

Effect of Hypertonic Saline Infusion on Postoperative Cellular Immune Function

A Randomized Controlled Clinical Trial

Jens A. Kølsen-Petersen, M.D., Ph.D.,* Jens-Ole D. Nielsen, M.D.,* Else M. Tonnesen, M.D., D.M.Sc.†

Background: Previous studies found hypertonicity to affect immune responses in intact laboratory animals and in human blood cell cultures. In this study, the authors investigated the cellular immune response to surgery after preoperative infusion of hypertonic saline in humans.

Methods: Sixty-two women scheduled to undergo abdominal hysterectomy were randomly assigned to single-blinded infusion of 4 ml/kg NaCl, 7.5%; 4 ml/kg NaCl, 0.9%; or 32 ml/kg NaCl, 0.9%, over 20 min. Blood was collected at baseline, during surgery, and 1, 24, and 48 h after surgery for the determination of leukocyte and differential counts, flow cytometric phenotyping of mononuclear cells, and natural killer cell activity against K 562 tumor cells. Phytohemagglutinin-induced lymphocyte proliferation, plasma elastase, and neutrophil chemotaxis were measured at the same time points except during surgery. The authors tested cell-mediated immune function *in vivo* by delayed-type hypersensitivity reaction in the skin.

Results: Surgery induced well-known changes in the cellular immune response, which were unrelated to the tonicity or volume of the infused fluids.

Conclusion: Infusion of a clinically relevant dose of hypertonic saline did not seem to modify the postoperative cellular immune response after elective abdominal hysterectomy.

HYPERTONIC saline solutions are effective in the treatment of hemorrhagic and septic shock, burn injury, and perioperative fluid deficits.¹ Infusion rapidly restores the macrocirculation and the microcirculation by a combination of osmotic-induced plasma volume expansion, arteriolar vasodilation, and capillary endothelial shrinkage.² Furthermore, hypertonicity may ameliorate the detrimental consequences on the immune function of trauma, shock, reperfusion, and major surgery. It was shown that lymphocytes proliferated at a greater rate under hypertonic circumstances³ and that hypertonicity reversed shock-induced suppression of T-cell function,⁴⁻⁶ leading to improved survival in an animal model.⁷ In addition, hypertonicity suppressed neutrophil functions.⁸⁻¹³ Activated neutrophils are thought to play a pivotal role in tissue injury and may contribute to organ dysfunction after ischemia-reperfusion.¹⁴ Consequently,

infusion of hypertonic saline may protect against neutrophil-mediated organ injury and at the same time boost other parts of the immune system.

Previous studies were performed in human blood cell cultures and different animal models. However, it is difficult to extrapolate from animal studies and laboratory experiments to the clinical situation, where immunocompetent cells communicate and are influenced by hormones and cytokines. Consequently, clinical studies are needed. Only one study addressed the immune effects of hypertonic saline infusion in humans.¹⁵ We conducted a study to test the hypothesis that infusion of a clinically relevant dose of hypertonic saline reverses the postoperative suppression of cellular immune responses.

Materials and Methods

Approval of Study Design

The Regional Ethical Review Committee (VN 2000/145) and The Danish Medicines Agency, Copenhagen, Denmark (2612-1491) approved this prospective, randomized, single-blinded study. The study was conducted according to the guidelines for Good Clinical Practice¹⁶ and was monitored by the Good Clinical Practice Unit, Aarhus University Hospital (2001/43), Aarhus, Denmark.

Eligibility

We assessed patients scheduled to undergo abdominal hysterectomy for possible enrollment according to the inclusion and exclusion criteria. The criteria for inclusion were age between 18 and 65 yr, nonmalignant disease, and American Society of Anesthesiologists physical status class I or II. The criteria for exclusion were body mass index greater than 35 kg/m², heart failure (New York Heart Association group III or IV), renal failure (P-creatinine > 125 μM), anemia (B-hemoglobin < 7.2 mm), diabetes mellitus, and use of medication known to affect the immune system, including nonsteroidal antiinflammatory drugs or histamine antagonists within 48 h of inclusion. We included patients after informed consent in compliance with the Helsinki Declaration.

Procedure

Sixty-two women were studied. After fasting overnight, they received 1 g acetaminophen perorally in addition to their daily medication. Patients aged 40 yr and older (n = 48) received 2,500 IE dalteparin subcutaneously for prophylaxis of thromboembolism accord-

* Department of Anesthesia and Intensive Care, Viborg County Hospital. † Professor, Aarhus University Hospital, Aarhus C, Denmark.

Received from the Department of Anesthesia and Intensive Care, Viborg County Hospital, Viborg, Denmark. Submitted for publication August 28, 2003. Accepted for publication December 11, 2003. Supported by The Western Danish Research Forum for Health Sciences, Aarhus University, Aarhus, Viborg County, Denmark; Rosa and Asta Jensen's Fund, Viborg, Denmark; The Danish Medical Association Research Fund, Copenhagen, Denmark; and The Laerdal Foundation for Acute Medicine, Stavanger, Norway.

Address reprint requests to Dr. Kølsen-Petersen: Department of Anesthesia and Intensive Care, Viborg County Hospital, Heibergs Alle 4, 8800 Viborg, Denmark. Address electronic mail to: jaakp@dadlnet.dk. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

ing to our institutional routine. Peroral diazepam, 5 mg, was allowed at the discretion of the attending nurses and was administered to 14 patients, with no difference between groups. The study was conducted in the operating room on the morning before surgery. A cubital vein of each arm was cannulated for blood sampling and for infusion of fluid. We randomly assigned the patients to receive 4 ml/kg NaCl, 7.5% (Central Pharmacy, Odense University Hospital, Odense, Denmark; HS group, n = 21); 4 ml/kg NaCl, 0.9% (NS4 group, n = 21); or 32 ml/kg NaCl, 0.9% (Hospital Pharmacies in Denmark, Copenhagen, Denmark; NS32 group, n = 20), given over 20 min at a constant rate *via* an infusion pump (IVAC 591; IVAC, San Diego, CA). The assignments were placed in sealed, opaque, randomly assorted envelopes, which were opened by a hospital staff member who was not one of the study investigators. The patients were blinded to the nature of the fluid. Because of ethical considerations, the maximal infused volume did not exceed a volume corresponding to 75 kg, which was the case in five, seven, and five patients in the HS, NS4, and NS32 groups, respectively. Between termination of the test fluid infusion and extubation, we infused 5 ml · kg⁻¹ · h⁻¹ saline, 0.9%, which was reduced to 1.5 ml · kg⁻¹ · h⁻¹ during the stay in the recovery room. Extra fluid could be infused if the patients showed signs of hypovolemia or if the blood loss exceeded 20% of the calculated blood volume. This was deemed necessary on two occasions when 500 ml saline, 0.9%, and 500 ml hydroxyethyl starch, 200/0.5, 6%, were infused in the HS and NS32 groups, respectively.

We recorded heart rate, blood pressure, and peripheral oxygen saturation every 2 min during the infusion and every 5 min during the succeeding anesthesia and surgery.

The patients were anesthetized with 3 µg/kg fentanyl and 3–5 mg/kg thiopental before intubation, which was facilitated by 0.6 mg/kg rocuronium. Anesthesia was maintained with 1–3% sevoflurane in oxygen-air at a fresh flow rate of 2 l/min and 100 µg supplementary fentanyl when judged necessary by the anesthetist, who was unaware of the treatment assignment. Cefuroxime, 1.5 g, was administered intravenously as an antibiotic prophylaxis to all patients.

Postoperative pain was treated with 1 g peroral acetaminophen four times daily, 50 mg tramadol three times daily, and intravenous morphine or ketobemidone. We treated nausea with 1 mg intravenous ondansetron and 0.5 mg droperidol.

Measurements

Cell-mediated immune function *in vivo* was tested by delayed-type hypersensitivity reaction in the skin with a commercially available kit (Multitest CMI; Institut Merieux, Lyon, France). Seven standardized recall antigens and a glycerol control were applied intracutane-

ously on the interior surface of the forearm after the induction of anesthesia and before the beginning of surgery. The antigens were toxoid from *Clostridium tetani* and *Corynebacterium diphtheriae*, tuberculin plus antigens from streptococcus (group C), *Candida albicans*, *Trichophyton mentagrophytes*, and *Proteus mirabilis*. For each antigen, the size of the induration was estimated after 48 h according to the manufacturer's instructions, as the average of two perpendicular diameters. A reaction less than 2 mm was considered negative. A compound score was calculated as the sum of the positive reactions divided by the number of positive reactions. If a reaction was positive, the maximal thickness of the induration was measured by ultrasound (Dermascan version 3; Cortex Technology, Hadsund, Denmark).

Venous blood was collected at baseline (T0), after infusion of saline (T-infusion), 25 min after incision (T-surgery), and 1 (T1), 11 (T11), 24 (T24), and 48 h (T48) after skin closure for the determination blood hemoglobin, plasma sodium, chloride, and potassium, which were all measured according to laboratory routines in our hospital by means of a Vitros950 (Ortho-Clinical Diagnostics, Birkerød, Denmark). Leukocyte and differential counts were determined by means of a SysmexXE2100 (Sysmex GmbH Denmark, Almind, Denmark) at these time points and in addition 6 h after surgery (T6). We measured plasma osmolality by freezing point depression at T0, T-infusion, T1, T24, and T48 (The Advanced Osmometer Model 3900; Advanced Instruments, Norwood, MA).

The immunologic parameters were determined at T0, T1, T24, and T48 except for natural killer (NK) cell-mediated cytotoxicity and mononuclear cell phenotyping, which were also measured 5 min after incision (T-incision). All reagents were from Invitrogen (Taastrup, Denmark) unless otherwise stated. Briefly, cells were isolated on the day of sampling by gradient centrifugation (Lymphoprep); washed; cryopreserved in medium containing 50% (vol/vol) heat-inactivated fetal calf serum, 40% RPMI 1640 (Roswell Park Memorial Institute media), and 10% dimethyl sulfoxide; and stored at -80°C. Three laboratory technicians, unaware of the treatment assignment, performed the further processing. After thawing and washing, the cells were suspended in growth medium (RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 67 µg/ml penicillin, and 100 µg/ml streptomycin) at a final concentration of 5 × 10⁶/ml. This mixture contained more than 99% mononuclear cells with a viability exceeding 95% as determined with the ethidium bromide-acridine orange solution (Central Pharmacy, Odense University Hospital). The mononuclear cell-growth medium suspension was used as a basis for measuring NK cell-mediated cytotoxicity, flow cytometric phenotyping of lymphocytes, and phytohemagglutinin-induced proliferation of T cells.

Natural Killer Cell-mediated Cytotoxicity. Natural killer cell-mediated cytotoxicity was tested against K 562 tumor cells in a 4-h ^{51}Cr -release assay. Mononuclear cells, treated as described in the previous section, were placed in microtiter plates (Nunc; Invitrogen) with ^{51}Cr -labeled (Amersham Biosciences, Hørsholm, Denmark) cultured leukemic target cells (K 562). The assays were performed in triplicate at effector:target cell ratios of 50:1, 25:1, 12.5:1, and 6.25:1. Furthermore, target cells were incubated in either growth medium or 5% Triton-X-100 (Central Pharmacy, Odense University Hospital) for the determination of spontaneous and maximal release of ^{51}Cr , respectively. After 4 h of incubation (37°C , 5% CO_2), the radioactivity was measured in the supernatant (Top-Count-NXT; Perkin Elmer Life Sciences, Hvidovre, Denmark). The NK cell activity was expressed as $100 \times [(\text{cpm experimental release} - \text{cpm spontaneous release})/(\text{cpm maximal release} - \text{cpm spontaneous release})]$, where cpm is counts/min. The spontaneous release averaged 10% of the maximal release. In a previous investigation, we estimated the sum of the sampling, isolation, thawing, and analytical variations to 18% calculated from measurements of duplicate blood samplings.

Flow Cytometric Phenotyping of the Mononuclear Cells. A 300- μl sample of the mononuclear cell-growth medium mixture was centrifuged, and the cell pellet was resuspended in 100 μl Cellwash (BD Biosciences, Palo Alto, California), followed by incubation in the dark at 4°C for 30 min with 20 μl monoclonal antibodies identifying CD3, CD4, CD8 (TriTEST), CD16+56 (Simultest), CD14, CD45 (LeucoGATE), CD19, and CD20 (all from BD Biosciences). After lysing remaining erythrocytes by FACS lysing 1:10 (BD Biosciences), washing (Cellwash), and resuspension in 500 μl Cellwash, 10^4 cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences). We used isotypic controls according to the manufacturer's instructions. The acquired data were processed using computer software (CellQuest; BD Biosciences). The coefficients of variation were estimated in a previous investigation, as described in the section about NK cell-mediated cytotoxicity, to 2% (CD3^+), 3% (CD4^+), 4% (CD8^+), 13% (CD14^+), 17% ($\text{CD16}^+/\text{CD56}^+$), and 14% ($\text{CD19}^+/\text{CD20}^+$). The phenotypes are shown in parentheses.

Phytohemagglutinin-induced Lymphocyte Transformation. Phytohemagglutinin-induced lymphocyte transformation was determined by the incorporation of [^3H]thymidine. Mononuclear cells suspended in growth medium were stimulated by phytohemagglutinin (assay concentration, 4 $\mu\text{g}/\text{ml}$) in wells of sterile cell culture plates (Nunc). The plates were incubated (37°C , 5% CO_2 -air) for a total of 5 days. We added 20 μl [^3H]thymidine in isotonic saline (1 μCi ; Amersham Biosciences) 24 h before harvesting (Filter Mate Harvester; Perkin Elmer Life Sciences). [^3H]thymidine uptake was measured by an automated beta-scintillation counter (Top-

Count-NXT; Perkin Elmer Life Sciences). We used the median values of triplicates and expressed the results as counts/min in stimulated cells minus counts/min in corresponding unstimulated cells. The coefficient of variation was estimated in a previous investigation to 18% as described in the section about NK cell-mediated cytotoxicity.

Neutrophil Chemotaxis. Neutrophil chemotaxis was measured by the under-agarose method applied to glass microscope slides as previously described in detail.¹⁷ In short, ethanol-cleaned and gelatin-coated object glasses were placed at the bottom of specially made chambers, which were filled with medium containing agarose (Sigma-Aldrich Denmark A/S, Brøndby, Denmark) and gelatin dissolved in RPMI 1640 with HEPES (Bie & Berntsen A/S, Åbyhøj, Denmark). Three times six holes were punched out after hardening. We used 10 mg/ml zymosan (Sigma-Aldrich Denmark A/S)-activated serum, containing the complement fragments C3a and C5a, as chemoattractant. Neutrophils were isolated by dextran sedimentation of whole blood followed by gradient centrifugation (Lymphoprep) and hypotonic lysis of remaining erythrocytes. The final cell suspension contained greater than 99% neutrophil granulocytes. We placed samples of 10 μl (5×10^4 cells) in the middle line of holes, 10 μl zymosan-activated serum in the first line, and 10 μl RPMI 1640 in the last line. The chambers were incubated for 2 h and 15 min at 37°C before killing the cells by adding methanol. We measured the chemotactic migration as the linear distance from the margin of the well to the fifth leading cell in the direction of the chemoattractant. In the same way, the spontaneous migration was measured in the direction of the control well. The analytical variation determined by duplicate measurements in two separate chemotactic chambers was 5%.

We measured the plasma concentration of elastase, a marker of neutrophile degranulation, by a particle-enhanced turbidimetric immunoassay (Ecoline; MERCK, Glostrup, Denmark) on an automated analyser (Hitachi 912; Roche A/S, Hvidovre, Denmark). The analytical variation was 4% at a mean of 27.8 $\mu\text{g}/\text{l}$ calculated from duplicate measurements of 20 blood samples.

Statistical Analysis

In a power analysis, we assumed that the difference in T-cell proliferation between groups was $30 \pm 15\%$ (mean \pm SD) at 310 mOsm.⁴ Therefore, we needed at least four patients in each group to detect a difference between groups with a power of 0.8 and a level of significance of 0.05. Baseline characteristics and data from the skin test were analyzed by means of the Kruskal-Wallis test, except the frequencies of anergy, which were compared by means of the chi-square test. All other data were natural log transformed to stabilize the variance between measurements. For each variable, the transformed data were analyzed by repeated-measures multivariate analysis of variance (MANOVA) using

Table 1. Baseline Characteristics

	4 ml/kg NaCl, 7.5%	4 ml/kg NaCl, 0.9%	32 ml/kg NaCl, 0.9%
No.	19	20	19
Age, yr	46 (39–53)	45 (32–53)	44 (39–53)
Weight, kg	73 (56–87)	71 (61–95)	60 (54–81)
BMI, kg/cm ²	25 (20–29)	26 (22–34)	23 (20–26)
Daily smoker	7	7	7
Daily medication	12	9	14
Indication for surgery			
Bleeding disorder	8	6	7
Fibroma uteri	7	6	6
Pelvic pain	1	1	3
Cervikal dysplasia, CIN I or II		3	1
Ovarian cyst		2	1
Other	3	2	1
Volume of test fluids, ml	292 (223–300)	282 (242–300)	1,920 (1,715–2,400)
Anesthesia			
Fentanyl, μ g	450 (390–610)	450 (350–600)	450 (350–700)
Thiopental, mg	400 (290–500)	400 (300–500)	350 (295–410)
Rocuronium, mg	50 (30–65)	50 (35–70)	50 (40–70)
Ephedrine administration, No. of patients	5	3	4
Atropine administration, No. of patients	3	0	1
0.9% NaCl infused,* ml/kg	10 (7–12)	9 (8–14)	11 (7–15)
Surgery			
Hemorrhage, † ml	220 (100–460)	300 (190–520)	400 (200–740)
Duration, min	50 (40–115)	65 (50–81)	77 (45–124)

Data are presented as median (10th–90th percentile).

* Between termination of test fluid infusion and extubation. † $P = 0.03$, Kruskal-Wallis Test.

BMI = body mass index; CIN = cervical intraepithelial neoplasia.

time as within factor and fluid treatment as between factor (SPSS 10.05; SPSS, Chicago, IL). *Post hoc* comparisons were performed at each time point and between each of two succeeding time points using one-way analysis of variance (ANOVA), only when the initial multivariate analysis of variance resulted in significance. The measured immunologic parameters may be influenced by age,¹⁸ body weight,¹⁹ and the extent of surgery.²⁰ These factors were taken into account by means of multiple linear regression in a model that included age, body mass index, intraoperative blood loss, duration of surgery, and total dose of fentanyl as covariates. The level of significance was 0.05 and was corrected by means of the Bonferroni method when more than two comparisons were made. Results are presented as geometric mean, an estimate of the median assuming log-normal distribution, with 95% confidence interval, unless otherwise indicated.

Results

Of 192 patients screened between December 2001 and January 2003, 62 patients were included and randomly assigned to treatment. Four patients were excluded from the final analysis. One patient (HS group) did not wish to finish the study, one patient (HS group) experienced an anaphylactoid reaction toward fentanyl or rocuronium (the patient later underwent testing and was found to be allergic to these drugs), one patient

(NS4 group) was transferred to another hospital before finishing follow-up, and one patient (NS32 group) underwent surgery again after 24 h because of postoperative bleeding. The study population baseline characteristics of the remaining 58 patients are shown in table 1. None of the included patients received any blood products during the study. The median stay in the hospital was 3 days in all groups. Three patients were treated for infections during the stay, two patients with cystitis and one with wound infection, all in the NS4 group.

Blood Chemistry and Osmolality

The changes in plasma electrolytes and blood hemoglobin are shown in figure 1, and the effects on plasma osmolality are shown in figure 2. The decrease in blood hemoglobin after infusion depended on the infused fluid. Blood hemoglobin decreased 13% (10–15%) in the NS32 group (median [95% confidence interval]) compared with 5% (3–7%) and 1% (–1–2%) in the HS and NS4 groups, respectively ($P < 0.001$, ANOVA). Between surgery and 1 h after surgery, blood hemoglobin increased in all groups, indicating plasma leakage. The increase was most pronounced in the hypertonic saline group ($P < 0.01$, ANOVA).

Cell-mediated Immune Function In Vivo

The manufacturer stopped producing the test kit during the study. Consequently, only 40 patients underwent testing before the date of expiration of the last kit. Two

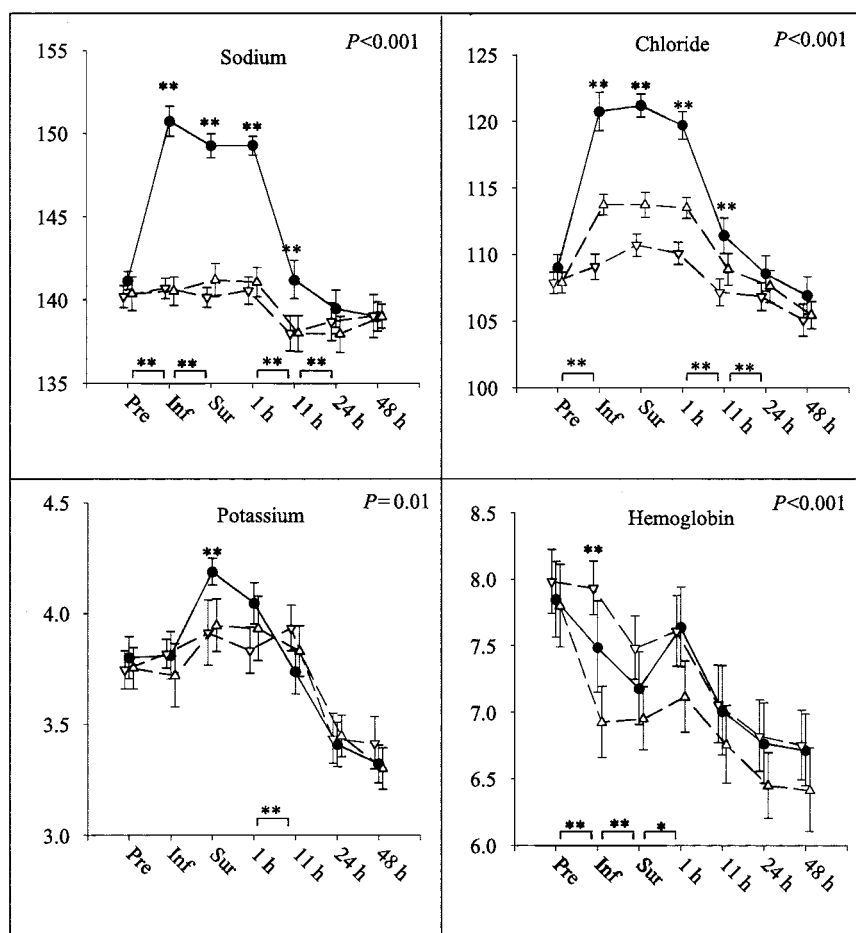


Fig. 1. Plasma electrolytes and blood hemoglobin (mm) versus time after infusion of 4 ml/kg NaCl, 7.5% (●); 4 ml/kg NaCl, 0.9% (▽); or 32 ml/kg NaCl, 0.9% (△). The changes over time depended on the infused fluid for all variables. *P* values are shown in the top right of each graph (multivariate analysis of variance, between groups). *Post hoc* comparisons were performed at each time point and between each of two succeeding time points using one-way analysis of variance (** $P < 0.001$ and * $P < 0.01$). The bars above the *x*-axes denote time intervals with significant changes. Data are presented as geometric mean with 95% confidence interval. 1, 11, 24, and 48 h = hours after surgery; Inf = after infusion; Pre = baseline; Sur = 25 min after incision.

of these patients did not finish the study as described in the first paragraph under Results. Overall, 71% of the positive reactions were against tuberculin ($n = 22$) or toxoid from clostridium tetani ($n = 17$), where also the largest responses were seen. No patients reacted against glycerin. Ten patients of 38 (26%) did not respond to any of the applied antigens (table 2). The frequency of anergy did not differ between groups ($P > 0.3$, chi-square test), nor was there any difference in the median number of positive reactions per patient, the sum of the positive diameters, the compound diameter, or the thickness of the indurations ($P > 0.5$ for all). Excluding the anergic patients from the analysis did not change the conclusion ($P > 0.1$ for all variables listed in table 2).

Leukocyte Count

Hysterectomy elicited neutrocytosis, monocytosis, and lymphopenia peaking 6 h after surgery in all groups (fig. 3). The differences between the groups were small.

Flow Cytometric Phenotyping of Mononuclear Cells

Generally, the numbers of lymphocyte subtypes changed over time ($P < 0.001$ for all, MANOVA, within groups) but with no significant differences between the

groups ($P > 0.05$ for all, between groups) (fig. 4). Figure 4 indicates that the numbers of B and T cells increased more between 1 and 24 h after surgery in the hypertonic saline group compared with both of the control groups. The number of NK cells increased 1 h after surgery in all groups followed by a decrease compared with baseline lasting at least 2 days, which paralleled the changes observed in NK cell-mediated cytotoxicity.

Natural Killer Cell-mediated Cytotoxicity

Cells from five or six patients were processed at a time. Unfortunately, the growth medium used in the first five patients was contaminated by bacteria, rendering results from these patients unusable. Furthermore, data from one patient were excluded because the spontaneous Cr-51 release was very close to and in some occasions exceeded the NK cell-induced release. Finally, data from one patient from 24 and 48 h were missing because of technical difficulties. Consequently, 7 patients were excluded in addition to the 4 patients mentioned previously, leaving 51 patients for the analysis. Generally, the NK cell-mediated cytotoxicity increased during surgery and 1 h after surgery, followed by a decrease below baseline 24 and 48 h after surgery ($P < 0.001$, MANOVA,

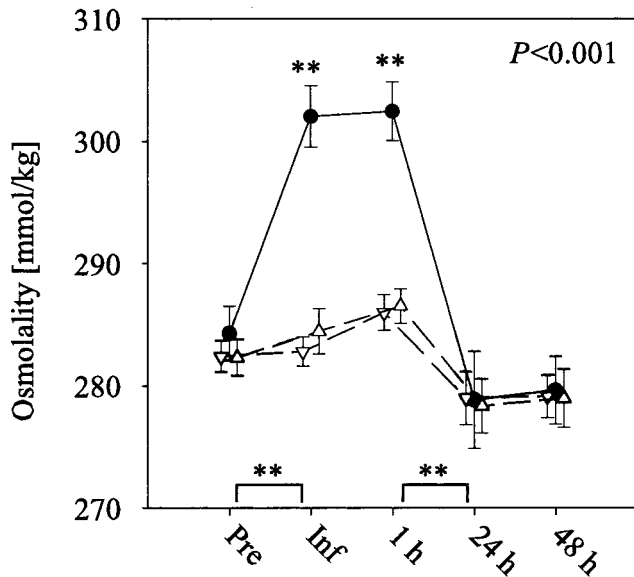


Fig. 2. Plasma osmolality (mmol/kg) versus time after infusion of 4 ml/kg NaCl, 7.5% (●); 4 ml/kg NaCl, 0.9% (▽); or 32 ml/kg NaCl, 0.9% (△). The changes over time depended on the infused fluid. $P < 0.001$, multivariate analysis of variance, between groups. *Post hoc* comparisons were performed at each time point and between each of two succeeding time points using one-way analysis of variance (** $P < 0.001$). The bars above the x-axis denote time intervals with significant changes. Data are presented as geometric mean with 95% confidence interval. Inf = after infusion; Pre = baseline; 1, 24, and 48 h = hours after surgery.

within groups), with no difference between groups ($P = 0.45$) (fig. 5).

Phytohemagglutinin-induced Lymphocyte Transformation

Surgery induced a decrease in proliferation after 1 h unrelated to the infused fluid ($P < 0.001$, MANOVA, within groups, $P = 0.45$, between groups). After 24 and 48 h, the proliferation had returned to baseline in the HS and NS32 groups, whereas in the NS4 group, there was only a partial normalization (fig. 6).

Neutrophil Migration

We chose to suspend the cells in the same isotonic medium during the laboratory procedures to investigate whether hypertonic stress *in vivo* affected the neutro-

phil migration. However, after inclusion of 15 patients, we discovered that neutrophil migration is immediately reestablished, even after prolonged hypertonicity (4 h), when the cells are suspended in isotonic medium.²¹ Hence, the method was judged inappropriate to investigate the effect of hypertonicity *in vivo*. Consequently, neutrophil chemotaxis was only measured in these 15 patients. The maximal difference between groups at the measured time points was approximately 1 mm. There were no changes within or between groups for chemotactic or spontaneous migration. The baseline chemotactic migration for all patients was 1.44 (1.29, 1.60) cm, which increased to 1.65 (1.60, 1.71) cm 1 h after surgery and decreased slightly to 1.62 (1.52, 1.72) and 1.62 (1.55, 1.69) cm after 24 and 48 h, respectively. The spontaneous migrations were 0.53 (0.43, 0.65), 0.56 (0.49, 0.63), 0.66 (0.57, 0.76), and 0.65 (0.59, 0.73) cm, respectively.

Plasma Elastase

Plasma elastase increased from 27 (25–29) $\mu\text{g/l}$ at baseline to 43 (40–46) $\mu\text{g/l}$ 48 h after surgery, a 60% (51–70%) increase, with no difference between the groups ($P < 0.001$, MANOVA, within groups and $P = 0.5$ between groups) (fig. 7). There was no difference in the maximal concentration ($P = 0.7$, ANOVA) or in the time to reach the maximal plasma concentration ($P = 0.15$, ANOVA).

Multiple Linear Regression Analysis

The data from the skin test, the increase in the number of T and B cells in the first 24 h after surgery, the immediate decrease in phytohemagglutinin-induced proliferation and the succeeding increase between surgery and 48 h after surgery, the maximal and minimal NK cell-mediated cytotoxicities, and the maximal elastase concentration for each patient were analyzed by multiple linear regression as described in the Statistical Analysis section. The conclusions, however, were the same as above.

Discussion

This is the first study to report the effects of hypertonic saline infusion on human immune function during and

Table 2. Delayed-type Hypersensitivity Measured in the Skin

	4 ml/kg NaCl, 7.5%	4 ml/kg NaCl, 0.9%	32 ml/kg NaCl, 0.9%
No. of anergic patients, %	4/14 (29%)	1/10 (10%)	5/14 (36%)
No. of positive reactions	1.5 (0,4)	1.5 (0,3)	1 (0,4)
Sum of positive reactions, mm	9.5 (0,19.5)	8.3 (0,16.5)	8 (0,24.5)
Compound score, mm	5 (0,10)	5 (0,9)	5 (0,9)
Compound thickness, mm	1.5 (0,2.3)	1.7 (0,2.3)	1.7 (0,2.4)

Data are presented as median (range). The sizes of the indurations with diameters larger than 2 mm were measured 48 h after intradermal application of seven recall antigens. The mean diameter for each response was calculated from two perpendicular diameters. The compound score was calculated for each individual as the sum of the mean diameters divided by the number of positive reactions. The thickness of the induration was measured by ultrasound for all positive reactions. The frequency of anergy did not differ between groups, $P > 0.3$, Chi-square test. $P > 0.5$ for the remaining variables, Kruskal-Wallis U test.

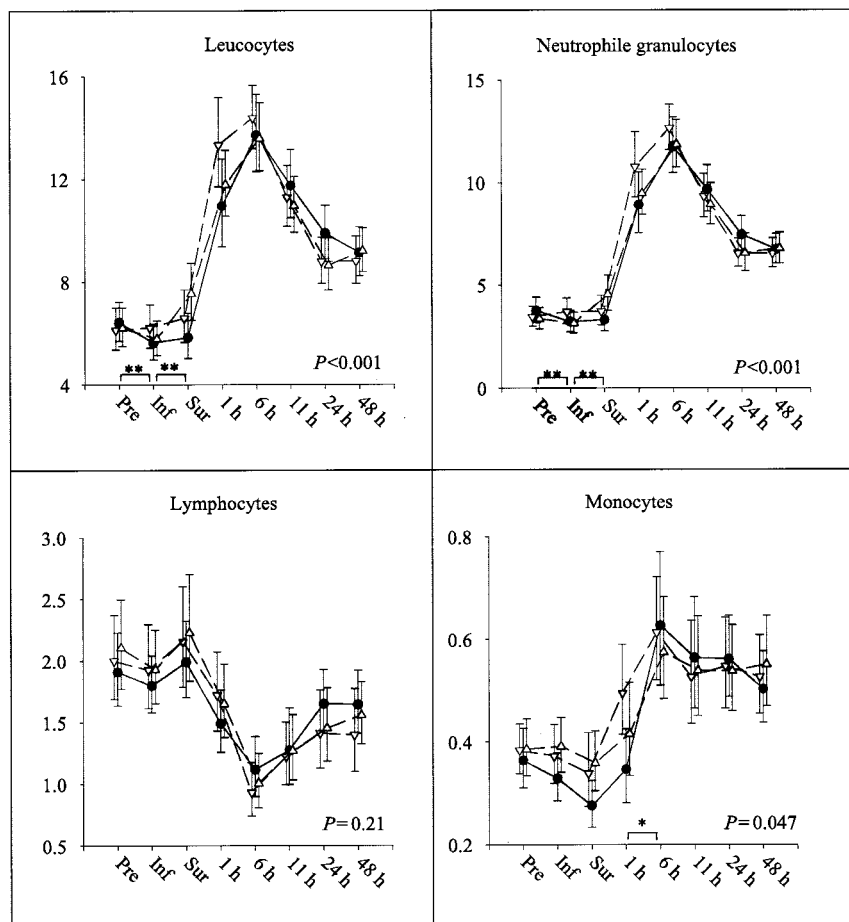


Fig. 3. Leukocyte count ($\times 10^9/l$) versus time after infusion of 4 ml/kg NaCl, 7.5% (\bullet); 4 ml/kg NaCl, 0.9% (∇); or 32 ml/kg NaCl, 0.9% (\triangle). The changes over time depended on the infused fluid for all cell types except lymphocytes. P values are shown in the bottom right corner of each graph (multivariate analysis of variance, between groups). The lymphocytes only changed over time ($P < 0.001$, multivariate analysis of variance, within groups). *Post hoc* comparisons were performed at each time point and between each of two succeeding time points using one-way analysis of variance (** $P < 0.001$ and * $P < 0.01$). The bars above the x -axes denote time intervals with significant changes. Data are presented as geometric mean with 95% confidence interval. 1, 6, 11, 24, and 48 h = hours after surgery; Inf = after infusion; Pre = baseline; Sur = 25 min after incision.

after surgery. Our results show that the immune-modulating properties of a hypertonic saline infusion seem to be unable to modify the immune response evoked by abdominal hysterectomy. Surgery elicited well-known changes in the number and distribution of leukocytes, NK cell activity, mitogen-induced lymphocyte proliferation, and plasma elastase concentration, with no effect, however, of hypertonic saline infusion. Furthermore, the test fluid infusion did not affect the overall cell-mediated immune function *in vivo* as evaluated by skin testing.

Lymphocyte proliferation is necessary for clonal expansion and consequently for the function of the adaptive immune system.²² Previous studies found increased proliferation of phytohemagglutinin-stimulated lymphocytes in hypertonic medium^{23,24} and restoration of hemorrhage-induced depression of mitogen-induced T-cell proliferation after hypertonic saline resuscitation of mice compared with lactated Ringer's solution.⁵ We, however, found a temporary decrease 1 h after surgery in phytohemagglutinin-induced proliferation of mononuclear cells, which was unrelated to the volume or tonicity of the infusion fluid. The lymphocytes in the peripheral blood circulation represent only 2–3% of the total amount in the body.²⁰ Furthermore, active cells are likely to be located in lymphoid organs and inflamed

tissues.²⁵ Therefore, the result of an immune test performed on peripheral blood cells does not necessarily reflect responses at the organ level.²⁰ To investigate the overall cellular immune function at the organ level, we measured the response to seven common antigens in the skin. The delayed-type hypersensitivity skin test measures a T cell-dependent immune response *in vivo* manifested as an inflammatory reaction that reaches peak intensity 24–48 h after antigenic stimulation.²⁶ Previous studies have shown enhanced cell-mediated immune function *in vivo* in normal rabbits²³ and hemorrhaged mice⁵ after hypertonic saline infusion, whereas we were unable to demonstrate an effect of infusion. The response was quantified by measuring the size of the induration. However, at times it was difficult to determine the exact demarcation. The variation thus induced may have decreased the power of the test compared with the animal experiments. Increasing the sensitivity of the test by applying ultrasonographic measurement of the thickness of the indurations and digital analysis did not change the results.

The number and cytotoxicity of the NK cells increased during and immediately after surgery, followed by a decrease lasting at least 48 h, with no relation to the infused fluid. The magnitude of the biphasic response was similar to previous studies in NK cells after hyster-

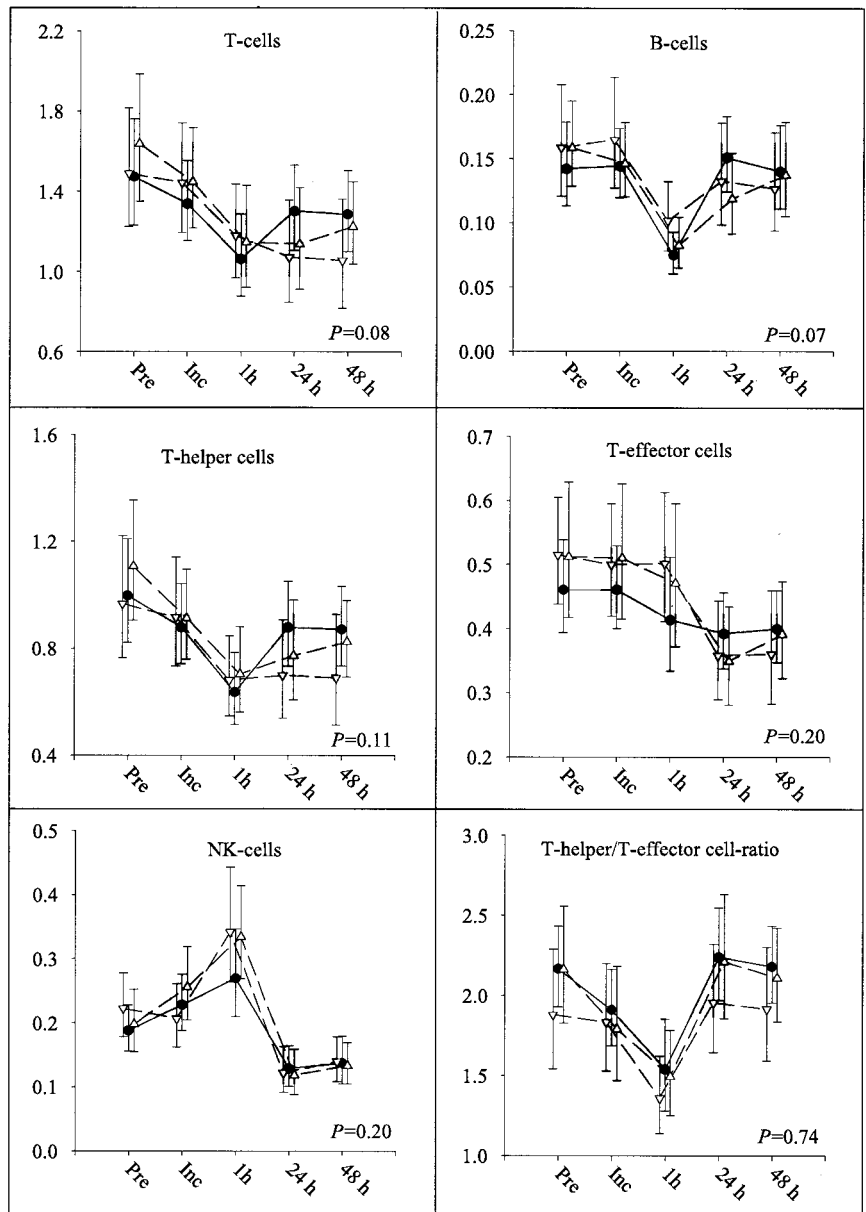


Fig. 4. Number of mononuclear cell subtypes ($\times 10^9/l$) as determined by flow cytometry versus time after infusion of 4 ml/kg NaCl, 7.5% (●); 4 ml/kg NaCl, 0.9% (▽); or 32 ml/kg NaCl, 0.9% (△). There were no differences between groups. *P* values are shown in the bottom right corner of each graph (multivariate analysis of variance, between groups). All subtypes changed over time ($P < 0.001$, multivariate analysis of variance, within groups). Data are presented as geometric mean with 95% confidence interval. 1, 24, and 48 h = hours after surgery; B cells = CD19/20⁺ cells; Inc = 5 min after incision; natural killer (NK) cells = CD16⁺/56⁺ cells; Pre = baseline; T cells = CD3⁺ cells; T-effector cells = CD3⁺/CD8⁺ cells; T-helper cells = CD3⁺/CD4⁺ cells.

ectomy.²⁷ Previous studies found that hypertonic saline resuscitation reversed hemorrhage-induced decrease in NK cell activity and reduced bacterial translocation in rats.²⁸ It is unclear, however, whether the effect was due to hypertonicity or resuscitation.

Hypertonicity has been shown to impede a plethora of neutrophil functions, including endothelial adhesion, chemotaxis, and degranulation.^{8,10,12,15,29,30} Reduced endothelial-neutrophil interactions may lead to neutrophilia after hypertonic saline resuscitation.^{8,31,32} We found no signs of neutrophilia compared with both control groups. The concentration of elastase, a marker of neutrophil degranulation, increased after surgery in agreement with other studies,³³ with no difference between groups. Previous studies with isolated stimulated human neutrophils found augmented³⁴ and reduced³⁰

elastase release, depending on whether the activation of the cells took place before or after hypertonic stress.¹² In agreement with these findings, we had expected an attenuation of the neutrophil elastase release because the infusion of hypertonic saline was performed before the surgery. There are several explanations for the discrepancy between our study and the previous investigations discussed below.

First, the effect of hypertonicity on isolated immune cells is dose and time dependent.^{4,23,30,34,35} Generally, immune cells are affected by plasma sodium concentrations of 10–20 mM above normal values, but the effect peaks at approximately 40–50 mM.^{4,23,30} It has been proposed that if the hypertonic solution is to benefit the patient with respect to the immune response, the level of hypertonicity should probably exceed 330 mOsm/l.²

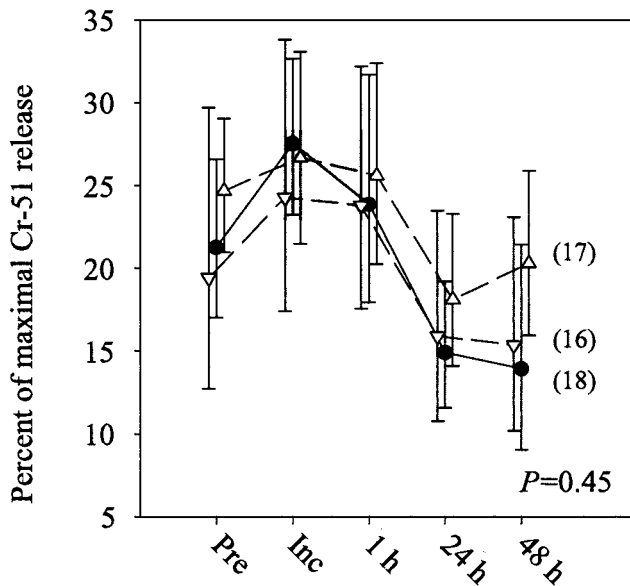


Fig. 5. Natural killer cell-mediated cytotoxicity in 51 patients against ^{51}Cr -labeled K 562 tumor cells at an effector:target cell ratio of 50:1 versus time after infusion of 4 ml/kg NaCl, 7.5% (●); 4 ml/kg NaCl, 0.9% (▽); or 32 ml/kg NaCl, 0.9% (△). Numbers in parentheses indicate the number of patients in each group. The activity changed over time ($P < 0.001$, multivariate analysis of variance, within groups) but with no difference between groups ($P = 0.45$). Data are presented as geometric mean with 95% confidence interval. 1, 24, and 48 h = hours after surgery; Inc = 5 min after incision; Pre = baseline.

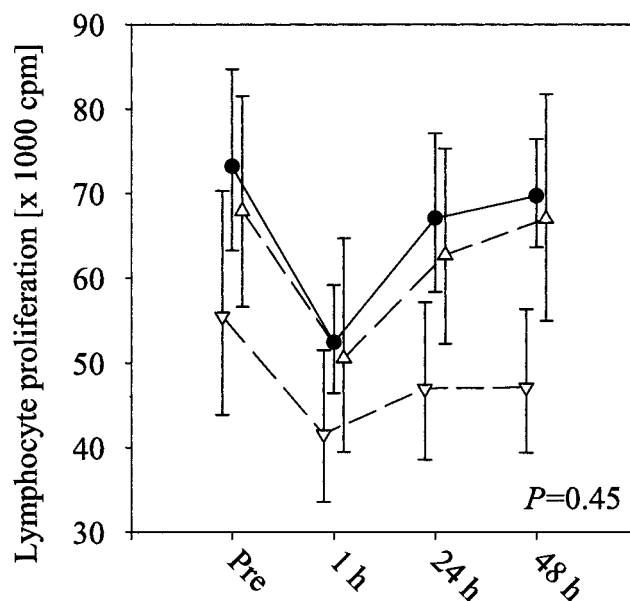


Fig. 6. T-lymphocyte proliferation measured by ^3H thymidine uptake in phytohemagglutinin-stimulated mononuclear cells versus time after infusion of 4 ml/kg NaCl, 7.5% (●); 4 ml/kg NaCl, 0.9% (▽); or 32 ml/kg NaCl, 0.9% (△). The ^3H thymidine uptake changed over time ($P < 0.001$, multivariate analysis of variance, within groups) but with no difference between groups ($P = 0.45$). Data are presented as geometric mean with 95% confidence interval. 1, 24, and 48 h = hours after surgery; Pre = baseline.

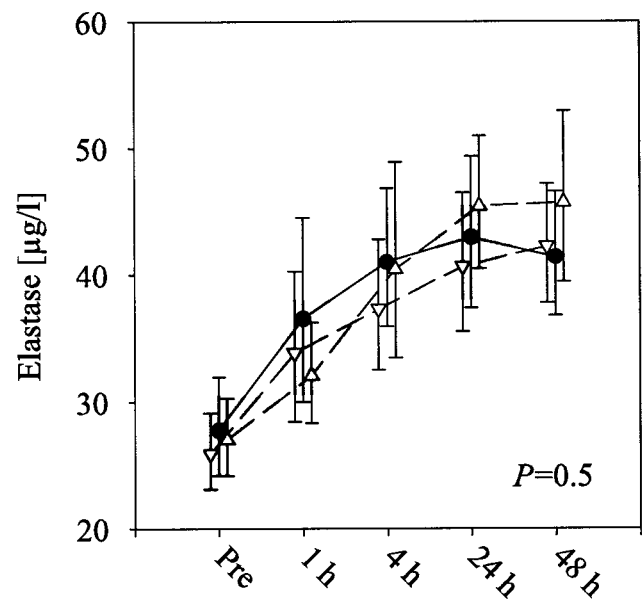


Fig. 7. Elastase measured in plasma versus time after infusion of 4 ml/kg NaCl, 7.5% (●); 4 ml/kg NaCl, 0.9% (▽); or 32 ml/kg NaCl, 0.9% (△). The concentration changed over time ($P < 0.001$, multivariate analysis of variance, within groups) but with no difference between groups ($P = 0.5$). Data are presented as geometric mean with 95% confidence interval. 1, 4, 24, and 48 h = hours after surgery; Pre = baseline.

We found a maximum plasma osmolality of 302 (300, 305) mOsm/kg (approximately 285 [283, 288] mOsm/l). Consequently, it may be that the level of hypertonicity reached in our study was too small to make any significant difference. Because of concerns of possible vasodilation, cardiac arrhythmia, and increased bleeding, a 10- to 20-min infusion time of 250 ml of 7.5% NaCl-6% Dextran70 has been recommended for trauma care in a recent review.² The administration regime in our study is thus identical to current recommendations, and the increase in plasma sodium and osmolality is likely to be clinically relevant. Furthermore, the average maximal measured plasma sodium concentrations were 152 ± 6 mm (mean \pm SD)³⁶ and 152 ± 9 mm³⁷ in trauma patients resuscitated with a 250-ml bolus of 7.5% NaCl-6% Dextran70, sodium concentrations that are comparable with the maximal concentration of 151 ± 2 mm in this study.

Second, except for one study,¹⁵ all other studies regarding the immunomodulating potentials of hypertonic saline have been performed on isolated cells or in animal models. The statistical variation is likely to be considerably larger in a clinical trial and may thus obscure a potential effect.

Third, we studied relatively healthy normovolemic women undergoing a standardized surgical procedure under general anesthesia. This population is not comparable with animals resuscitated from hemorrhagic shock. The study population was chosen to reduce variation due to age, sex, and preexisting diseases while at the same time offer an opportunity to study the effects of

surgery as a model for trauma. Abdominal hysterectomy induces well-described changes in the immune response similar to those seen after trauma and other kinds of major surgery.²⁰ Our study confirms that abdominal hysterectomy offers a valid and reproducible means of studying the immune response to trauma. However, the immunologic changes after trauma depends on the amount of tissue injury,³⁸ blood loss,³⁹ and possibly sex.^{40,41} Consequently, the results may not be extrapolated to male patients or situations with major trauma, ischemia-reperfusion, or bleeding. Furthermore, there may be effects on immunologic parameters that we did not measure. Thus, infusion of 4 ml/kg 7.5% NaCl in healthy volunteers increased the expression of L-selectin on unstimulated neutrophils, whereas the expression of CD11b decreased.¹⁵

Fourth, the anesthesia may influence the immune function. It is generally accepted, though, that the extent of surgery is more important.^{20,42} To minimize bias from these factors, the surgery and anesthesia were standardized and performed by persons blinded to the treatment allocation.

A difference in the plasma volume-expanding properties of the infused fluids, as indicated by the decrease in hemoglobin after infusion, may have influenced the results. However, we found no differences between the extremes in our study, the groups receiving 4 and 32 ml/kg isotonic saline, which indicates that the amount of plasma dilution did not significantly affect the results.

This study was not designed to investigate potential effects on clinical endpoints. However, several clinical trials on hypertonic saline-colloid resuscitation, including a total of 739 hypotensive trauma patients, found no difference in the overall rate of complications related to dysfunction of the immune system, such as acute respiratory distress syndrome, infections, or sepsis.^{37,43,44}

In conclusion, abdominal hysterectomy induced well-known changes in the cellular immune response unrelated to the tonicity or volume of the preoperatively infused fluids. Therefore, as opposed to animal and cell culture studies, the immune-modulating properties of hypertonic saline infusion seem to be limited in clinical practice, at least in situations with moderate tissue trauma and bleeding. It is unlikely that infusion of hypertonic saline in the current recommended dose will significantly reduce complications due to dysfunction of the immune system.

The authors thank Niels Trolle Andersen (Biostatistician, Department of Biostatistics, Aarhus University, Aarhus, Denmark) for advice regarding the statistical analysis. They also thank Ellen Esbjerg (Laboratory Technician, Department of Clinical Immunology, Odense University Hospital, Odense, Denmark), Ingrid Qualmann (Laboratory Technician, Department of Clinical Immunology, Odense University Hospital), Birgitte Døllerup (Laboratory Technician, Department of Clinical Biochemistry, Viborg County Hospital, Viborg, Denmark), and Lillian Meyer (Laboratory Technician, Department of Clinical Biochemistry, Viborg County Hospital) for their skillful laboratory assistance. Erlend J. Erlandsen, M.S.C. (Research Fellow, Department of Clinical Biochemistry, Viborg County

Hospital), and Bodil Andersen (Senior Technician, Department of Clinical Biochemistry, Viborg County Hospital) are thanked for their support.

References

1. Kreimeier U, Messmer K: Small-volume resuscitation: From experimental evidence to clinical routine. Advantages and disadvantages of hypertonic solutions. *Acta Anaesthesiol Scand* 2002; 46:625-38
2. Kramer GC: Hypertonic resuscitation: Physiologic mechanisms and recommendations for trauma care. *J Trauma* 2003; 54:S89-99
3. Junger WG, Coimbra R, Liu FC, Herdon-Remelius C, Junger W, Junger H, Loomis W, Hoyt DB, Altman A: Hypertonic saline resuscitation: A tool to modulate immune function in trauma patients? *Shock* 1997; 8:235-41
4. Coimbra R, Junger WG, Liu FC, Loomis WH, Hoyt DB: Hypertonic/hyperoncotic fluids reverse prostaglandin E2 (PGE2)-induced T-cell suppression. *Shock* 1995; 4:45-9
5. Coimbra R, Junger WG, Hoyt DB, Liu FC, Loomis WH, Evers MF: Hypertonic saline resuscitation restores hemorrhage-induced immunosuppression by decreasing prostaglandin E2 and interleukin-4 production. *J Surg Res* 1996; 64: 203-9
6. Loomis WH, Namiki S, Hoyt DB, Junger WG: Hypertonicity rescues T cells from suppression by trauma-induced anti-inflammatory mediators. *Am J Physiol Cell Physiol* 2001; 281:C840-8
7. Coimbra R, Hoyt DB, Junger WG, Angle N, Wolf P, Loomis W, Evers MF: Hypertonic saline resuscitation decreases susceptibility to sepsis after hemorrhagic shock. *J Trauma* 1997; 42:602-6
8. Pascual JL, Ferri LE, Seely AJ, Campisi G, Chaudhury P, Giannias B, Evans DC, Razek T, Michel RP, Christou NV: Hypertonic saline resuscitation of hemorrhagic shock diminishes neutrophil rolling and adherence to endothelium and reduces in vivo vascular leakage. *Ann Surg* 2002; 236:634-42
9. Rizoli SB, Kapus A, Fan J, Li YH, Marshall JC, Rotstein OD: Immunomodulatory effects of hypertonic resuscitation on the development of lung inflammation following hemorrhagic shock. *J Immunol* 1998; 161:6288-96
10. Rosengren S, Henson PM, Worthen GS: Migration-associated volume changes in neutrophils facilitate the migratory process in vitro. *Am J Physiol* 1994; 267:C1623-32
11. Hampton MB, Chambers ST, Vissers MC, Winterbourn CC: Bacterial killing by neutrophils in hypertonic environments. *J Infect Dis* 1994; 169:839-46
12. Ciesla DJ, Moore EE, Zallen G, Biffl WL, Silliman CC: Hypertonic saline attenuation of polymorphonuclear neutrophil cytotoxicity: Timing is everything. *J Trauma* 2000; 48:388-95
13. Pascual JL, Khwaja KA, Chaudhury P, Christou NV: Hypertonic saline and the microcirculation. *J Trauma* 2003; 54:S133-40
14. Partrick DA, Moore FA, Moore EE, Barnett CC Jr, Silliman CC: Neutrophil priming and activation in the pathogenesis of postinjury multiple organ failure. *New Horiz* 1996; 4:194-210
15. Angle N, Cabello-Passini R, Hoyt DB, Loomis WH, Shreve A, Namiki S, Junger WG: Hypertonic saline infusion: Can it regulate human neutrophil function? *Shock* 2000; 14:503-8
16. The European Agency for the Evaluation of Medical Products: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95). London, The European Agency for the Evaluation of Medical Products, 1997
17. Petersen T, Jensen A, Aaes H: Chemotaxis of canine polymorphonuclear granulocytes using the underagarose method applied to glass microscope slides. *Comp Haematol Int* 1998; 8:31-6
18. Pieri C, Recchioni R, Moroni F, Marcheselli F, Damjanovich S: The response of human lymphocytes to phytohemagglutinin is impaired at different levels during aging. *Ann N Y Acad Sci* 1992; 673:110-9
19. Tanaka S, Isoda F, Ishihara Y, Kimura M, Yamakawa T: T lymphopenia in relation to body mass index and TNF-alpha in human obesity: Adequate weight reduction can be corrective. *Clin Endocrinol (Oxf)* 2001; 54:347-54
20. Salo M: Effects of anaesthesia and surgery on the immune response. *Acta Anaesthesiol Scand* 1992; 36:201-20
21. Rizoli SB, Kapus A, Parodo J, Fan J, Rotstein OD: Hypertonic immunomodulation is reversible and accompanied by changes in CD11b expression. *J Surg Res* 1999; 83:130-5
22. Medzhitov R, Janeway C Jr: Innate immunity. *N Engl J Med* 2000; 343: 338-44
23. Junger WG, Liu FC, Loomis WH, Hoyt DB: Hypertonic saline enhances cellular immune function. *Circ Shock* 1994; 42:190-6
24. Junger WG, Hoyt DB, Hamreus M, Liu FC, Herdon-Remelius C, Junger W, Altman A: Hypertonic saline activates protein tyrosine kinases and mitogen-activated protein kinase p38 in T-cells. *J Trauma* 1997; 42:437-43
25. Delves PJ, Roitt IM: The immune system: II. *N Engl J Med* 2000; 343: 108-17
26. Kruisbeek AM, Shevach E: Assays for T cell function. *Current Protocols in Immunology*. New York, Wiley & Sons, 1991, pp 3.12.1-14
27. Tonnesen E, Wahlgreen C: Influence of extradural and general anaesthesia on natural killer cell activity and lymphocyte subpopulations in patients undergoing hysterectomy. *Br J Anaesth* 1988; 60:500-7
28. Hirsh M, Dyugovskaya L, Bashenko Y, Krausz MM: Reduced rate of bacte-

rial translocation and improved variables of natural killer cell and T-cell activity in rats surviving controlled hemorrhagic shock and treated with hypertonic saline. *Crit Care Med* 2002; 30:861-7

29. Ciesla DJ, Moore EE, Biffl WL, Gonzalez RJ, Silliman CC: Hypertonic saline attenuation of the neutrophil cytotoxic response is reversed upon restoration of normotonicity and reestablished by repeated hypertonic challenge. *Surgery* 2001; 129:567-75

30. Junger WG, Hoyt DB, Davis RE, Herdon-Remelius C, Namiki S, Junger H, Loomis W, Altman A: Hypertonicity regulates the function of human neutrophils by modulating chemoattractant receptor signaling and activating mitogen-activated protein kinase p38. *J Clin Invest* 1998; 101:2768-79

31. Angle N, Hoyt DB, Coimbra R, Liu F, Herdon-Remelius C, Loomis W, Junger WG: Hypertonic saline resuscitation diminishes lung injury by suppressing neutrophil activation after hemorrhagic shock. *Shock* 1998; 9:164-70

32. Angle N, Hoyt DB, Cabello-Passini R, Herdon-Remelius C, Loomis W, Junger WG: Hypertonic saline resuscitation reduces neutrophil margination by suppressing neutrophil L selectin expression. *J Trauma* 1998; 45:7-12

33. Wanscher M, Knudsen F, Antonsen S, Hansen SH: Release of granulocyte elastase and complement changes during hysterectomy. *Scand J Clin Lab Invest* 1989; 49:255-8

34. Partrick DA, Moore EE, Offner PJ, Johnson JL, Tamura DY, Silliman CC: Hypertonic saline activates lipid-primed human neutrophils for enhanced elastase release. *J Trauma* 1998; 44:592-7

35. Murao Y, Hoyt DB, Loomis W, Namiki S, Patel N, Wolf P, Junger WG: Does the timing of hypertonic saline resuscitation affect its potential to prevent lung damage? *Shock* 2000; 14:18-23

36. Vassar MJ, Perry CA, Holcroft JW: Prehospital resuscitation of hypotensive trauma patients with 7.5% NaCl versus 7.5% NaCl with added dextran: A controlled trial. *J Trauma* 1993; 34:622-32

37. Mattox KL, Maningas PA, Moore EE, Mateer JR, Marx JA, Aprahamian C, Burch JM, Pepe PE: Prehospital hypertonic saline/dextran infusion for post-traumatic hypotension: The U.S.A. Multicenter Trial. *Ann Surg* 1991; 213:482-91

38. Sauaia A, Moore FA, Moore EE, Lezotte DC: Early risk factors for postinjury multiple organ failure. *World J Surg* 1996; 20:392-400

39. Chaudry IH, Ayala A, Ertel W, Stephan RN: Hemorrhage and resuscitation: Immunological aspects. *Am J Physiol* 1990; 259:R663-78

40. Wichmann MW, Muller C, Meyer G, Adam M, Angele MK, Eisenmenger SJ, Schildberg FW: Different immune responses to abdominal surgery in men and women. *Langenbecks Arch Surg* 2003; 387:397-401

41. Yokoyama Y, Schwacha MG, Samy TS, Bland KI, Chaudry IH: Gender dimorphism in immune responses following trauma and hemorrhage. *Immunol Res* 2002; 26:63-76

42. Tønnesen E: Immunological aspects of anesthetic management. *Adv Neuroimmunol* 1993; 3:255-67

43. Younes RN, Aun F, Accioly CQ, Casale LP, Szajn bok I, Birolini D: Hypertonic solutions in the treatment of hypovolemic shock: A prospective, randomized study in patients admitted to the emergency room. *Surgery* 1992; 111:380-5

44. Younes RN, Aun F, Ching CT, Goldenberg DC, Franco MH, Miura FK, Santos SS, Sequeiros IM, Rocha ESM, Fujimura I, Birolini D: Prognostic factors to predict outcome following the administration of hypertonic/hyperoncotic solution in hypovolemic patients. *Shock* 1997; 7:79-83