Inhibition of Autonomic Storm by Epidural Anesthesia Does Not Influence Cardiac Inflammatory Response After Brain Death in Rats

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ABSTRACT

Background.   After brain death (BD) donors usually experience cardiac dysfunction, which is responsible for a considerable number of unused organs. Causes of this cardiac dysfunction are not fully understood. Some authors argue that autonomic storm with severe hemodynamic instability leads to inflammatory activation and myocardial dysfunction.

Objectives.   To investigate the hypothesis that thoracic epidural anesthesia blocks autonomic storm and improves graft condition by reducing the inflammatory response.

Methods.   Twenty-eight male Wistar rats (250–350 g) allocated to four groups received saline or bupivacaine via an epidural catheter at various times in relation to brain-death induction. Brain death was induced by a sudden increase in intracranial pressure by rapid inflation of a ballon catheter in the extradural space. Blood gases, electrolytes, and lactate analyses were performed at time zero, and 3 and 6 hours. Blood leukocytes were counted at 0 and 6 hours. After 6 hours of BD, we performed euthanasia to measure vascular adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, Bcl-2 and caspase-3 on cardiac tissue.

Results.   Thoracic epidural anesthesia was effective to block the autonomic storm with a significant difference in mean arterial pressure between the untreated (saline) and the bupivacaine group before BD ($P < .05$). However, no significant difference was observed for the expressions of VCAM-1, ICAM-1, TNF-α, IL-1β, Bcl-2, and caspase-3 ($P > .05$).

Conclusion.   Autonomic storm did not seem to be responsible for the inflammatory changes associated with BD; thoracic epidural anesthesia did not modify the expression of inflammatory mediators although it effectively blocked the autonomic storm.

NOWADAYS, AN IMPORTANT BARRIER to cardiac transplantation is the donor scarcity.[1–3] After brain death (BD), donors experience a variety of cardiac derangements. In some cases, they are so pronounced as to preclude the use of these hearts for transplantation.[4–8]

The so-called "autonomic storm"[9] is usually seen with BD. It is marked by a catecholamine surge; the concentrations of norepinephrine and epinephrine increase 100- to 1000-fold above normal values.[9,10] Clinically, marked tachycardia and hypertension documented for few minutes are replaced by vasoplegia and hypotension.[11,12] We hypothesized that abolishing autonomic storm would reduce the inflammatory response after BD in cardiac allografts precluding exclusion of organs as unsuitable for transplantation. In the present study, we used thoracic epidural anes-

thesia (TEA) with bupivacaine, a local anesthetic that produces a reversible sympathectomy,[13,14] to inhibit autonomic storm in a rat model of BD.

MATERIALS AND METHODS
Animals and Surgical Procedures

The experimental protocol was approved by our Animal Subject Committee. All animals received humane care in compliance with the "Guide for Care and Use of Laboratory Animals" (prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996).[32]

Twenty-eight male Wistar rats, weighting 250 to 350 g, were allocated to four groups: group 1 (saline group) received an infusion of 20 μL of saline solution to the epidural space prior to group 2 (pre-bup), 20 μL of bupivacaine prior to group 3 (bup-20), 20 μL of bupivacaine 20 minutes after; and group 4 (bup-60), 20 μL of bupivacaine 60 minutes after BD induction.

Animals were anesthetized in a chamber with 5% isoflurane, intubated and ventilated wiith a rodent ventilator (Harvard Apparatus, model 683, USA) at a 10 mL/kg, tidal volume and 70 breaths/min frequency. They were maintained by inhalation of 2% isoflurane until BD induction. The carotid artery was cannulated for continuous blood pressure monitoring and blood sampling. Another jugular venous line was used for continuous saline infusion (2 mL/h). No intravenous drugs were administered.

BD Model and Thoracic Epidural Anesthesia

A Fogarty 4-French catheter (Baxter Health Care, Deerfield, Ill, USA) was placed into the intracranial cavity of rats through a drilled parietal burr hole. The ballon catheter was rapidly inflated with 0.5 mL of saline to increased the intracranial pressure until BD, which was confirmed via maximal pupil dilatation, an absence of reflexes, and a drop in mean arterial pressure (MAP). Anesthesia was stopped after BD confirmation. Through an incision over T11–L1 vertebrae and angulation of column a polyethylen catheter 10 catheter placed into epidural space was advanced to the T5 level.

All animals were maintained for 6 hours. Thereafter, they were exsanguinated via the abdominal aorta, and the heart removed, immersed in hexane, and frozen with liquid nitrogen. Blood samples obtained from the abdominal aorta were centrifuged (1500g, 25°C) and plasma stored at −80°C for subsequent analyses.

Measurements

Blood gases, electrolytes, lactate, hematocrit, and white blood cell counts. Blood gases, electrolytes, and lactate analyses were performed on blood samples obtained from the carotid artery at baseline (0 minutes) as well as at 3 and 6 hours after the surgical procedures using a gas analyzer (Radiometer ABL 555, Radiometer Medical, Denmark). Hematocrit and white blood cell counts were performed in blood samples obtained from the cut tip of the tail at the baseline and 6 hours. Hematocrit was measured by microcapillary tube centrifugation. Total white blood cell counts were determined using a Neubauer chamber.

Tumor necrosis factor-α and interleukin-1β. Quantification of interleukin (IL)-1β and tumor necrosis factor (TNF)-α on cardiac tissue and serum was performed by enzyme-linked immunosorbent assays using the Quantikine kit (R & D Systems Inc., Minneapolis, Min, USA) in compliance with the manufacturer's recommenda-

tions. Cytokine basal reference values were obtained from three naïve rats.

Intercellular adhesion molecule-1 and vascular adhesion molecule-1. Quantification of intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1 in heart tissue was performed by immunohistochemistry. Frozen samples of serial 6-μm-thick sections of heart tissue were fixed in cold acetone for 10 minutes. The nonspecific sites were blocked with buffer (Super-Block Buffer, Pierce Biotechnology, Rockford, Ill, USA) for 12 hours at 4°C. For immunodetection of ICAM-1 and VCAM-1, monoclonal antibodies anti-rat ICAM-1 (Abcam Inc, Cambridge, Mass, USA) or anti-mouse VCAM-1 (Abcam Inc), which were previously conjugated to biotin were diluted 1:50 in phosphate-buffer saline (PBS) containing 0.3% Tween-20. After incubation for 12 hours at 4°C, sections washed successively with PBS were sequentially incubated for 1 hour at room temperature with streptavidin-fluorescein complex (Amersham Pharmacia Biotech, London, UK) diluted in PBS at 1:200. After washing the slides with PBS, samples were coated with a solution of propidium iodide (Vector, Burlingame, Calif, USA) to preserve fluorescence. The analysis was performed using an image acquisition system with CoolSNAP-Pro digital camera (Nikon, Tokyo, Japan) coupled to a fluorescence microscope (Nikon, Tokyo, Japan). Images were analyzed with software Image-Pro Plus 4.1 (Media Cybernetics, Bethesda, Md, USA). Results were expressed as mean fluorescence intensity.

Bcl-2 and caspase-3. Frozen heart tissue samples fixed in cold acetone for 10 minutes were used to measure Bcl-2 and caspase-3. After blocking endogenous peroxidase with phosphate buffered saline (PBS) and H_2O_2 nonspecific sites were blocked by incubation in biotin-free buffer (Super Block Blocking Buffer, Pierce Biotechnology, Rockford, Ill, USA). For immunodetection of caspase-3 and Bcl-2, samples were incubated for 16 hours at 4°C with rabbit polyclonal antibodies anti-caspase 3 (Abcam Inc) or anti-Bcl-2 (Abcam Inc) that were previously conjugated with biotin and diluted (1:50) in PBS with 0.3% tween-20. Samples were incubated for 1 hour at room temperature with streptavidin diluted in PBS (1:500), developed with 3,3'-diaminobenzidine, and counterstained with hematoxylin. After dehydration by immersion in solutions of increasing ethanol concentrations and diaphanization by immersion in xylene, samples were covered with Canada's balsam. The image analysis employed in a system using Axiovision software, version 4.7.2. (Carl Zeiss Inc, Hallbergmos-München, Germany). Results were expressed as the percentage of positively stained cells per five fields of 5000 μm^2 each.

Statistical Analysis

Data were analyzed using the GraphpadPrism 5.2 statistical program. Results were expressed as mean values ± standard errors of the means. To compare means between groups we employed the two-way analysis of variance, controlled by Levene's test of homogeneity, followed by Bonferroni's test. The level of significance was set at .05.

RESULTS
MAP, Blood Gas, Electrolytes, Lactate, Hematocrit, and Leukocyte Counts

BD induction was associated with a sudden increase in MAP over the first minute following catheter inflation. However the hemodynamic alterations were not seen among the pre-bup group, namely animals that received a
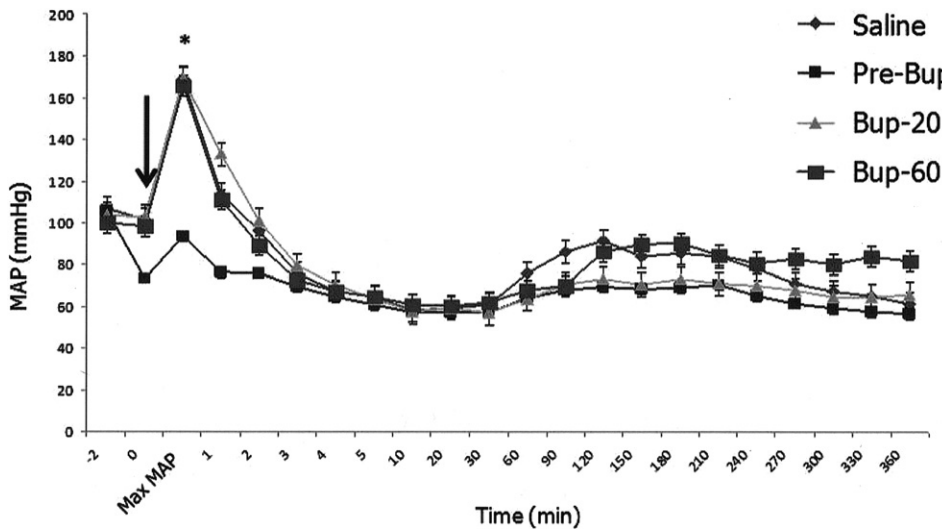
Fig 1. Mean arterial pressure (MAP) of brain-dead rats treated with saline, bupivacaine before brain death induction (pre-bup), and bupivacaine after 20 and 60 minutes (bup-20 and bup-60, respectively). Arrow represents induction of brain death. The peak after brain-death induction is statistically significant. Note that pre-bup group had no peak. The data are presented as the mean ± standard error of the mean.

bolus of bupivacaine before BD induction, suggesting inhibition of sympathetic release (Fig 1).

Blood gases, electrolytes, lactate, and hematocrit analysis did not show significant differences among the groups (data not shown). Comparing total leukocyte counts at basal and 6 hours, leukopenia was noted in all groups, without a significant difference among the groups. In contrast, statistical significance was reached when each group was analyzed at basal versus 6 hours, Fig 2 shows the mean values of total leukocyte counts.

TNF-α and IL-1β

An intense local and systemic inflammatory response was evidenced by high levels of serum and cardiac TNF-α and IL-1β versus their concentrations among naïve animals, which were below detection values. Significant differences were not observed among the groups (Fig 3) confirming the
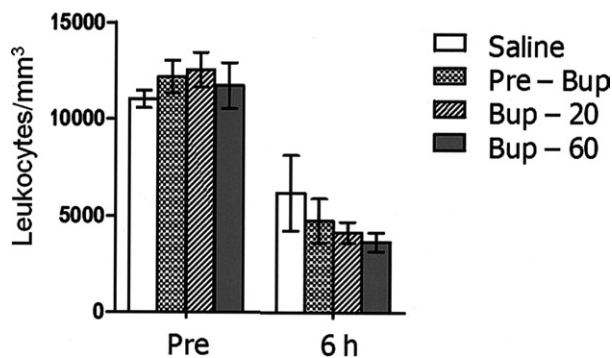

Fig 2. Number of total white blood cell counts obtained before (pre) and 6 hours after brain death. Brain-dead rats were treated with saline, bupivacaine before brain death induction (pre-bup), and bupivacaine after 20 and 60 min (bup-20 and bup-60, respectively). The data are presented as mean ± standard error of the mean.

hypothesis that a pronounced inflammatory response took place associated with BD.

Immunohystochemistry of Cardiac Tissue

Fig 4, shows expression of adhesion molecules ICAM-1 and VCAM-1 in the heart did not differ among the groups. Apoptosis was evaluated in the myocardium by the expression of caspase-3 and Bcl-2, which are pro-apoptotic and anti-apoptotic molecules respectively. As illustrated in Fig 5, there were no differences in the expression of either molecule among the groups.

DISCUSSION

Numerous studies have evaluated the role of BD on graft outcomes in experimental and clinical studies. Acute evaluation of human BD has shown catecholamine levels above normal values immediately after the BD event. Increased inflammatory responses and apoptosis have been observed in various organs.[15–17] In a rodent model of heart transplantation, a longer duration of BD led to increased leukocyte infiltration and expression of adhesion molecules on the endothelial cells of the transplanted graft, thereby resulting in accelerated acute rejection.[18]

Previous results reports by our group[19] demonstrated important inflammatory activation in a rat BD model, showing classical hemodynamic alterations soon after balloon catheter inflation. In the present study, TEA was used in a rat BD model in an attempt to attenuate the hemodynamic alterations that are triggered by the catecholamine storm.

Since the donor scarcity[3] is an emerging problem in the entire world, understanding causes of graft failure and interventions to render grafts more likely to be viable are important endeavors. Our data showed that all but one group evolved with a high mean arterial peak pressure soon after the Fogarty catheter insufflation leading to BD induction, namely the group receiving TEA with bupivacaine

Serum

Tissue

Fig 3. Enzyme-linked immunosorbent assay of cytokines in the serum and cardiac tissue after 6 hours of brain-death induction. Brain-dead rats were treated with saline, bupivacaine before brain-death induction (pre-bup), and bupivacaine after 20 and 60 min (bup-20 and bup-60, respectively). The data are presented as the mean $\pm$ standard error of the mean. The reference values obtained from normal rats were below the limit of detection of the assay. IL, interleukin; TNF, tumor neurosis factor.
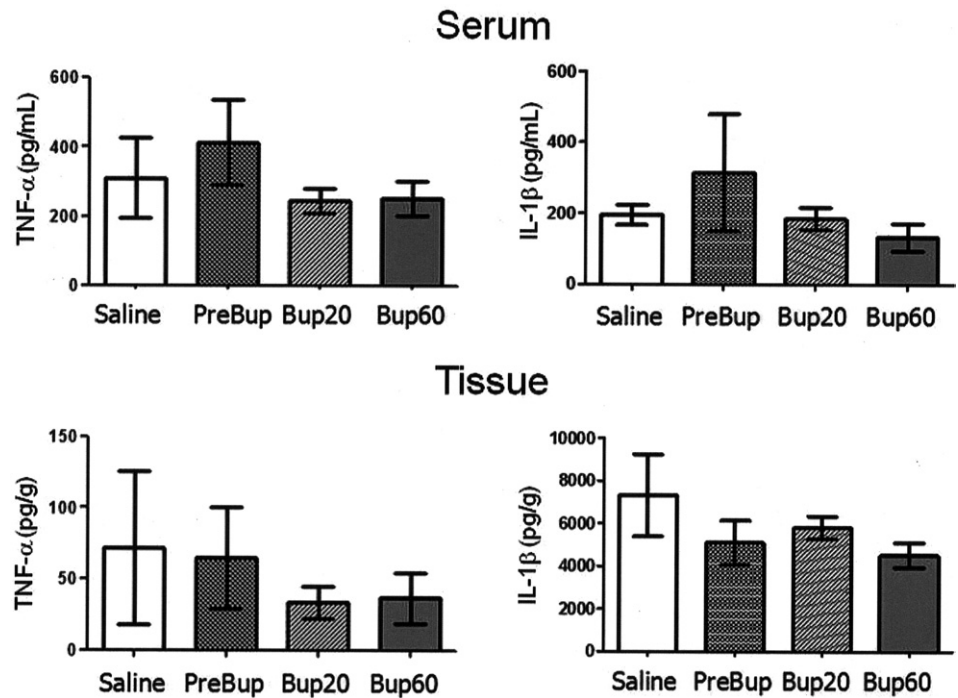
before BD induction. TEA obtained a chemical sympathectomy precluding the hemodynamic derangements usually seen with acute BD. Similar results have been reported using administration of intravenous drugs like propranolol,[20] xylazine,[21] and labetalol.[22] Another way to acheive a sympathectomy surgically in baboons has been reported by Novitzky et al.[12] However, their animals showed a classical hypertensive crisis indicating that the surgical procedure was not sufficient to block the autonomic storm.

Studies investigating the relationship between BD and the activation of peripheral organs have demonstrated that cytokines act as signaling molecules during the inflammatory process.[15] Pro-inflammatory cytokines, such as TNF-α and IL-1β, induce endothelial activation resulting in leukocyte interactions with adhesion molecules expressed on the cell surface.[23,24] Their data demonstrated TNF-α and IL-1β to be highly expressed in all animals, either in serum or in the myocardium, indicating an intense inflammatory activity

in all groups, suggesting that the autonomic storm was not linked to cytokine release. There was no difference among the groups.

Studying the mesentery, our group[19] reported increased ICAM-1 expression triggered by BD consistent with other studies performed in various organs.[25–29] Herein, the abolition of the autonomic storm by TEA did not change the expression of adhesion molecules on cardiac endothelial cells.

Apoptosis is another mechanism measured in our BD model. The data presented herein showed that the number of caspase-3 and Bcl-2-positive cells did not differ among the groups. Birks et al[30] measured pro-caspase-3 and cleavage products of caspase-3 leves in BD human hearts that had been used versus discarded for transplantation observing a high expression among grafts considered unsuitable for transplantation due to poor pretransplant function.
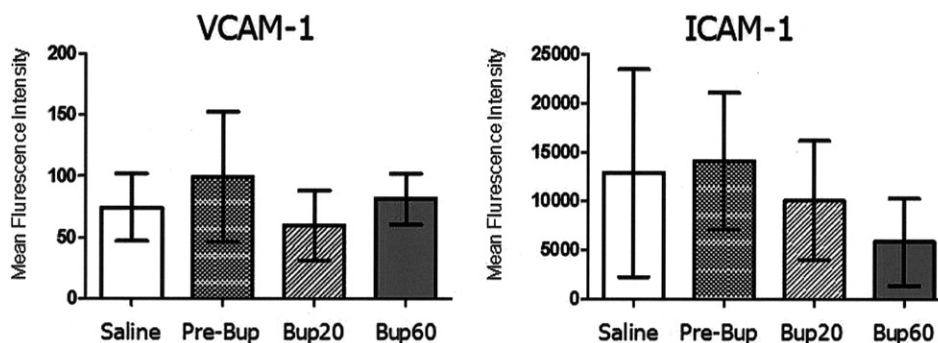

Fig 4. Expression levels of vascular adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 in the cardiac tissue in brain dead rats treated with saline, bupivacaine before brain death induction (pre-bup), and 20 or 30 minutes thereafter (bup-20 and bup-60, respectively). The data are presented as mean $\pm$ standard error of the mean.
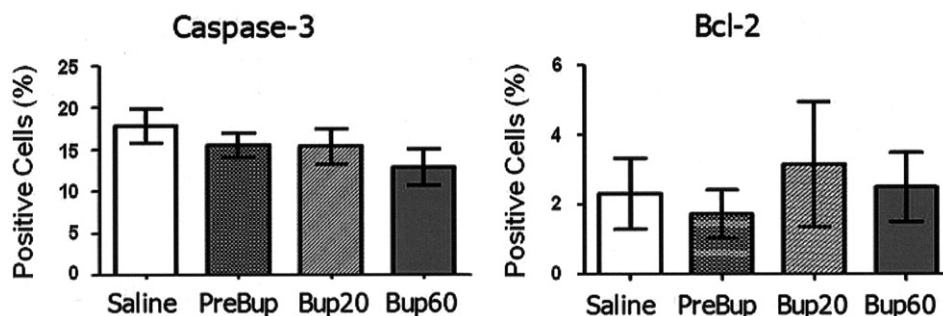
Fig 5. Expression of pro- and anti-apoptotic molecules (caspase-3 and Bcl-2, respectively) in the myocardium. Brain-dead rats were treated with saline, bupivacaine before brain-death induction (pre-bup), and bupivacaine after 20 and 60 minutes (bup-20 and bup-60, respectively). The data are presented as mean ± standard error of the mean.

The number of white blood cells was significantly reduced 6 hours after BD in all animals, including those that had inhibited hemodynamic storm. Thus the reduced number of leukocytes, at least in this BD model, was not a sympathetic-dependent mechanism, since animals with hemodynamic changes after BD induction showed the same evolution. The reduction in blood leukocyte counts among BD rats agreed with previously reported results.[19,31]

In conclusion, we demonstrated taht it was possible to block the hemodynamic derangements associated with acute BD by TEA with bupivacaine. However, TEA did not influence the inflammatory response associated with BD in cardiac tissue at 6 hours thereafter. Furthermore, BD was associated with reduced white blood cell counts, irrespective of the autonomic storm. Finally, the hemodynamic effects of autonomic storm associated with acute BD seemed to not be responsible for the subsequent development of the inflammatory response. Therefore, further studies are needed to elucidate the mechanisms responsible for inflammation and myocardial dysfunction among BD patients.

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