

Deleterious Effects of Mild Hypothermia in Septic Rats Are Ameliorated by Granulocyte Colony-stimulating Factor

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Background: The authors studied the effects of mild hypothermia on the outcome in a rat model of intraabdominal sepsis and tested whether granulocyte colony-stimulating factor (G-CSF) augments the host response and improves outcome during mild hypothermia.

Methods: A rat model of peritoneal contamination and infection with human stool bacteria was used to simulate clinical trials that included increasing complexity. In trial 1, postoperative hypothermia (32°C) was compared with normothermia (38°C), without supportive treatment (10 rats per group). In trial 2, with a more severe infection, rats were given antibiotic prophylaxis. Using 20 rats per group, the authors compared postoperative hypothermia (32°C), normothermia, and postoperative hypothermia (32°C) with 20 µg/kg G-CSF prophylaxis given 12 h before surgery and 12 h and 36 h after surgery. The primary endpoint was death at 120 h. Secondary endpoints were systemic cytokine concentrations, leukocyte counts, and the phagocytic activity of granulocytes and monocytes.

Results: In trial 1, 50% of the normothermia group and 10% of the postoperative hypothermia group survived. In trial 2, 50% of the normothermia group, 20% of the hypothermia group, and 60% of the hypothermia plus G-CSF group survived. Postoperative hypothermia plus G-CSF reduced plasma concentrations of interleukin-6 (hypothermia group, 511 ± 104 pg/ml; hypothermia plus G-CSF group, 247 ± 51 pg/ml) and macrophage inflammatory protein-2 (hypothermia group, 239 ± 43 pg/ml; hypothermia plus G-CSF group, 178 ± 21 pg/ml).

Conclusions: In this rat model of intraabdominal sepsis, postoperative hypothermia was deleterious. However, G-CSF treatment, initiated before contamination, reduced the mortality rate, increased the neutrophil count, and downgraded the systemic cytokine response.

THERE is overwhelming evidence in animals that mild hypothermia improves outcome from cerebral and cardiac ischemia.¹⁻³ Hypothermia has been shown to im-

prove outcome from out-of-hospital cardiac arrest in humans,⁴ and other human studies of stroke and acute myocardial infarction are in progress. Even without conclusive evidence of benefit, hypothermia is increasingly being used therapeutically in neurosurgery and in patients who have had strokes.⁵ It is also occasionally used in an effort to reduce oxygen consumption in septic patients.⁶

Mild perioperative hypothermia, though, is known to provoke numerous serious complications, including morbid myocardial events,⁷ coagulopathy and increased transfusion requirement,⁸ and delayed drug metabolism^{9,10} leading to prolonged recovery¹¹ and admission to the hospital. Hypothermia also impairs immune function by decreasing oxidative killing by polymorphonuclear granulocytes (PMNs), which is the most important host defense against bacterial pathogens. In pigs, for example, hypothermia decreases leukocyte and neutrophil concentration and suppresses bone marrow and neutrophil function. In fact, even an endotoxin challenge fails to stimulate neutrophil release from bone marrow in pigs at a body temperature of 29°C.¹² Hypothermia also reduces interleukin-2 production, which is central in various immune responses,¹³ and thus may increase susceptibility for infections. Consistent with these observations, perioperative hypothermia triples the risk of surgical wound infections.¹⁴

Clinicians thus must balance the putative benefits of therapeutic hypothermia against the risks, including infectious complications. A potential approach is to moderate the infectious risks of therapeutic hypothermia by combining it with granulocyte colony-stimulating factor (G-CSF). G-CSF stimulates host defenses against microbes¹⁵⁻¹⁷ and reduces proinflammatory cytokine responses.^{18,19} Therefore, as might be expected, G-CSF is effective in certain types of infection, such as diabetic foot ulcers²⁰ and high-risk febrile neutropenia.²¹

However, whether G-CSF is beneficial in intraabdominal contamination and infection with concomitant hypothermia is unknown. For modeling these complex clinical interactions, clinic modeling randomized trials^{22,23} are applicable (table 1). In these clinic modeling randomized trials, we tested the hypothesis that prophylactic G-CSF would improve survival rate, decrease cytokine release, and enhance phagocytosis of granulocytes and monocytes in rats submitted to mild postoperative hypothermia after peritoneal contamination and infection (PCI).

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Table 1. Clinic Modeling Trials: Rationale and Characteristics

Modeling the Clinical Situation	Modeling Randomized Trials
<ul style="list-style-type: none"> ● Modeling clinical trials (scenario and methodology) before or after conducting a clinical trial ● Modeling clinical complexity is more important than species differences ● Modeling treatment effects as expected or warranted in the clinical scenario (δ, α, and β; e.g., high sample size) ● Searching for positive and negative results 	<ul style="list-style-type: none"> ● Sample size calculation ($\delta = 0.25$, $2\alpha = 0.05$, $1 - \beta = 0.9$) ● Randomized allocation to the groups ● Double-masked design ● Clinically relevant endpoint (5 day mortality) ● Evaluation of morbidity ● Search for positive and negative results ● "Intent to treat" rule ● Adequate statistical analysis

Materials and Methods

Our study was performed with permission of the regional animal welfare committee in Gießen, Hessen, Germany. We used 80 male Wistar rats, 220–280 g (Charles River Wiga, Sulzfeld, Germany), in two separate trials. They were given a standard diet (Altromin, Lage, Germany) and water *ad libitum*.

Two independent trials were performed. In the first, the effects of mild postoperative hypothermia (32°C) were compared with normothermia after PCI without antibiotic prophylaxis or other supportive treatment. In the second, we evaluated the effects of a more severe infection combined with antibiotic prophylaxis during postoperative mild hypothermia (32°C), during postoperative mild hypothermia with G-CSF prophylaxis, and during normothermia.

Protocol

The rats were deprived of food for 12 h before surgery. In the appropriate animals, 20 μ g/kg of G-CSF (Filgrastim; Amgen, Munich, Germany) was given as a subcutaneous injection three times: 12 h before surgery and again 12 and 36 h after surgery (PCI). This dose and administration schedule was based on our previous studies.²⁴ Control groups were given equal volumes of a placebo consisting of lactated Ringer's solution.

One hour before surgery, the animals were anesthetized with 0.08 mg/kg fentanyl and 4 mg/kg droperidol (Janssen-Cilag, Neuss, Germany), both given intraperitoneally. Ventilation was spontaneous. Subsequently, a tail vein was cannulated and 2 ml lactated Ringer's solution was given. Animals in trial 2 were then given intravenous antibiotic prophylaxis with 10 mg/kg cefuroxime (Fresenius, Bad Homburg, Germany) and 3 mg/kg metronidazole (Serag-Wiessner, Naila, Germany).

Using antiseptic technique, a 2-cm midline incision was made and 1.0 ml/kg (trial 1, without antibiotic) or 1.9 ml/kg (trial 2, with antibiotic) standardized human stool inoculum (diluted 1:2.5 in lactated Ringer's solution) was injected into the pelvic region. This dose

results in a mortality rate of approximately 50% in the control groups. The wound was closed in two layers using an interrupted Vicryl 3-0 suture.

Postoperative analgesia consisted of 20 mg/kg tramadol (Mundipharma, Limburg, Germany) given subcutaneously once daily. After the operation, the animals received food and water *ad libitum*. Postoperative temperature management is described later. At the end of each trial (after 120 h), survivors were killed by inhalation of CO₂.

Trial 1. Animals were assigned by simple random permutation to two groups using ear marks ($n = 10$ rats per group): postoperative hypothermia or postoperative normothermia. The surgeon was blinded to the postoperative temperature management. Core temperature in the postoperative hypothermia group was maintained at $32 \pm 1^\circ\text{C}$ for 1 h after surgery by surface cooling of the animals using ice-filled plastic bags. The animals were subsequently warmed to 38°C with an infrared heating lamp. In the normothermia group, an infrared lamp maintained postoperative core temperature at 38°C.

Trial 2. All animals received antibiotic prophylaxis as soon as the tail vein was cannulated, at least 1 h before surgery. They were randomly assigned to three groups ($n = 20$ rats per group): postoperative mild hypothermia (32°C), postoperative mild hypothermia (32°C) with G-CSF prophylaxis before and after surgery, or postoperative normothermia. In designated animals, 20 μ g/kg G-CSF was given as a subcutaneous injection three times: 12 h before surgery and again 12 and 36 h after surgery. Control groups were given equal volumes of lactated Ringer's placebo.

Measurements

Animals were weighed the day before surgery. Before operation, a digital thermometer was inserted 3 cm into the rectum for continuous core temperature measurement throughout surgery and the postoperative period until rewarming was completed in the appropriate animals.

Ten rats, randomly selected from each trial 2 group,

had 1.5 ml blood taken from the retroorbital (after supplemented analgesia with fentanyl/droperidol) 1 h before and 1 h after PCI. The blood was replaced intravenously with 3 ml lactated Ringer's solution. By using only 10 rats from each group, half of each group was left unstressed by blood sampling. Heparinized whole blood was used for determination of phagocytosis of fluorescein-isothiocyanate opsonized *Escherichia coli* by granulocytes and monocytes in flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany) with a phagotest kit (Orpegen-Pharma, Heidelberg, Germany).

The leukocyte count was determined in an automated blood cell counter optimized for rat blood (Coulter Max-M, Krefeld, Germany). For cytokine determinations, blood was immediately centrifuged and the resulting plasma was stored at -70°C until assayed. An enzyme-linked immunosorbent assay technique (Pharmingen/Becton Dickinson, Heidelberg, Germany; Biosource, Camarillo, CA) was used to determine interleukin-6 (IL-6), tumor necrosis factor (TNF- α), and macrophage inflammatory protein-2 (MIP-2) concentrations.

Statistical Analysis

The primary endpoint for both trials was survival of rats at 120 h after surgery. For trial 1, the sample size of 10 rats per group was calculated with the formula of Friedman,²⁵ estimating a 50% survival difference between the normothermia and hypothermia groups with an α error of 0.025 and a power of 0.9. For trial 2, 20 rats per group were used for sample size calculation, but based on a 25% difference in the survival rates. Survival rates were analyzed with the chi-square test and survival curves with the log-rank test. Ordinal data were analyzed with the Kruskal-Wallis test using SPSS software.²⁶ *Post hoc* testing included a Bonferroni-Holm correction. Ordinal data are presented as means \pm SEMs; $P < 0.05$ was considered statistically significant.

Results

The rats in all groups were of similar weight (250 ± 30 g). There were no complications related to surgery, but one rat in trial 2 that was assigned to G-CSF died before the hypothermia phase of the experiment was completed. Data from this animal were included in the analysis.

In the low-complexity initial trial, only 10% (1 of 10) of the septic rats in the hypothermia group survived 120 h after surgery. In contrast, 50% (5 of 10) of the rats in the normothermia group survived ($P < 0.05$) (fig. 1).

In the more complex second trial, with a more severe infection but antibiotic prophylaxis, the survival rate in the normothermia group was also 50% (10 of 20) compared with 20% (4 of 20) in the hypothermia group (fig. 2). However, prophylaxis with G-CSF combined with

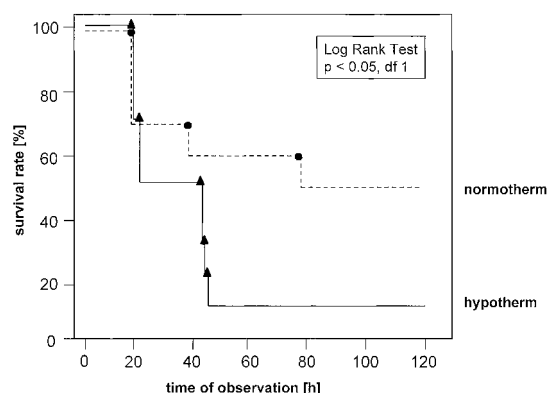


Fig. 1. Kaplan-Meier survival analysis of rats without supportive treatment in trial 1, comparing mild postoperative hypothermia (32°C) and normothermia (38°C) in septic rats for 120 h ($n = 10$ per group, $P < 0.05$). Contamination and infection was performed with 1.0 ml/kg standardized human stool.

postoperative hypothermia improved the survival rate to 60% (12 of 20), which was significantly better than that in the hypothermia alone group ($P < 0.05$) (fig. 2). The survival rate of the animals stressed by blood sampling did not differ significantly from rats, who were otherwise treated comparably.

Preoperative cytokine concentrations of TNF- α , IL-6, and chemokine MIP-2 levels were at the detection limit of the assays (data not shown). Postoperative plasma TNF- α concentrations were similar in the three groups (fig. 3). However, plasma concentrations of IL-6 and MIP-2 were significantly less in the G-CSF prophylaxis group than in the hypothermia only group; MIP-2 concentrations were 68 ± 11 pg/ml in the normothermia group, 239 ± 43 pg/ml in the hypothermia group, and 178 ± 21 pg/ml in the G-CSF group ($P < 0.001$) (fig. 3). IL-6 concentrations were 100 ± 26 pg/ml in the normothermia group, 511 ± 104 pg/ml in the hypothermia group, and 247 ± 51 pg/ml in the G-CSF group ($P < 0.05$).

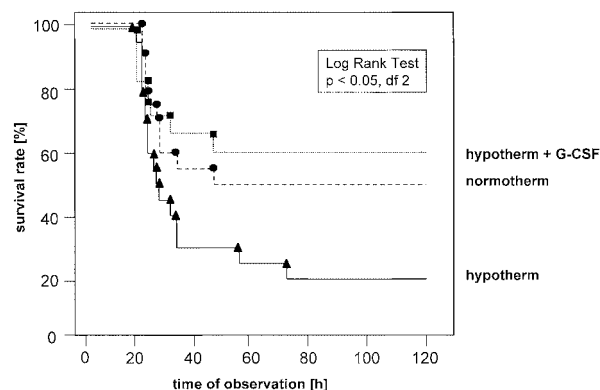


Fig. 2. Kaplan-Meier survival analysis of rats (all with a cefuroxime-metronidazole antibiotic prophylaxis) in trial 2 for 120 h, comparing mild postoperative hypothermia (32°C) alone versus mild postoperative hypothermia (32°C) plus G-CSF prophylaxis versus postoperative normothermia (38°C). Contamination and infection was performed with 1.9 ml/kg standardized human stool ($n = 20$ per group, $P < 0.05$, mild hypothermia plus G-CSF, $P < 0.05$).

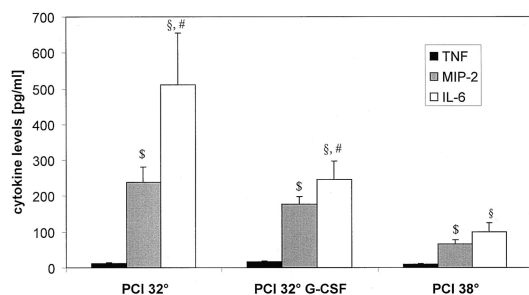


Fig. 3. Tumor necrosis factor, macrophage inflammatory protein-2 (MIP-2), and interleukin-6 (IL-6) protein concentrations in plasma of rats 1 h after contamination and infection in trial 2. Cytokine concentrations were determined by rat enzyme-linked immunosorbent assay. The Kruskal-Wallis test and the *post hoc* analysis were performed for IL-6 and for MIP-2 ($n = 10$ per group; $§, §P < 0.001$; $\#P < 0.05$ between hypothermia with and without G-CSF for IL-6). Data are presented as mean \pm SEM.

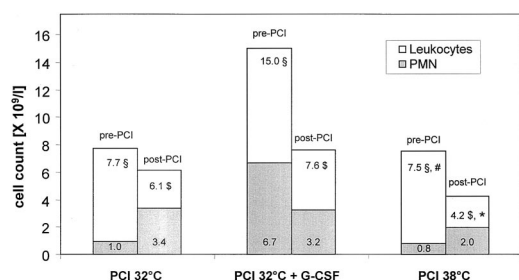


Fig. 4. Counts of leukocytes and polymorphonuclear granulocytes ($10^9/l$) determined with an automated blood cell counter 1 h before and 1 h after contamination and infection in trial 2 ($§, §P < 0.01$; $\#P < 0.05$ versus mild hypothermia with G-CSF before peritoneal contamination and infection, $*P < 0.05$ versus mild hypothermia with G-CSF peritoneal contamination and infection).

0.001) (fig. 3). The IL-6 concentration in the G-CSF group was significantly less than in the hypothermia alone group ($P < 0.05$).

As expected, the leukocyte and the PMN count were increased in the G-CSF prophylaxis group ($15.0 \times 10^9/l$ and $6.7 \times 10^9/l$, respectively) compared with the normothermia ($7.5 \times 10^9/l$ and $0.8 \times 10^9/l$, respectively) and the hypothermia group ($7.7 \times 10^9/l$ and $1.0 \times 10^9/l$, respectively) before surgery ($P < 0.01$ for leukocytes and PMN) (fig. 4). After surgery, the leukocyte and PMN counts remained significantly greater in the hypothermia

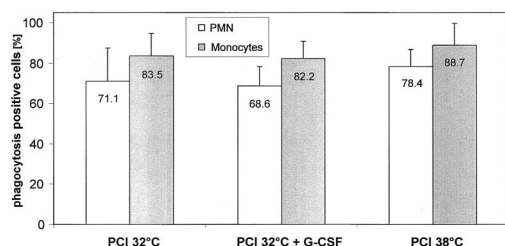


Fig. 5. Flow cytometric determination of the granulocyte and monocyte phagocytic activity in trial 2. Blood was sampled 1 h after contamination and infection ($n = 10$, $*P = 0.388$ with chi-square test). Data are presented as mean \pm SEM.

groups than in the normothermia group ($P < 0.05$). One hour after surgery, the phagocytic activity of granulocytes and monocytes was similar in all groups (fig. 5).

Discussion

Therapeutic use of mild hypothermia ($32^\circ\text{--}33^\circ\text{C}$) was first reported to be beneficial for patients with severe head injury in 1993.^{27,28} Although the animal evidence supporting therapeutic use of hypothermia is overwhelming, only a few prospective, randomized trials have been reported in humans. The results remain equivocal, with mild hypothermia providing substantial benefit after out-of-hospital cardiac arrest,⁴ but not for traumatic brain injury.²⁹ Major trials of therapeutic hypothermia for aneurysm surgery and acute myocardial infarction are in progress.

Assuming hypothermia is proven beneficial for various ischemic conditions, there will be at least two major problems with applying the treatment in humans. The first is, that nonanesthetized humans precisely regulate core temperature.³⁰ Efforts to induce therapeutic hypothermia thus provoke vigorous thermoregulatory defenses, such as vasoconstriction and shivering, both of which activate the sympathetic nervous system resulting in hypertension, tachycardia, and substantial increases in circulating catecholamine concentrations.³¹

The second major problem with therapeutic hypothermia is impairment of host immune defenses, resulting in infectious complications. Many *in vitro* and animal studies indicate that mild hypothermia *per se* suppresses systemic inflammatory responses,^{32,33} which may contribute to death in hypothermic patients. Our results are consistent with these observations in that postoperative hypothermia compared with normothermia reduced the survival rate from 50% to 10% in trial 1 with a low complexity level (no antibiotic prophylaxis) and from 50% to 20% in trial 2 with a higher complexity level (more severe infection, but with antibiotic prophylaxis). In contrast, though, we found significantly increased concentrations of proinflammatory cytokine IL-6 and chemokine MIP-2, when hypothermia was initiated after infection. This findings can be interpreted as a state of hyperinflammation triggered by hypothermia, which disturbs the delicate proinflammatory and antiinflammatory cytokine balance,³⁴ resulting in the deleterious outcome in this group. To our knowledge, there are so far only two cases of patients with accidental hypothermia reported, in whom increased expression of IL-6 was reported.³⁵

Antibiotics are routinely given for sepsis prophylaxis in clinical practice. Adequate intravenous antibiotic prophylaxis has nonetheless been neglected in many animal experiments. Trials were performed without antibiotic prophylaxis³⁶ or with a clinically unusual application

(e.g., intramuscular application) of antibiotics.³⁷ From previous studies, it is known that with a small amount of infectious material, as in the first trial, antibiotic prophylaxis results in an approximate 100% survival rate of the animals.²⁴ However, when the infectious challenge is nearly doubled, as in the second trial, with the same antibiotic prophylaxis, only 50% of the animals survive. Comparing across the trials, we may state that antibiotic prophylaxis alone contributes to an improvement in the survival rate of approximately 40%, when stool inoculum is nearly doubled. The inclusion of an antibiotic prophylaxis is only one important feature of our clinic modeling randomized trials for modeling the clinical complexity. Others include appropriate use of anesthesia, volume loading, laparotomy, PCI with human stool bacteria, suitable postoperative analgesia, and complicating risk factors such as hypothermia. In addition, study conditions similar to those of randomized clinical trials are applied (table 1). We therefore think our model of peritoneal sepsis is highly relevant and applicable from a clinical point of view. Furthermore, it was validated and confirmed extensively by our group in terms of microbiologic characterization and reproducibility, as shown by a dose-mortality relationship.²⁴

G-CSF prophylaxis may stimulate cellular immune defense mechanisms and alter the cytokine network. Migration, phagocytosis, and production of superoxide anions are normalized in septic animals by G-CSF prophylaxis.^{17,38} G-CSF also induces expression of adhesion molecules CD11b/c and CD18 of circulating granulocytes and enhances other leukocyte functions.³⁹ In the second trial, G-CSF antagonized the deleterious effect of postoperative hypothermia on survival and reduced the excessive proinflammatory cytokine production, but not beyond that of septic rats, which were normothermic. From our previous work²³ and that of others, it is known that G-CSF prophylaxis before sepsis increases the survival rate in normothermic rats. We also tested optimal dosing and time schedule for G-CSF before performing the trials.²⁴ It is known that in animals, approximately 10-fold higher doses are necessary to achieve immune stimulation than in humans.²⁴ However, even doses up to 200 $\mu\text{g/kg}$ were given in rats.⁴⁰

A potential explanation for increased survival rates with G-CSF is that it reduces the concentrations of proinflammatory cytokines (*i.e.*, IL-6 and TNF- α) not only systemically, but also at the site of infection (peritoneum), as we have measured in normothermic animals previously.⁴¹ In trial 2, G-CSF suppressed the excessive release of the proinflammatory cytokine IL-6 and chemokine MIP-2 after hypothermia. MIP-2 enhances PMN recruitment and migration into infected tissues. Thus, we also saw a decrease in circulating PMNs in the animals treated with G-CSF. Given the findings that neutrophil phagocytic activity is reduced in hypothermia,⁴² we suggest that our dose of G-CSF was sufficiently large to

increase absolute neutrophil count and recruitment, but may have been insufficient to augment neutrophil and monocyte phagocytosis of circulating cells in rats with hypothermia. This hypothesis is supported by the results of a clinical trial showing beneficial immunologic effects of G-CSF, which was given to patients with severe head injuries, in whom mild therapeutic hypothermia was initiated.⁴³

Our model is clinically relevant for studying the development of postoperative intraabdominal sepsis and accompanying risk factors. Thus, current and previous work indicates that postoperative hypothermia impairs the host immune response to an infectious challenge. Most surgical patients therefore should be kept normothermic perioperatively. If mild hypothermia is proven to ameliorate tissue ischemia, however, it may nonetheless be indicated in selected patients. Our results suggest that the administration of G-CSF may improve the host response to bacterial peritonitis in such patients.

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