression [2].

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Recently, it has become apparent that macrophage migration inhibitory factor (MIF) plays a central role in several immune responses, including the modulation of several cytokines and T-cell proliferation [3,4]. By controlling immune and inflammatory responses, MIF is thought to play an important role in the pathophysiology of septic shock and chronic inflammatory diseases [5-7]. The role of MIF mediating HTS restoration of T cell dysfunction is unknown.

Therefore, we hypothesize, that T cell immune restoration by HTS occurs, at least in part, by a MIF-mediated mechanism. The proposed experiments will provide the first evidence supporting MIF as the primary mechanism for restoring T cell dysfunction by HTS.

## METHODS

### Cells culture and cell stimulation

Jurkat cells clone E6-1 (ATCC, Manassas, VA, USA) were maintained in Roswell Park Memorial Institute-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/mL penicillin/streptomycin at 37°C in a 5% carbon dioxide incubator. Cells were cultured to a density of  $5 \times 10^5$  cell/mL. Cell viability, as determined by trypan blue dye exclusion, was >99%. For the protein extracts, cells were plated at a density of  $2.5 \times 10^6$  cell/mL in a 6-well flat-bottom culture plates, and were stimulated with PGE<sub>2</sub> (1 µg/mL) (Sigma-Aldrich Co., St. Louis, MO, USA) in the presence or absence of HTS at 20 mmol/L (HTS20) and 40 mmol/L (HTS40) above isotonicity, resulting in sodium concentrations of 160 mmol/L and 180 mmol/L, which were measured by a GEM Premier 3000 (Instrumentation Laboratory, Lexington, MA, USA), respectively.

### Protein extracts

After incubation for 24 hours at 37°C, the cells were washed 2 times in cold phosphate buffered saline (PBS) and then centrifuged for 10 minutes. Cells pellet were resuspended in 10 µL per  $2 \times 10^6$  cell/mL of superlysis buffer (protease inhibitors, 1 M HEPES, 5 M NaCl, 0.5 M ethyl-

enediaminetetraacetic acid, 1 mM NaOAc, 20% Triton X-100, 50 mM phenylmethylsulfonylfluoride), incubated on ice for 7 minutes, and then centrifuged at  $3,000 \times g$  for 15 minutes at 4°C. The supernatant was then transferred to an eppendorf tube and used for assay. The total protein concentration was determined by the Bradford method using a commercially available assay kit (Thermo Fisher Scientific, Rockford, IL, USA) [8]. The prepared protein lysates were aliquoted and used for Western blot analysis.

### T-cell proliferation assay

Jurkat cells were plated in 96-well flat-bottom tissue culture plates to attain a final concentration of  $2.5 \times 10^6$  cell/mL. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were conducted by the quantity of PGE<sub>2</sub> 0.01, 0.1, 1, 10 µg/mL and MTT assay value was the lowest at PGE<sub>2</sub> of 1 µg/mL (have not shown). Therefore, PGE<sub>2</sub> 1 µg/ml were used. The effect of HTS on T-cell proliferation following PGE<sub>2</sub>-induced suppression was evaluated in Jurkat cells with 1 µg/mL PGE<sub>2</sub>, a concentration that suppresses T-cell proliferation, and HTS at 20 mmol/L and 40 mmol/L above isotonicity was added at the same time as PGE<sub>2</sub>. After incubation for 24 hours at 37°C, the resultant T-cell proliferation was determined by the MTT cell proliferation assay (ATCC).

### Enzyme-linked immunosorbent assay (ELISA) for MIF

MIF concentration in culture supernatants was measured by sandwich enzyme-linked immunosorbent assay. Briefly, 2 µg/mL of monoclonal capture antibody (R&D Systems, Minneapolis, MN, USA) was added to a 96-well plate and incubated for 2 hours at room temperature. After incubation, the plates were incubated in blocking solution comprised of PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 for 2 hours at room temperature. The test samples and standard recombinant MIF (R&D Systems) were added to the plates, and the plates were incubated for overnight at 4°C. The plates were then washed four times with PBS containing Tween 20, 200 ng/mL of biotinylated detection monoclonal antibodies (R&D Systems) was added, and the plates were incubated for 2 hours at room temperature. Subsequently, the plates

were washed, streptavidin-alkaline-phosphatase (1:2,000; Sigma-Aldrich Co.) was added, and the reaction was allowed to proceed for 2 hours at room temperature. The plates were washed four times, and 1 mg/mL of p-nitrophenylphosphate dissolved in diethanolamine (Sigma-Aldrich Co.) was added to induce the color reaction, which was stopped by adding 50  $\mu$ L of 1 N NaOH. The optical density at 405 nm was measured on an automated microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). A standard curve was generated by plotting optical density versus the log of the MIF concentration. The experiments were conducted 10 times.

### Western blot analysis for MIF expression

Expression of MIF protein was quantified by Western blot analysis. Proteins (20  $\mu$ g/sample) were fractionated on an 15% sodium dodecylsulfate-polyacrylamide gel (Bio-Rad Laboratories Inc.) and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 hour in 5% BSA (Sigma-Aldrich Co.), and then incubated with a primary antibody, anti-human MIF (1:250; R&D systems). After washing, membranes were incubated with 1:2000 horseradish peroxidase-labeled goat anti-mouse antibody (R&D systems) as the secondary antibody. The proteins were detected using SuperSignal (Thermo Fisher Scientific Inc., Rockford, IL, USA) chemiluminescence kit.

### Statistical analysis

One-way analysis of variance was performed to evaluate the significance of difference between the experimental groups. For a single comparison of two groups, a Student's t-test was used with SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean  $\pm$  SD. Values of  $P < 0.05$  were considered significant. All experiments were performed in triplicate.

## RESULTS

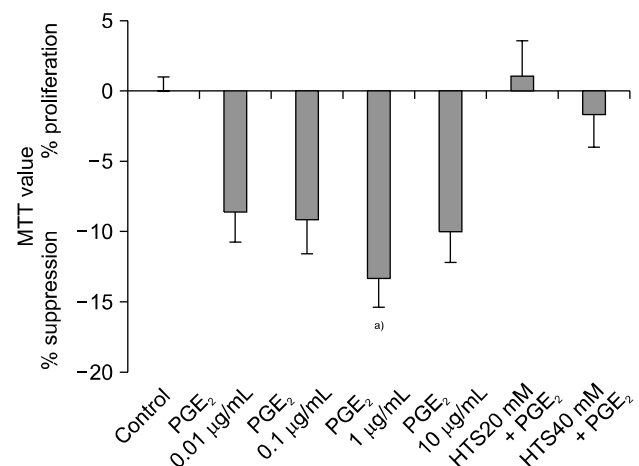
### The effect of HTS on PGE<sub>2</sub>-induced Jurkat cell suppression

Jurkat cells were plated on 96-well culture plates at a concentration of  $2.5 \times 10^6$  cell/mL in cell culture media. PGE<sub>2</sub> at 1  $\mu$ g/mL inhibited Jurkat cell proliferation by

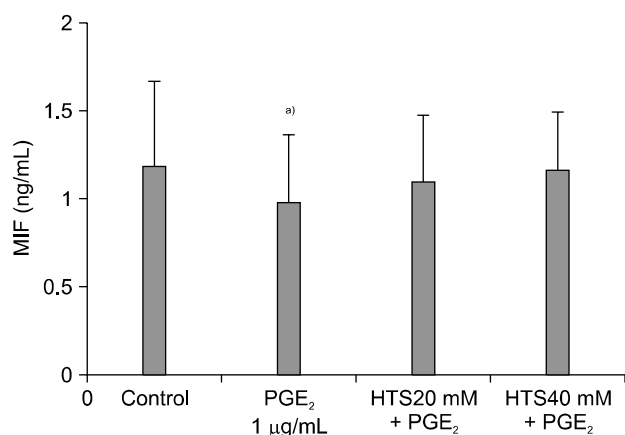
14.7% ( $P < 0.05$ ) (Fig. 1). The addition of HTS restored Jurkat cell proliferation suppressed by PGE<sub>2</sub> to control levels, as measured using an MTT cell proliferation assay ( $P < 0.05$ ). There was no statistical difference in restoration by either HTS20 or HTS40 (Fig. 1).

### The effect of HTS on MIF concentrations in the cell supernatant

To determine the relation between HTS and MIF on Jurkat cell proliferation, MIF levels were measured in the cell supernatant. The MIF level was decreased by 0.98 ng/mL  $\pm$  0.38 in the supernatant of PGE<sub>2</sub> stimulated cells, when compared to control levels (1.19 ng/mL  $\pm$  0.48). On the other hand, HTS20 restored and increased the MIF level (1.09 ng/mL  $\pm$  0.38) in PGE<sub>2</sub>-suppressed Jurkat cells ( $P < 0.05$ ). PGE<sub>2</sub>-suppressed Jurkat cells treated with HTS40 had a higher MIF level (1.16 ng/mL  $\pm$  0.33) than HTS20-treated cells, however, there was not a statistically significant difference of MIF levels in the supernatant of HTS20 or HTS40-treated cells (Fig. 2).



**Fig. 1.** Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) inhibited Jurkat cell proliferation by 14.7% ( $P < 0.05$ ). The addition of HTS restored Jurkat cell proliferation suppressed by PGE<sub>2</sub> to control levels, as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell proliferation assay ( $P < 0.05$ ). There was no statistical difference in restoration by either HTS20 or HTS40. The experiments were conducted 10 times and conducted by MTT. The experiments were also done by EZ-Cytox Cell viability assay kit (Daeil Lab, Seoul, Korea) and the results were the similar. ANOVA, analysis of variance. <sup>a)</sup>Mean  $\pm$  SD (ANOVA, Paired t-test)  $P < 0.05$ ,  $2.5 \times 10^6$  cell/mL 1 day incubation number: 10 times.



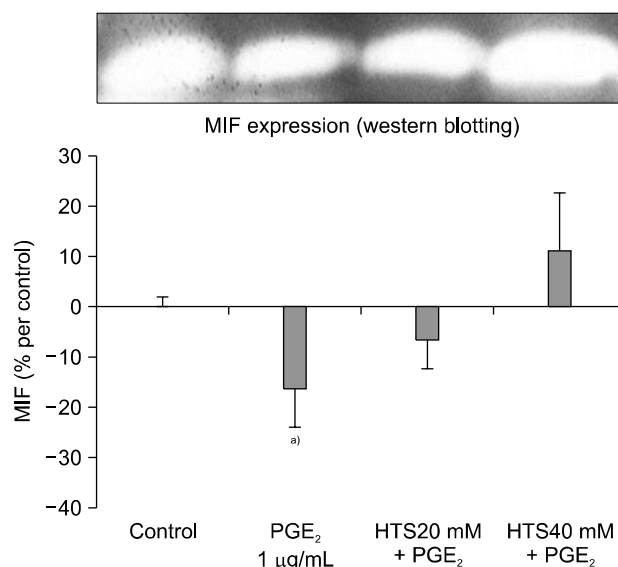
**Fig. 2.** Migration inhibitory factor (MIF) levels in cell supernatants were measured. Jurkat cells in presence of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) had a decrease in MIF level (0.98 ng/mL ± 0.38), when compared with Jurkat cells alone (1.19 ng/mL ± 0.48). HTS20 restored and increased MIF level (1.09 ng/mL ± 0.38) when compared with PGE<sub>2</sub>-suppressed Jurkat cells ( $P < 0.05$ ). PGE<sub>2</sub>-suppressed Jurkat cells treated with HTS40 had the highest MIF level (1.16 ng/mL ± 0.33); however, there was no statistically significant difference in MIF levels between the HTS20- and HTS40-treated groups. The experiments were conducted 10 times. ANOVA, analysis of variance. <sup>a)</sup>Mean ± SD (ANOVA, Paired t-test)  $P < 0.05$ ,  $2.5 \times 10^6$  cell/mL 1 day incubation number: 10 times.

### The effect of HTS on MIF expression

To determine the HTS effect on MIF expression, western blot analysis was performed. Correlating with the ELISA, levels of MIF protein expression were lower in PGE<sub>2</sub>-stimulating cells (16% decrease in band density) ( $P < 0.05$ ). The addition of HTS to PGE<sub>2</sub>-stimulated cells increased MIF protein expression, with the highest expression in the HTS40-treated cells, however, there was not a statistically significant difference of MIF protein expression in the HTS20 or HTS40-treated cells (Fig. 3).

## DISCUSSION

Trauma-induced suppression of cellular immune function likely contributes to sepsis, MODS, and death. This suppression is secondary to several immunomodulating factors including PGE<sub>2</sub>, transforming growth factor beta (TGFβ), interleukin 4 (IL-4), and IL-10, which block certain intracellular signaling events in T-cells [1]. T-cell dysfunction after traumatic stress is characterized by a decrease in



**Fig. 3.** Levels of migration inhibitory factor (MIF) protein expression were lower in prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)-stimulated cells (16% decrease in band density) ( $P < 0.05$ ). The addition of hypertonic saline (HTS) to PGE<sub>2</sub>-stimulated cells increased MIF protein expression with the highest expression in the HTS40-treated cells. ANOVA, analysis of variance. <sup>a)</sup>Mean ± SD (ANOVA, Paired t-test)  $P < 0.05$ ,  $2.5 \times 10^6$  cell/mL 1 day incubation MIF MW: 12.5 kDa number: 5 times.

T-cell proliferation, an aberrant cytokine profile, decreased T-cell monocyte interactions, and attenuated expression of the T-cell Receptor Complex [9-11]. The exact mechanism involved in the development of T-cell dysfunction is unknown. Under stressful conditions, macrophage are triggered to rapidly produce and release PGE<sub>2</sub>, a powerful endogenous immune suppressant [12]. PGE<sub>2</sub> interacts with the corresponding membrane receptor of T cells, blocking T cell function by interfering with IL-2 gene expression at multiple stages [13-15]. In addition, PGE<sub>2</sub> is a potent activator of cAMP-dependent protein kinase, which regulates expression of p27<sup>kip1</sup> and cyclin D3 to suppress proliferation of leukemic T cell lines, including Jurkat cells, by arresting cells in the G1 phase of the cell cycle [15]. Therefore, the experiments were performed with PGE<sub>2</sub>, as it has been shown that PGE<sub>2</sub>, at a concentration of 1 µg/mL, causes inhibition of T cell proliferation by about 15%. Moreover, HTS restored suppressed PGE<sub>2</sub>-induced T cell proliferation. Hyperosmolality may play an important role in the HTS-enhancement of T-cell proliferation and hypertonicity increased IL-2 expression in T-cell pro-

liferation [16-18]. However, the mechanism explaining the HTS-mediated restoration of T cell function is still unclear.

MIF may serve as a general marker for systemic inflammation in septic and non-septic acute critical illness [5-7]. Furthermore, MIF is a cytokine that is secreted by the anterior pituitary and immune cells in response to surgical stress, injury, and sepsis. This cytokine appears to be a critical regulator of the inflammatory pathways, leading to systemic inflammatory response syndrome and subsequent MOSF [4,19]. Besides, activated T cells produce MIF, and neutralizing anti-MIF antibodies inhibit T-cell proliferation and IL-2 production. T cells also release MIF in response to glucocorticoid stimulation and MIF acts to override glucocorticoid inhibition of T-cell proliferation, as well as interleukin 2 and interferon  $\gamma$  production [3]. And MIF also reduces pro-oxidative stress-induced apoptosis and nitric oxide induced apoptosis. As to a contribution by MIF to the regulation of cell survival, the inhibitory effect of MIF on apoptosis could correlate with its reported stimulatory effects on cell proliferation, even though cellular apoptosis and cell proliferation do not simply oppose each other [20-22].

That is to say, MIF exhibits pro- and anti-inflammatory activities, and regulates cell proliferation and survival. Therefore, we know that T cell immune restoration by HTS occurs, at least in part, by a MIF-mediated mechanism. In our experiment, PGE<sub>2</sub> inhibited Jurkat cell proliferation and decreased MIF levels, when compared to control levels. The addition of HTS increased MIF production, as compared with PGE<sub>2</sub>-stimulated Jurkat cells in concordance with restored PGE<sub>2</sub>-suppressed Jurkat cell proliferation. Considering side effect of HTS, HTS at sodium concentrations of 160 mM showed sufficient result in this experiment. It has been suggested that HTS could be a proliferation regulator by a MIF-mediated mechanism. In other words, PGE<sub>2</sub> reduces expression of cyclin D and induces expression of p27<sup>kip1</sup>, while MIF regulates the expression of the cell cycle regulators cyclin and p27 [15,23,24]. Therefore, HTS could restore PGE<sub>2</sub>-suppressed Jurkat cell proliferation through MIF production. Cho et al. [25] showed that NF- $\kappa$ B inhibition leads to increased synthesis and secretion of MIF in human CD4<sup>+</sup> T cells. Notably, HTS also inhibits NF- $\kappa$ B, as compared to isotonic

saline, in our experiment (unpublished results). However, the mechanism of MIF regulation of HTS is not yet explained.

Unfortunately, the present study is associated with certain limitation. First, PGE<sub>2</sub> were used alone as immunosuppressant in present study. If another immunosuppressant (IL-4, IL-10, et al.) were used, the present study might have more interesting outcome. Second, This study was done about MIF only, so other cytokine were not used. And ELISA and western blot analysis were performed for MIF levels. However, if another cytokine and another method for MIF levels (realtime PCR on the expression of mRNA for MIF) are used, the present study might have more interesting outcome, therefore, we will consider conducting further experiments by using other cytokine and methods in the future.

The role of HTS in restoring Jurkat cell proliferation suppressed by PGE<sub>2</sub>, at least in part, should be mediated through a MIF pathway. Further studies are needed to determine the effects of HTS and MIF.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

## ACKNOWLEDGEMENTS

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