The Effect of Hydroxyethyl Starches (HES 130/0.42 and HES 200/0.5) on Activated Renal Tubular Epithelial Cells

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Abstract

Background: Acute renal failure is a frequent complication of sepsis. Hydroxyethyl starch (HES) is widely used in the treatment of such patients. However, the effect of HES on renal function during sepsis remains controversial. We established an in vitro model of tumor necrosis factor-alpha (TNF-alpha)-stimulated human proximal tubular epithelial (HK-2) cells to assess the possible effects of HES 130/0.42 and HES 200/0.5 on these activated cells. Methods: HK-2 cells were stimulated with TNF-alpha in the presence or absence of HES 130/0.42 or 200/0.5. After 4, 10, and 18 h of incubation, monocyte chemoattractant protein-1 (MCP-1), a key chemoattractant for neutrophils and macrophages, was measured. In addition, viability and cytotoxicity assays were performed. Results: MCP-1 expression was doubled upon TNF-alpha exposure. In the presence of 2% and 4% HES 200/0.5 in 98% (96%) medium over a stimulation time period of 10 h and 18 h, the MCP-1 concentration was decreased between 26% and 56% (P < 0.05). TNF-alpha stimulation resulted in a significant decrease of viability by 53%-63%, whereas viability decreased by only 32%-40% in coincubation with HES 130/0.42 (P < 0.005) and remained even less affected by TNF-alpha in the presence of HES 200/0.5 (P < 0.001). The TNF-alpha-induced cell death rate was attenuated in the presence of HES 200/0.5 (P < 0.05). Conclusions: This in vitro study shows that both HES products modulate cell injury upon inflammatory stimulation. The effect was more pronounced in the HES 200/0.5 group than for HES 130/0.42, suggesting a possible biological difference between the HES types.
Effect of hydroxyethyl starches (HES 130/0.42 and HES 200/0.5) on activated renal tubular epithelial cells

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IMPLICATIONS STATEMENT

In an *in vitro* model of tubular epithelial cell injury, HES 130/0.42 and HES 200/0.5 quantitatively and qualitatively modulate the inflammatory response upon TNF-α stimulation.

The influence seems to be different for the two HES products.
ABSTRACT

**Background:** Acute renal failure is a frequent complication of sepsis. Hydroxyethyl starch (HES) is widely used in the treatment of such patients. However, the effect of HES on renal function during sepsis remains controversial. An *in vitro* model of tumor necrosis factor-α (TNF-α)-stimulated human proximal tubular epithelial cells (HK-2 cells) was established to assess possible effects of HES 130/0.42 and HES 200/0.5 on these activated cells.

**Methods:** HK-2 cells were stimulated with TNF-α in the presence or absence of HES 130/0.42 or 200/0.5. After 4, 10, and 18 h of incubation monocyte chemoattractant protein-1 (MCP-1), a key chemoattractant for neutrophils and macrophages, was determined. In addition, viability- and cytotoxicity assays were performed.

**Results:** MCP-1 expression was doubled upon TNF-α exposure. In the presence of 2% and 4% HES 200/0.5 in 98% (96%) medium over a stimulation time period of 10 h and 18 h, MCP-1 concentration was decreased between 26% and 56% (**p** < 0.05). TNF-α stimulation resulted in a significant decrease of viability by 53% - 63%, while viability decreased by only 32% - 40% in co-incubation with HES 130/0.42 (**p** < 0.005), and remained even less affected by TNF-α in the presence of HES 200/0.5 (**p** < 0.001). TNF-α-induced cell death rate was attenuated in the presence of HES 200/0.5 (**p** < 0.05).

**Conclusions:** This *in vitro* study shows that both HES products modulate cell injury upon inflammatory stimulation. The effect was more pronounced in the HES 200/0.5 group compared to HES 130/0.42, suggesting a possible biological difference between both HES types.
INTRODUCTION

Systemic inflammatory response syndrome (SIRS) and sepsis with multiple organ failure remain leading causes of death in intensive care units, despite substantial research in this field over several decades (1). Pathophysiological changes in patients with SIRS or sepsis are characterized by enhanced expression of inflammatory mediators, accumulation of neutrophils and increased vascular permeability with capillary leakage, resulting in interstitial edema formation, decreased intravascular volume and poor organ perfusion. Adequate fluid management is therefore a key issue in the treatment of these patients. Clinically, colloids are frequently used for volume replacement when attempting to maintain or improve tissue perfusion in patients experiencing infection, sepsis, trauma, shock, or surgical stress (2-4). Compared to crystalloids, colloids have the advantage of augmenting colloid oncotic pressure and minimizing edema formation, therefore improving oxygen delivery and organ function (5).

Hydroxyethyl starches (HES) are among the most widely used compounds because their volume-expanding effect is both large and long-lasting. There has been extensive research on general efficacy and safety of colloid administration in septic patients (2,6). Both deleterious and protective effects of HES compounds on the kidney have been described (7). These reports, however, are based on clinical observations and not on basic research data (8).

Therefore, the aim of this study was to construct an *in vitro* model of tubular epithelial cell injury. Tumor necrosis factor-α was chosen as stimulating agent as previously described (9). The choice of this cytokine represents the crucial role of this mediator in the inflammatory cascade during sepsis. We were specifically interested to determine the effect of HES 130/0.42 and HES 200/0.5 on stimulated tubular epithelial cells. The inflammatory response (quantitatively assessed by the expression of monocyte chemoattractant protein-1, MCP-1), cell viability and TNF-induced cell death were determined. Additionally, fluorescence labeled-HES was localized in injured cells. We assumed that both HES products would interfere with the inflammatory response of tubular epithelial cells, but that HES 200/0.5 would have a more pronounced negative effect on tubular epithelial cells compared to HES 130/0.42.
MATERIAL AND METHODS

Cell Culture
HK-2 cells, a human proximal tubule cell line, were used for these experiments (10). Cells were cultured under standard conditions, including temperature (37°C), CO₂ concentration (5%), and humidity (95%).

Cultivation was performed in 24-well plates (Corning Inc., New York, NY), in 96-well cell plates (Nunclon™ Surface, NUNC, Wiesbaden, Germany), or in 96-strip well plates (Vitaris, Baar, Switzerland). Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Basel, Switzerland), with 10% fetal bovine serum (FBS, Invitrogen, Basel, Switzerland), 1% Hepes 1 M (Life Technologies, Basel, Switzerland), 1% Penicillin-Streptomycin (Life Technologies, Basel, Switzerland) and 0.1 μg/l epidermal growth factor (Invitrogen, Basel, Switzerland) was used as medium for cultivation.

Cells were used with a 90% confluence. 12 h before the start of the experiments medium was changed to DMEM/1% FBS with 1% sodium pyruvate (Invitrogen, Basel, Switzerland).

Experimental Design
Inflammation of HK-2 cells was induced by recombinant tumor necrosis factor alpha (recombinant human TNF-α, BD Pharmingen, Basel, Switzerland) at concentrations between 0.1 and 10 ng/ml. HES 130/0.42 and HES 200/0.5 were provided by BBraun, Melsungen, Germany. Experiments were performed in four different groups: control (co), HES, TNF-α (TNF), TNF/HES.

After 2 h of “pre-stimulation” medium of control cells (Co) was changed, while HES cells (HES) were given new medium with HES. TNF-α stimulation was renewed as well after an initial stimulation of 2 h (TNF) or, in the TNF/HES group medium containing TNF-α and HES was replaced. Supernatants and/or cells were collected after 4, 10 or 18 h of stimulation, and cytotoxicity assays and/or viability tests were performed. Supernatants were aliquoted and frozen at -20°C.

In addition to HES preparations Ringer’s lactate (BBraun, Melsungen, Germany) and physiogel (BBraun, Melsungen, Germany) were used in a setup of 2 h pre-stimulation with TNF-α, followed by a 18 h experiment.
HES concentrations of 1%, 2% and 4% were calculated based on a 100% HES solution (for a 1% concentration 10 µl HES was added to 990 µl medium). Control medium was supplemented with the same amount of normal saline. Concentrations of 1%, 2% and 4% were used, based on the following previous results: After application of 15 ml/kg HES to volunteers, plasma concentrations of 10 g/l were found (11). In another study, using a hemodilution protocol in pigs, HES plasma concentrations were 1% after 50 ml/kg HES (12). HES concentrations of also 2% and 4% were used for this study, to evaluate the possible effect of higher dosages of HES applications.

**ELISA (Enzyme-linked immunosorbent assay)**

MCP-1 released from HK-2 cells into culture media was determined by enzyme-linked immunosorbent assay (ELISA). Sandwich ELISA was performed according to the manufacturer’s protocol assessing human MCP-1 (R&D Systems Europe Ltd., Abingdon, England). The detection range for MCP-1 was 0 - 500 pg/ml.

**Cell viability assay**

The MTT-assay is a well-known and acknowledged method to measure cell viability *in vitro* (13). The method is based on the reduction of the yellow tetrazoliumsalt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) to purple formazan crystals by mitochondrial dehydrogenases. Dehydrogenases are active in living cells only. Conversion of MTT is therefore directly related to cell viability.

MTT was purchased from Sigma (Buchs, Switzerland). A stock solution was prepared diluting 5 mg of MTT in 1 ml PBS. MTT stock solution was added 4 h before the end of each time course. Plates were incubated for 4 h at 37°C and supernatants were removed thereafter. Reduction of MTT was stopped by adding MTT solubilization solution from Sigma (Buchs, Switzerland). Data were collected spectrophotometrically using a standard 96-well plate reader at a wavelength of 570 nm (Labsystems Multiskan RC-Elisa reader, BioConcept, Allschwil, Switzerland).
**LDH-Assay**

Cell death rate (cytotoxicity) was determined using a nonradioactive standard lactate dehydrogenase (LDH) assay (Promega, Madison, WI). We used the LDH assay to measure membrane integrity of the HK-2 cells by quantitatively assessing lactate dehydrogenase (LDH), a stable cytosolic enzyme, which is released upon cell lysis.

Cells were cultivated in 96-well-plates. At the end of each time course cells of some wells of each group were lysed with 10 μl 1% Triton X-100 (Sigma, Basel, Switzerland) to determine total LDH of the cells. 50 μl of supernatants of each well were transferred to a new 96-well plate. 50 μl of the LDH substrate reagent were added to each well already containing 50 μl of the transferred supernatant. The plate was incubated for 30 min a room temperature and protected from light. Reaction was stopped by 50 μl of 1 M acetic acid. Absorbance was read at a wavelength of 492 nm.

**Labeling of HES with Fluorescein Isothiocyanat (FITC)**

Preperations were made as follows: 1.19 g HES (dried at 70°C over P2O5 in oil pump vacuum) was added to 15 ml dimethylsulfoxide (DMSO, Sigma, Basel, Switzerland) and heated at 95°C. After HES was fully dissolved, 125 mg fluorescein isothiocyanate (FITC, Fluka, Buchs, Switzerland) was added. After 6 h the solution was cooled to room temperature and added to 100 ml ethanol, to precipitate the labeled HES. To eliminate any free FITC, the precipitated HES was dissolved in 20 ml deionized water and dialysed against water for five days. The exclusion volume of the membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA) was 3.5 g/mol. Final isolation of the labeled HES was done by lyophilisation. FITC-labeled HES was then resuspended in 0.9% NaCl to obtain the original concentration of 6%.

**Cellular HES Uptake**

HK-2 cells were cultured on LabTek chamber slides as described above. Inflammation was induced by stimulation with TNF-α (10 ng/ml). After 2 h medium was changed and experimental groups were set up as described for 18 h with 4% FITC-HES. Control experiments were performed at 4°C. Cells were washed with PBS, fixed with 3% paraformaldehyde in PBS for 2 min, washed again and a 4,6-diamidino-2-phenylindole-staining (DAPI) was performed. After a final washing step, cover slips were mounted by
using Dako-glycerol containing 2.5%-diazybicyclo-(2.2.2)-octane (Sigma, Basel, Switzerland). For quantification of HES uptake, conventional fluorescent images were taken by using an inverted microscope (Eclipse TE 300/200; Nikon, Düsseldorf, Germany) equipped with a 60x oil immersion objective. To better localize fluorescence dye within the cells, confocal images were taken on a Leica TCS SP 5 UV CLSM (Wetzlar, Germany) confocal laser scan microscope equipped with a 63x oil immersion objective. Three dimensional reconstructions were calculated by Imaris Bitplane 6.0.0 software (Bitplane AG, Zürich, Switzerland).

**Statistics**

Expression of MCP-1, viability and cytotoxicity are presented as mean ± standard deviation (SD) and were compared between TNF and TNF/HES groups using analysis of variance for repeated measures with experiment plates (=experiment) as random within factor. The difference between the two types of HES was analyzed using analysis of variance for repeated measures with between factors group (TNF and TNF/HES) and HES type. Plate was included as random within factor nested into HES type. Addressing the fact that absolute values at different plates are not directly comparable, the interaction between group and HES type is used to indicate differences between effects of HES type. SPSS 13 (SPSS Inc., Chicago, IL) was used for statistical analyses. P-values less or equal to 0.05 were considered statistically significant.
RESULTS

MCP-1-Expression

_HES 130/0.42_

To evaluate expression of MCP-1 upon stimulation, HK-2 cells were incubated with recombinant TNF-α (control with PBS). After 2 h of stimulation, cells were co-incubated with 1% HES 130/0.42 or PBS, thereby 1% HES representing a solution of 990 µl medium with 10 µl HES. MCP-1 increased from 14±6 pg/ml to 28±6 pg/ml upon TNF-α stimulation (Tab. 1). Treatment with 1% HES 130/0.42 in the presence of TNF-α did not change MCP-1 expression. Increasing HES 130/0.42 concentrations to 2% and 4% also did not influence MCP-1 expression in the presence of TNF-α.

Similar data were found when stimulating HK-2 cells with TNF-α for 10 h, adding HES 130/0.42 2 h after initiating the TNF-α incubation. While MCP-1 concentration was changed from 14±3 pg/ml to 35±6 pg/ml upon TNF-α stimulation, 1%, 2%, and 4% HES 130/0.42 did not have any impact on MCP-1 production in co-incubation with TNF-α (Tab. 1).

18 h stimulation with TNF-α increased MCP-1 concentration from 11±3 pg/ml to 36±6 pg/ml, while 1%, 2%, and 4% HES 130/0.42 again did not alter TNF-α-stimulated MCP-1 expression (Tab. 1).

_HES 200/0.5_

The effect of HES 130/0.42 was compared with HES 200/0.5. The same experiments as mentioned above were performed with HES 200/0.5.

Again, MCP-1 increased from 5±2 pg/ml to 31±6 pg/ml upon TNF-α stimulation (Tab. 2). Interestingly, while a 4 h co-incubation with 1% and 2% HES 200/0.5 did not alter expression of MCP-1 in the presence of TNF-α, 4% HES 200/0.5 significantly decreased MCP-1 expression from 30±5 pg/ml to 19±6 pg/ml (p< 0.001).

The 10 h time course showed the following change of MCP-1 expression: while 1% HES 200/0.5 did not influence TNF-α-induced production of MCP-1, 2% and 4% decreased MCP-1 concentration from 29±6 pg/ml to 21±5 pg/ml (p= 0.002) and from 30±5 pg/ml to 20±5 pg/ml (p< 0.001), respectively (Tab. 2).
The 18 h TNF-α stimulation with 1% HES 200/0.5 had no effect on MCP-1 expression as compared with the TNF-α exposure alone, but with 2% HES 200/0.5 MCP-1 concentration was changed from 30±6 pg/ml to 21±5 pg/ml (p= 0.001), and with 4% HES 200/0.5 from 30±7 pg/ml to 13±5 pg/ml (p< 0.001) (Tab. 2).

HES 130/0.42 and HES 200/0.5

To evaluate the exact impact of TNF-α stimulation, we altered TNF-α concentrations from 0.1 to 1, 5, and 10 ng/ml TNF-α. Co-incubation of TNF-α in different concentrations with 4% HES 130/0.42 did not lead to significant changes of MCP-1 expression in comparison to the TNF-α group (Tab. 3).

While TNF-α concentrations of 0.1 and 1 ng/ml in co-incubation with HES 200/0.5 did not show any differences in MCP-1 expression compared to TNF-α alone, TNF-α concentrations of 5 or 10 ng/ml in co-incubation with HES 200/0.5 induced less MCP-1 expression than with TNF-α alone (5 ng/ml TNF-α: 29±4 pg/ml vs. 16±6 pg/ml (p< 0.001); 10 ng/ml TNF-α: 32±4 pg/ml vs. 14±3 pg/ml (p< 0.001 for both concentrations) (Tab. 3).

The following results were found, comparing the effect of both HES groups for MCP-1 expression with a 10 ng/ml TNF-α stimulation using different HES concentrations for different time intervals: a significant intergroup difference was observed with 2% HES at 10 h stimulation (p= 0.002) as well as 18 h (p= 0.003). With 4% HES at 4, 10, and 18 h stimulation a significantly different effect was observed (p< 0.001, p= 0.29, and p< 0.001). With different TNF-α concentrations a significant intergroup difference was seen with 5 and 10 ng/ml TNF-α (p< 0.001 for both conditions). All these experiments indicate a greater attenuating effect of HES 200/0.5 than HES 130/0.42.

Ringer’s lactate and physiogel

To further define interaction of fluids with stimulated tubular epithelial cells, experiments were performed with 4% Ringer’s lactate in 18 h co-incubation with TNF-α. No difference in MCP-1 expression was observed between the TNF-α and TNF-α/Ringer’s lactate group (data not shown). Similar results were found for physiogel in co-incubation with TNF-α.
**Determination of Cell Viability**

*HES 130/0.42*

Another goal of this study was to evaluate cell viability upon TNF-α stimulation with and without co-incubation of 4% HES 130/0.42. A TNF-α stimulation of 18 h was chosen with varying TNF-α concentrations between 0.1, 1, 5, and 10 ng/ml. Viability decreased by 63% upon stimulation with 0.1 ng/ml TNF-α. (Fig. 1A). In the presence of 4% HES 130/0.42, viability decreased by only 40% resulting in a difference of 17% between TNF-α and TNF/HES (p< 0.001). With a 1 ng/ml TNF-α stimulation, viability decreased by 55%, in the presence of 4% HES 130/0.42, however, only by 36% (p= 0.003 between TNF-α and TNF/HES). With 5 ng/ml TNF-α stimulation, viability decreased by 58%, and by 33% in the presence of 4% HES 130/0.42 (p< 0.001 between TNF-α and TNF/HES). With 10 ng/ml TNF-α stimulation finally, viability decreased by 53% and by 32% in co-incubation with 4% HES 130/0.42 (p< 0.001 between TNF-α and TNF/HES) (Fig. 1A).

*HES 200/0.5*

Experiments were repeated, co-incubating HK-2 cells with 4% HES 200/0.5 in the presence of 0.1, 1, 5, and 10 ng/ml TNF-α. TNF-α- induced decrease of cell viability was 54% upon stimulation with 0.1 ng/ml, 49% with 1 ng/ml, and 57% with 5 and 10 ng/ml. A stimulation with 0.1 ng/ml TNF-α in co-incubation with HES 200/0.5 decreased viability by only 26%. In co-incubation with 1 ng/ml TNF-α, viability remained unchanged in comparison to control cells. 5 ng/ml TNF-α with HES compromised viability by 18%, 10 ng/ml with HES by 9% compared to control cells (p< 0.001 between TNF-α and TNF/HES for all situations) (Fig. 1B).

Comparing the effect of HES130/0.42 and HES 200/0.5 on viability the following results were found: at 1, 5, and 10 ng/ml TNF-α stimulation the attenuating effect of HES 200/0.5 was greater than the attenuating effect of HES 130/0.42 (p< 0.001, p= 0.001, p< 0.001).
Ringer’s lactate and physiogel

Viability was also determined in cells stimulated with TNF-α in the presence of Ringer’s lactate and physiogel. While no improvement of viability was observed in the presence of Ringer’s lactate (Tab. 4), physiogel clearly showed a positive effect on TNF-α-impaired viability (Tab. 5).

Determination of Cytotoxicity

HES 130/0.42

The same experiments were repeated to determine cytotoxicity. Co-incubation of TNF-α with HES 130/0.42 significantly attenuated cytotoxicity in the presence of 0.1 ng/ml TNF-α (p = 0.043) (Fig. 2A). For higher TNF-α concentrations, HES co-incubation did not further change cytotoxicity (p values n.s. between TNF-α and TNF/HES).

HES 200/0.5

Incubating HK-2 cells with different concentrations of TNF-α and co-incubating them with 4% HES 200/0.5 gave similar results as with HES 130/0.42. For 0.1 and 1 ng/ml TNF-α, no difference in cytotoxicity was observed between the TNF-α and TNF/HES group. However, HES 200/0.5 blocked increase of cytotoxicity at the higher doses of TNF-α (p = 0.003 between 5 ng/ml TNF-α and TNF/HES and p = 0.026 between 10 ng/ml TNF-α and TNF/HES) (Fig. 2B).

Ringer’s lactate and physiogel

Ringer’s lactate nor physiogel had an impact on cell death (data not shown).

Cellular Uptake of FITC-labeled HES

Cellular Uptake of FITC-labeled HES was shown for both HES 130/0.42 and HES 200/0.5. However, a visible difference in the quantity of HES uptake regarding both HES compounds was observed when comparing TNF-α stimulated cells with unstimulated cells (Fig. 3A-D). To demonstrate an intracellular uptake, excluding a cellular adhesion of HES molecules to the outer membrane of the cells only, confocal
microscopy was performed. Three-dimensional reproduction showed a clear intracellular accumulation of HES-FITC (Fig. 4A, B and C).
DISCUSSION

HES has been shown to be beneficial in several inflammatory conditions *in vitro* and *in vivo* by attenuating chemotaxis of white blood cells through endothelial cells, down-regulating inflammatory mediators in blood during sepsis, and improving pulmonary function during endotoxemia (14-16). Additionally, recent data suggest that in sepsis HES may decrease production of hepatic inflammatory mediators (17).

Acute renal failure (ARF) is a common complication of severe sepsis and septic shock (18,19). Correction of volume depletion is crucial to the prevention of acute tubular necrosis. It has been shown that colloids may provide advantages in maintaining kidney function in comparison to crystalloid volume replacement (20). The choice of administered colloid, however, might impact on the renal function in severely ill patients. Clinical trials have pointed out a negative effect of HES 200 on kidney function in sepsis (21,22).

Possible deleterious renal effects of HES have been described even in the absence of an inflammatory condition. In two patients receiving HES 200/0.5 deterioration of pre-existing renal impairment has been described (23). Two cases of ARF were found after administration of HES 450/0.7 (24). Renal insufficiency with biopsy-proven osmotic nephrosis-like lesions was attributed to HES in additional cases (25,26). Evidence has been reported that the use of HES in organ donors may be associated with osmotic-like lesions of both the proximal and the distal renal tubules in kidney-transplant recipients (27,28). These data are supported by another study including 69 kidney transplants from organ donors, which showed that the need of hemodialysis or hemodiafiltration was significantly higher in the group receiving HES 200/0.62 plus gelatin than in the gelatin group (27). In another controlled study, urinary output of 24 renal transplant patients was lower in the group receiving HES compared to receiving no HES (29).

No data exist so far evaluating the impact of HES products on stimulated tubular epithelial cells *in vitro*. We therefore evaluated some aspects of the inflammatory response of human tubular epithelial cells upon stimulation with TNF-α and the co-incubation with HES 130/0.42 or 200/0.5. This model has some limitations: 1) It is an *in vitro* model with no elimination mechanisms and no dynamics. 2) Stimulation with TNF-α does not represent a sepsis model, but creates a inflammatory condition with TNF-α being a key mediator in the activation cascade of the inflammation in sepsis (30). 3) The two products differ in their colloid osmotic pressure, which might also have an impact on tubular epithelial cell.
In a first experimental setup the inflammatory response was evaluated for expression of MCP-1. This chemokine was primarily chosen as the main molecular target, playing a pivotal role not only in monocyte, but also in polymorphonuclear cell (PMN) recruitment in various experimental systems (31-35). Interestingly, HES 130/0.42 did not have an impact on MCP-1 expression in combination with TNF-α stimulation. HES 200/0.5, however, attenuated TNF-α -induced MCP-1 production. This observation indicates a difference between HES 130/0.42 and HES 200/0.5 in the potential for interference with the inflammatory cascade. The beneficial or harmful character of the downregulation of MCP-1 has to be further evaluated. Whether these observed effects are merely due to the fact that HES molecules cover the cell surface and thereby prevents TNF-α interaction with the receptor, or can be attributed to endocytosis and some yet unknown intracellular target effect will be a point of further investigations.

Previous *in vitro* studies on endothelial cell activation suggested a possible beneficial role of HES in the inhibition of endothelial cell activation, preventing neutrophil adhesion upon stimulation with endotoxin. Another study found that HES treatment of PMNs significantly altered tethering to and transmigration through stimulated cultured human endothelial cell monolayers (36). Results from a study, performed with human microvascular endothelial cells under 2% oxygen for 48 h, indicate that hypoxia-induced increases in vascular leakage and acute inflammation are attenuated by HES treatment (37). Similar results were also achieved in several animal models, showing that HES had an anti-inflammatory effect through suppression of inflammatory mediators. Feng et al. induced lung inflammation in a sepsis model by performing cecal ligation und puncture (CLP) (38). Animals were randomly assigned to receive saline or HES 130/0.4. Fluid replacement with HES 130/0.4 significantly attenuated the CLP-induced increase of cytokine- and chemokine levels and recruitment of neutrophils. This was also confirmed by Lv et al (17). In the CLP model, HES showed beneficial effects in pulmonary injury through downregulation of inflammatory mediators and suppression of NF-kappaB. Our study provides information regarding a different impact of both HES products on stimulated tubular epithelial cells, but no qualitative conclusions can be made.

Beside the inflammatory response we were also interested in the effect of both HES products on cell viability. Increased viability of tubular epithelial cells in the presence of HES 130/0.42 and 200/0.5 upon stimulation with TNF-α was observed. Both HES preparations restored the mitochondrial activity, which was impaired by exposure to TNF-α. Thereby, HES 200/0.5 showed a greater protective effect on viability
than HES 130/0.42. The mechanism of HES-induced cell protection is not clear and has to be further investigated.

Another important goal of this study was to evaluate if tubular epithelial cells ingest HES as observed in other cells such as monocytes, keratinocytes, or perivascular histiocytes (39,40). Our experiments with FITC-labeled HES preparations indicate an increased cellular uptake of FITC-HES by TNF-α stimulated cells in comparison to unstimulated cells.

In summary, our in vitro findings of HES-exposed TNF-α-injured tubular epithelial cells suggest that the two HES preparation have different qualitative and quantitative effects on the inflammatory reaction and viability of tubular epithelial cells. Well-designed in vivo studies, followed by clinical studies are needed to elucidate whether these results can be transfered from basic research into clinical practice.
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REFERENCES


FIGURE LEGEND

Fig. 1A:
HES 130/0.42 4% administration and cell viability. Confluent cell layers were pre-treated with TNF-α at concentrations of 0.1, 1, 5, or 10 ng/ml (or PBS for control) for 2 h followed by co-incubation of cells with HES 130/0.42 4% with or without TNF-α 0.1, 1, 5, or 10 ng/ml for 18 h. Values are mean ± SD from 5 experiments.

Fig. 1B:
HES 200/0.5 4% administration and cell viability. Confluent cell layers were pre-treated with TNF-α at concentrations of 0.1, 1, 5, or 10 ng/ml (or PBS for control) for 2 h followed by co-incubation of cells with HES 200/0.5 4% with or without TNF-α 0.1, 1, 5, or 10 ng/ml for 18 h. Values are mean ± SD from 5 experiments.

Fig. 2A:
HES 130/0.42 4% administration and cell death. Confluent cell layers were pre-treated with TNF-α at concentrations of 0.1, 1, 5, and 10 ng/ml (or PBS for control) for 2 h, followed by co-incubation of cells with HES 130/0.42 4% with or without TNF-α 0.1, 1, 5, or 10 ng/ml for 18 h. Values are mean ± SD from 5 experiments.

Fig. 2B:
HES 200/0.5 4% administration and cell death. Confluent cell layers were pre-treated with TNF-α at concentrations of 0.1, 1, 5, and 10 ng/ml (or PBS for control) for 2 h, followed by co-incubation of cells with HES 200/0.5 4% with or without TNF-α 0.1, 1, 5, or 10 ng/ml for 18 h. Values are mean ± SD from 5 experiments.
Fig. 3A-D:

A. Stimulation of HK-2 cells with phosphate-buffered saline for 2 h, followed by exposure to 4% HES-FITC 130/0.42 (green) for 18 h. B. Exposure of HK-2 cells to TNF-α for 2 h, followed by stimulation with TNF-α for 18 h in the presence of 4% HES-FITC 130/0.42 (green). C. Stimulation of HK-2 cells with phosphate-buffered saline for 2 h, followed by exposure to 4% HES-FITC 200/0.5 (green) for 18 h. D. Exposure of HK-2 cells to TNF-α for 2 h, followed by stimulation with TNF-α for 18 h in the presence of 4% HES-FITC 200/0.5 (green). Blue DAPI staining shows cell nuclei.

Fig. 4A, B:

Confocal microscopy showing cellular uptake of 4% HES-FITC (green).

A. Exposure of HK-2 cells to TNF-α for 2 h, followed by stimulation with TNF-α for 18 h in the presence of 4% HES-FITC 130/0.42 (green). Blue DAPI staining shows cell nuclei. Top view (large picture), front view (small picture bottom), side view (small right picture). B. Three-dimensional reconstruction showing cellular uptake of 4% HES-FITC 130/0.42 (green). Five independent experiments were performed.

C. Exposure of HK-2 cells to TNF-α for 2 h, followed by stimulation with TNF-α for 18 h in the presence of 4% HES-FITC 200/0.5 (green). Blue DAPI staining shows cell nuclei. D. Three-dimensional reconstruction showing cellular uptake of 4% HES-FITC 200/0.5 (green). Five independent experiments were performed.