ORIGINAL ARTICLE

Selective inhibition of polymorphonuclear neutrophils by resuscitative concentration of hypertonic saline

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Objectives: This study investigated the effect of hypertonic saline on the role of polymorphonuclear neutrophils (PMNs) in the inflammatory response and the effect of hypertonic saline infused at different phases in relation to an inflammatory stimulus.

Materials and methods: PMNs were isolated from peripheral blood of healthy volunteers (Boyum's method) and cultured in three different media ($[Na^+] = 140$ mmol/l, 180 mmol/l, and 200 mmol/l). PMNs were then stimulated with fMLP (*N*-formyl-methionyl-leucyl-phenylalanine) and H_2O_2 synthesis was quantified by flow cytometry at 5, 30, 60, 120, and 180 minutes. PMNs were treated with hypertonic saline before, simultaneously with, and after fMLP stimulation, and H_2O_2 synthesis quantified again.

Results: H_2O_2 synthesis was two or three times higher in fMLP stimulated than in non-stimulated PMNs, and it reached maximum level at 120 minutes. In the absence of fMLP stimulation, there was no significant difference between control and hypertonic saline with regard to activity of H_2O_2 synthesis. In the presence of fMLP stimulation, H_2O_2 synthesis significantly decreased in PMNs treated with hypertonic saline. There was no significant difference between the two hypertonic saline solutions ($[Na^+] = 180$ mmol/l and 200 mmol/l) with regard to H_2O_2 synthesis. However, H_2O_2 synthesis decreased in PMNs treated with hypertonic saline before and simultaneously with fMLP stimulation, but was not significantly decreased in the cells treated with hypertonic saline after fMLP stimulation.

Conclusions: Hypertonic saline appears to decrease H_2O_2 in stimulated neutrophils. This may be a further beneficial role of hypertonic saline when used clinically as an early resuscitation fluid.

Secondary tissue injury causes serious clinical outcomes in patients with trauma and haemorrhage. Polymorphonuclear neutrophils (PMNs) are key players with a crucial role in an early phase in tissue injury. In other words, activated PMNs not only carry out phagocytosis but also secrete enzymes to defend against external pathogens. In the inflammatory response, however, activated PMNs can also cause tissue injury by producing oxygen radicals.

A strong inflammatory peptide, fMLP (*N*-formyl-methionyl-leucyl-phenylalanine) triggers chemotaxis, cytoplasmic vacuolisation, lysosomal enzyme secretion, and oxidative metabolism in neutrophils.¹ In the presence of various proinflammatory stimuli, PMNs activate NADPH (nicotinamide adenine dinucleotide phosphate) oxidase. NADPH oxidase mediates the transformation of oxygen (O_2) to superoxide anion (O_2^-). Then superoxide dismutase mediates the transformation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), a precursor of the oxygen radical with strong cytotoxicity. These oxygen radicals can cause tissue damage.

Recent studies have shown that hypertonic saline is more effective than isotonic saline in lowering the incidence of pulmonary and hepatic injury in animals with haemorrhagic shock. According to other studies, however, hypertonic saline accelerated the inflammatory response of PMNs.² In a clinical setting, hypertonic saline has been used for the prehospital treatment.

We postulated that hypertonic saline affects the early inflammatory response of neutrophils. On the basis of our hypothesis, we compared the effect of hypertonic saline with that of isotonic saline. In addition, we investigated whether hypertonic saline would affect the inflammatory response of neutrophils when it was infused with fMLP in different sequences.

METHODS

The Korea University Ethics Committee approved this study. Prior to the study, we recruited 20 healthy volunteers. Then we took blood samples to isolate the neutrophils. To monitor the changes in H_2O_2 synthesis, using isotonic Krebs–Ringer bicarbonate and 2 mol/l NaCl solutions, we prepared three types of neutrophil culture medium with concentrations $[Na^+]$ approximately = 140 mmol/l, 180 mmol/l, and 200 mmol/l. To investigate whether hypertonic saline would affect the inflammatory response of neutrophils when it was infused with fMLP in different sequences, the concentration of the medium was set at 140 mmol/l.

Preparation of the buffer solution and isolation of PMNs

Krebs–Ringer bicarbonate buffered saline (Sigma, St Louis, MO) was dissolved in distilled water. The resulting solution was treated with sodium bicarbonate and 1N HCl to make pH = 7.4 and $[Na^+] = 140$ mmol/l.

The PMNs were isolated by Boyum's method as described previously.^{3–4} Purified neutrophils were stored in a test tube with Krebs–Ringer solution at 37 °C. The PMNs were counted using a haemocytometer, which revealed the concentration of PMNs was set at 2×10^6 PMN/ml. The cell viability was maintained over 95% as determined by Trypan blue (Sigma, St Louis, MO).

Preparation of fMLP

Commercially available fMLP (Sigma, St Louis, MO) 43.76 mg was dissolved in 10 ml dimethyl sulphoxide

Abbreviations: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; H_2O_2 , hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate; PMN, polymorphonuclear cell

(DMSO, Sigma, St Louis, MO). Then the resulting solution was diluted using Krebs–Ringer solution in a stepwise manner, making the final concentration 10^{-6} mol/l.

H₂O₂ synthesis in the PMNs

The magnitude of inflammatory response was quantified based on Bass' method in which H₂O₂ synthesis is measured by flow cytometry.^{3–6} Purified PMNs were transferred to a test tube with Ca²⁺ free Krebs–Ringer bicarbonate buffer saline, in which the total concentration was 10^6 PMN/0.5 ml. PMNs were treated with 5×10^{-6} mol/l DCFH-DA (2',7'-dichloro-fluorescein diacetate) (Sigma, St Louis, MO) and then cultured at 37 °C for 15 minutes. To inhibit the aggregation of PMNs, EDTA (ethylene diamine tetraacetic acid) (Sigma, St. Louis, MO) 5×10^{-3} mol/l was added. Then, Krebs–Ringer bicarbonate buffer saline and 2 mol/l NaCl were added to this test tube, making [Na⁺] = 140 mmol/l, 180 mmol/l, and 200 mmol/l. In each medium, both fMLP stimulated neutrophils and non-stimulated neutrophils were cultured at 37 °C for 5, 30, 60, 120, and 180 minutes. Then H₂O₂ synthesis was measured in these neutrophils.

Following this, the differences in the amount of H₂O₂ synthesis were compared in the infusion phase. To do this, we treated the PMNs with 2 mol/l NaCl and fMLP in different sequences: (1) 2 mol/l NaCl followed by fMLP; (2) 2 mol/l NaCl and fMLP at the same time; and (3) 2 mol/l NaCl after fMLP. To measure the fluorescence, these PMNs were cultured at 37 °C for 5, 30, 60, 120, and 180 minutes. To halt the reaction, centrifugation was done at 450 *g* for 2 minutes. After removing the supernatant with no cell layers, we measured [Na⁺] using RapidLab (Bayer, Sudbury, UK) and confirmed if each sodium concentration was correct. After treating with cold Ca²⁺ free Krebs–Ringer solution, the purified PMNs were stored at 0–4 °C.

Flow cytometry analysis

H₂O₂ synthesis was quantified by flow cytometry (we used FACScan, Becton-Dickinson, San Jose, CA and LYSIS II version 1.0). Using forward angle light scatter and right angle side scatter, the neutrophil gate was isolated from cell debris, red blood cells, and lymphocytes. Data were obtained from 10 000 cells, and analysed in the PMN gate using Hewlett-Packard series 9153 computer.

Statistical analysis

All data were expressed as mean (standard error of mean (SEM)), and were compared with those of normal controls.

Figure 1 Effect of fMLP stimulation. Control: PMN + buffer (*t* test) **p* < 0.05.

Figure 2 Effect of hypertonic saline without fMLP stimulation. Control: PMN + buffer (ANOVA *t* test) **p* < 0.05.

Student's *t* test and analysis of variance were done using Jandel sigma (Jandel Scientific Corp. San Raphael, CA). Statistical significance was set at *p* < 0.05.

RESULTS

Effect of fMLP stimulation

In PMNs stimulated with fMLP, the mean fluorescence was 80.9 at 5 minutes, 170.75 at 30 minutes, and 181.11 at 60 minutes. Mean fluorescence at all instances was increased compared with the control with the rise significant at even 5 minutes. It reached maximum level at 120 minutes (*p* < 0.05) (fig 1).

Effect of hypertonic saline without fMLP stimulation

In the absence of fMLP stimulation, PMNs were treated with control ([Na⁺] = 140 mmol/l) and hypertonic saline ([Na⁺] = 180 mmol/l and 200 mmol/l). Then mean fluorescence was measured and compared with that of normal controls. At [Na⁺] = 180 mmol/l, the mean fluorescence was slightly but significantly decreased compared with that of normal controls ([Na⁺] = 140 mmol/l) at 30 and 60 minutes. At [Na⁺] = 200 mmol/l, the mean fluorescence was slightly but significantly decreased compared with that of normal controls ([Na⁺] = 140 mmol/l) at 30 minutes. However, there was no significant difference in the mean fluorescence between [Na⁺] = 180 mmol/l and 200 mmol/l (fig 2).

Effect of hypertonic saline with fMLP stimulation

In the presence of fMLP stimulation, PMNs were treated with control ([Na⁺] = 140 mmol/l) and hypertonic saline ([Na⁺] = 180 mmol/l and 200 mmol/l). Then mean fluorescence was measured by the method described earlier and compared with that of normal controls. In neutrophils treated with hypertonic saline of two concentrations ([Na⁺] = 180 mmol/l and 200 mmol/l), mean fluorescence decreased compared with that of normal controls ([Na⁺] = 140 mmol/l) (*p* < 0.05). However, there was no significant difference between [Na⁺] = 180 mmol/l and 200 mmol/l with regard to mean fluorescence (fig 3).

Effect of hypertonic saline with fMLP stimulation in different sequences

PMNs were treated with hypertonic saline ([Na⁺] = 180 mmol/l) and fMLP in different sequences: (1) hypertonic saline ([Na⁺] = 180 mmol/l) followed by fMLP; (2) hypertonic saline ([Na⁺] = 180 mmol/l) and fMLP at the same time; and (3) hypertonic saline ([Na⁺] = 180 mmol/l) following fMLP. Treatment with hypertonic saline prior to

Figure 3 Effect of hypertonic saline with fMLP stimulation. Control: PMN + fMLP + buffer (ANOVA *t* test) **p*<0.05.

and simultaneously with fMLP stimulation decreased the mean fluorescence, and this was statistically significant (*p*<0.05). When treatment followed fMLP stimulation, mean fluorescence decreased slightly, but this was not statistically significant except at 60 minutes (*p*<0.05) (fig 4).

DISCUSSION

Haemodynamically, secondary tissue injury can be explained as the findings seen during ischaemia-reperfusion. Tissue injury is more severe and neutrophils are infiltrated to a greater degree during reperfusion than in ischaemia. Particular attention has been paid to the pathophysiology of secondary tissue injury due to reperfusion.⁷ In ischaemia-reperfusion injury accompanying the re-influx of oxygen molecules, hypoxanthine is transformed into xanthine with the mediation of xanthine oxidase. In the mean time, toxic oxidants are generated in large amounts. In the presence of these toxic oxidants, activated PMNs are mobilised to respond to ischaemia-reperfusion injury.⁷ In response to the stimuli, with the mediation of NADPH oxidase, PMNs release oxygen metabolites as well as specific granules including microbial peptides, protein, and enzymes.⁸

By acting on approximately 55 000 neutrophil receptors, fMLP triggers a number of transduction pathways responsible for various neutrophil functions as detailed above. After binding with fMLP, the activity of the neutrophil receptor is attenuated, and thereafter shows the constant pattern because of the absence of free neutrophil receptors (termed downregulation). In PMNs stimulated with fMLP, proinflammatory responses are triggered, which include generation of superoxide anions, increased oxidative metabolism, increased chemotaxis, cytoplasmic degranulation, and vacuolisation.⁹⁻¹¹

In the present study, we examined the effect of fMLP on neutrophils and showed that mean fluorescence reached maximum level at 120 minutes and decreased thereafter. Similar to studies using phorbol-myristate-acetate (PMA), our results demonstrate that fMLP was appropriate for the induction of inflammatory response in neutrophils.¹²

Haemodynamically, there is still controversy regarding the feasibility of initial treatment using hypertonic saline, and many studies have been conducted using 7.5% NaCl solution ([Na⁺] = 1.283 mmol/l, [Cl⁻] = 1.283 mmol/l, pH = 5.7, and osmolality = 2.567 mmol/l).¹³ In patients with hypovolaemic shock, hypertonic saline has been shown to be effective in elevating blood pressure and increasing urine output. Moreover, side effects were reported to be negligible in these patients.¹⁴⁻¹⁶ In patients with head trauma and hypotension,

Figure 4 Effect of hypertonic saline with fMLP stimulation in different sequences. Control: PMN + fMLP (ANOVA *t* test) **p*<0.05. PMN + fMLP, fMLP stimulation after hypertonic saline treatment; PMN + fMLP + hypertonic saline treatment at the same time; PMN + fMLP + hypertonic saline treatment after fMLP stimulation.

hypertonic saline has been reported to be effective in elevating blood pressure and enhancing the survival rate.¹⁷⁻¹⁹ Recent studies have focused on the immunological effect of hypertonic saline, in addition to the haemodynamic effect mentioned above.

In our study, following treatment with hypertonic saline, compared with isotonic saline, H₂O₂ synthesis decreased to a greater extent in fMLP stimulated neutrophils than in non-stimulated neutrophils (see figs 2 and 3). It may be that hypertonic saline affects fMLP itself, the binding between fMLP and neutrophils, cytoplasmic degranulation of activated neutrophils, or the involvement of NADPH-oxidase. This is analogous to the findings that by activating cAMP within PMNs, hypertonic saline inhibits extracellular signal regulated kinase (ERK) and thereby prevents oxidative bursts due to fMLP stimulation.²⁰ In comparison with the studies using neutrophils stimulated with receptor dependent fMLP or receptor independent PMA, prior to the treatment with hypertonic saline, our results are analogous to the findings that p38 mitogen activated protein kinase (p38 MARK) was inactivated and the synthesis of superoxide anion decreased in fMLP stimulated neutrophils only.^{21, 22} It may be that hypertonic saline causes cytoskeletal reorganisation, which is essential for receptor dependent signal transduction. However, H₂O₂ synthesis was slightly but significantly decreased in non-stimulated neutrophils treated with hypertonic saline at 30 and 60 minutes (fig 2). On the basis of these results, it can be inferred that hypertonic saline is involved in other processes of H₂O₂ synthesis within neutrophils. Further studies are warranted to ascertain whether this inference is correct.

There was no significant difference between the two hypertonic concentrations ([Na⁺] = 180 mmol/l and 200 mmol/l) with regard to H₂O₂ synthesis. This implies that even [Na⁺] = 180 mmol/l is sufficient for the inhibition of H₂O₂ synthesis. Following treatment with hypertonic saline, the concentration of blood Na⁺ undergoes change over time, ranging between 150 mmol/l and 160 mmol/l. There is still controversy about whether the change of blood [Na⁺] is associated with the effect of hypertonic saline.¹⁵⁻¹⁷ In animal experiments, however, the concentration of blood [Na⁺] was reported as 180 mmol/l,²² and clinical studies have reported that blood [Na⁺] = 180 mmol/l is haemodynamically effective.²⁰ On the basis of these results, it is important that the concentration of serum Na⁺ should be maintained at

180 mmol/l. However, further studies are warranted to examine the effect of hypertonic saline with concentrations lower than 180 mmol/l.

In the present study, we examined whether hypertonic saline would affect H₂O₂ synthesis in neutrophils when it was infused with fMLP in different sequences. When hypertonic saline was administered prior to or simultaneously with fMLP stimulation, H₂O₂ synthesis decreased. Following fMLP stimulation, however, H₂O₂ synthesis did not decrease. This suggests that hypertonic saline affects fMLP itself or the binding between fMLP and neutrophils. Perhaps hypertonic saline may not affect H₂O₂ synthesis after fMLP binds to the neutrophil receptors. However, in our study H₂O₂ synthesis decreased slightly after 60 minutes of treatment with hypertonic saline in fMLP stimulated neutrophils. This may be mediated by downregulation and deserves to be studied further.

CONCLUSIONS

To summarise, synthesis of H₂O₂ increased in neutrophils stimulated with fMLP. Clinically, this is analogous to the inflammatory response seen in patients with severe trauma or haemorrhagic shock. In the present study we have demonstrated a significant effect of hypertonic saline on stimulated neutrophils, resulting in attenuation of the inflammatory response. Since there was no significant difference between the [Na⁺] = 180 mmol/l and 200 mmol/l concentrations with regard to H₂O₂ synthesis, considering the side effects seen at [Na⁺] = 200 mmol/l, the use of [Na⁺] = 180 mmol/l may be recommended. However, since H₂O₂ synthesis did not decrease in neutrophils treated with hypertonic saline after an inflammatory stimulus, early resuscitation may have a further beneficial effect on patients in clinical practice.

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