

Levosimendan up-regulates transforming growth factor-beta and Smad signaling in the aorta in the early stage of sepsis

Levosimendan erken dönem sepsiste aortada “transforming growth factor beta” ve Smad işaretlenmesini up-regüle eder

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BACKGROUND

This prospective, controlled experimental study was planned to investigate the effects of levosimendan on transforming growth factor (TGF)- β 3 and Smad1, Smad2 and Smad3 expression in the early stages of sepsis.

METHODS

Twenty-four rats were randomized into four groups: 1) sham-operated controls, 2) dobutamine group - subjected to abdominal hypertension and peritonitis-induced sepsis using cecal ligation and puncture (CLP), then treated with 10 $\mu\text{g.kg}^{-1}\text{min}^{-1}$ intravenous (IV) dobutamine infusion, 3) levosimendan group - as in 2, then treated with levosimendan IV bolus 200 $\mu\text{g.kg}^{-1}$ followed by 200 $\mu\text{g.kg}^{-1}\text{min}^{-1}$ IV infusion, and 4) a control group as in 2, with no treatment. All rats were killed 8 hours after CLP. Aorta tissue samples were analyzed by immunohistochemical staining.

RESULTS

CLP caused mild interleukin (IL)-1 immunostaining in both control and dobutamine groups. Immunoreactivity of tumor necrosis factor (TNF)- α was mild in both sham and control groups. TGF- β 3 immunostaining was mildly increased in groups sham, control and dobutamine, whereas it was found moderate in group levosimendan. Smad1, Smad2 and Smad3 were found moderately increased only in group levosimendan.

CONCLUSION

Beneficial effects of levosimendan on hemodynamics and global oxygen transport were reported in experimental and clinical trials. Besides its potency on Ca^{++} ion sensitivity, it should influence inflammatory cytokine production by diminishing TGF- β 3 and Smad1, Smad2 and Smad3 expression.

Key Words: Levosimendan; sepsis; Smad; transforming growth factor- β .

AMAÇ

Bu prospektif kontrollü deneysel çalışma, levosimendanın erken dönem sepsiste *transforming growth factor* (TGF)- β 3 ve Smad1, Smad2 ve Smad3 oluşumuna etkilerini araştırmak amacıyla planlandı.

GEREÇ VE YÖNTEM

Yirmi dört sıçan rasgele dört gruba ayrıldı: 1) sham kontrol grubu 2) dobutamin grubu - abdominal hipertansiyon, çekal ligasyon ve delme (ÇLD) tekniği ile peritonit modeli prensibiyle sepsis oluşturuldu, sonra sıçanlar 10 $\mu\text{g.kg}^{-1}\text{dk}^{-1}$ intravenöz (IV) dobutamin infüzyonu ile tedavi edildi, 3) levosimendan grubu - 2. grupta olduğu gibi sepsis modeli gerçekleştirildi sonra sıçanlar levosimendan IV bolus 200 $\mu\text{g.kg}^{-1}$ takiben, 200 $\mu\text{g.kg}^{-1}\text{dk}^{-1}$ IV infüzyonu ile tedavi edildi, 4) kontrol grubundakilere tedavi yapılmadı. Tüm sıçanlar ÇLD'den 8 saat sonra öldürüldü. Aort doku örnekleri immünohistokimyasal olarak boyandı.

BULGULAR

ÇLD hem kontrol, hem de dobutamin gruplarında ılımlı IL-1 boyanmasına neden oldu. *Tumor necrosis factor* (TNF)- α immünreaktivitesi sham ve kontrol grubunda ılımlıydı. Sham, kontrol ve dobutamin gruplarında TGF- β 3 ılımlı derecede yüksek bulunurken, bu parametre levosimendan grubunda orta derecede yüksek saptandı. Smad1, Smad2 ve Smad3 levosimendan grubunda orta derecede yükselmiş olarak saptandı.

SONUÇ

Levosimendanın hemodinami ve global oksijen taşınmasına sağladığı olumlu etkiler, deneysel ve klinik çalışmalarda bildirilmiştir. Levosimendanın Ca^{++} iyon duyarlılığı üzerine etkileri yanı sıra, TGF- β 3, Smad1, Smad2 ve Smad3 oluşumunu etkileyerek enflamasyonda sitokin salınımına da etkileri olabilir.

Anahtar Sözcükler: Levosimendan; sepsis; Smad; transforming growth factor- β .

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Uncontrolled release of inflammatory molecules such as nitric oxide, cytokines, reactive oxygen species and endothelins precede major changes in hemodynamics and tissue hypoperfusion, contributing to multiorgan failure. Support of tissue perfusion with adequate blood pressure and organ blood flow is a crucial component of sepsis treatment. Management of sepsis-induced circulatory failure aims at restoring cardiac output and arterial blood pressure by fluid resuscitation and inotrope/vasopressor administration.^[1] Inadequate tissue perfusion persists even after effective volume replacement^[2] and vasoactive therapy.^[3]

The use of a new calcium sensitizer, levosimendan, in patients with septic shock was first described in a case report in 2005,^[4] and since then, case reports,^[5] case series,^[6] randomized controlled trials^[7,8] and experimental studies^[9-11] have reported improvements in hemodynamics, global oxygen transport, pulmonary circulation, metabolism vasopressor requirements, and tissue blood flow and oxygenation.

Abdominal compartment syndrome (ACS) is an important complication for septic patients, and as a cause of abdominal hypertension, ACS has adverse effects on the circulation, threatening the function and viability of tissues.^[12,13] Pathophysiologic effects include release of cytokines, formation of oxygen free radicals, and decreased cellular production of adenosine triphosphate, leading to the translocation of bacteria from the gut and intestinal edema predisposing to multiorgan dysfunction syndrome that affects vital body systems.

We previously demonstrated that proinflammatory cytokine (tumor necrosis factor (TNF)- α , interleukin (IL)-1 β) production was diminished in a levosimendan-treated cecal ligation and puncture (CLP)-induced sepsis experimental model,^[14] and we aimed to investigate if transforming growth factor- β 3 (TGF- β 3) and R-Smads (Smad1, Smad2, Smad3) were affected.

In the present study, we complicated the CLP-induced sepsis model with abdominal hypertension to mimic the clinical situation of sepsis, and our hypothesis is that levosimendan should attenuate inflammation by up-regulating TGF- β 3 and R-Smads in the early stages of sepsis. To test this hypothesis, we investigated and compared the immunohistochemical staining of aorta tissue in the early stage of an experimental model of peritonitis-induced sepsis. We compared levosimendan with dobutamine because systemic and regional tissue perfusion effects of these two drugs had been investigated and compared in some experimental endotoxemia models previously,^[15] but the effects of these two drugs on the aorta in peritonitis-induced early stages of sepsis has not been studied.

MATERIALS AND METHODS

The study was conducted in the Experimental Animal Research Laboratory of University Hospital and approved by the local ethical committee. The study conformed to the recommendations of the Helsinki Declaration. Twenty-four male adult Wistar albino rats weighing 200-250 g were housed in plastic cages in a controlled environment with free access to water and food.

Anesthesia and Preparation

All animals were anesthetized with intramuscular (IM) 75 mg.kg⁻¹ ketamine and one femoral artery was catheterized. For measurement of mean arterial blood pressure (MAP), the arterial catheter was connected to a pressure transducer (Sasan pressure set, Sasan Medical Disposable Products, Mönchengladbach, Germany). MAP was monitored continuously (Datex Engstrom Ohmeda, Finland) and recorded every 15 minutes (min). The contralateral femoral vein was catheterized for fluid administration. A saline infusion was started as described in the study protocol and adjusted to maintain the MAP within 10 mmHg of initial levels.

Cecal Ligation and Puncture - Abdominal Hypertension

The rats' peritoneal cavities were opened and CLP was performed according to a previously standardized technique described by Wichterman and colleagues.^[15] The cecum was devascularized, ligated in a way that preserves intestinal continuity and perforated in a standardized fashion with an 18-gauge needle in two locations on the antimesenteric surface and left open. Then, two 20 gauge catheters were placed in two sides of the abdominal cavity parallel to each other and their tips were kept outside the abdominal cavity. Subsequently, the laparotomy incision was closed in two layers. The catheters were connected to a sphygmomanometer's pump, and intraabdominal pressure (IAP) was increased in a 5 min period by means of air pumping, until an intraperitoneal pressure of 15 mmHg was obtained. This level of IAP was sustained for the whole experiment with air pumping on demand.

Study Design

The surgical preparation was followed by 30 min of stabilization and then the animals were randomized into four equal groups. All rats were killed eight hours after stabilization.

Group S (Sham): Femoral arterial and venous catheterization was done, intraabdominal catheters were placed, but IAP was not elevated. MAP was monitored; 20 mL.kg⁻¹.h⁻¹ saline infusion was started.

Group C (Control): Femoral arterial and venous catheterization was done, CLP was applied, intraab-

dominal catheters were placed, MAP was monitored, and 20 mL.kg⁻¹ h⁻¹ saline infusion was started. Four hours later, the intravenous (IV) saline infusion rate was increased to 30 mL.kg⁻¹ h⁻¹ and the IAP was elevated to 15 mmHg.

Group D (Dobutamine): Treated as in C, with the addition of 10 μ g.kg⁻¹ min⁻¹ IV dobutamine (Dobutamine hydrochloride 250 mg.20mL⁻¹, Mayne Pharma, Mulgrave, Australia) infusion at the 4-hour time point, which continued during the study.

Group L (Levosimendan): Treated as in C, with the addition of 200 μ g.kg⁻¹ levosimendan (Simdax 2.5 mg.mL⁻¹, Abbott Laboratories, Abbott Park, IL, USA) as an IV bolus begun at the 4-hour time point, and continued as a 200 μ g.kg⁻¹ min⁻¹ IV infusion during the study.

Histological Evaluation

Tissue collection and fixation: Samples from all groups were fixed in 10% formalin solution for up to 48 hours (h). They were then washed with tap water and embedded in paraffin following a routine embedding procedure. Cross-sections of 5 μ were taken from the blocks and prepared for both histochemical and immunohistochemical staining.

Histochemical Observation

Sections dewaxed under 60 °C overnight were immersed in xylene for one hour and then rehydrated through a graded series of ethanol (100%, 95%, 80%, 70%, and 60%) for 2 min in each concentration and they were then washed in tap water. Sections were stained with either hematoxylin-eosin (H-E) according to routine protocols. Slides were mounted using Entellan, covered with glass cover slips prior to viewing, and photographed under the Olympus BX-40 (Tokyo, Japan) light microscopy.

Immunohistochemical Staining

The sections were incubated at 60 °C overnight and then dewaxed in xylene for 30 min. After soaking in a decreasing series of ethanol, sections were washed with distilled water. They were then treated with 2% trypsin in 50 mM Tris buffer (pH 7.5) at 37 °C for 15 min, and washed with phosphate-buffered saline (PBS). Sections were delineated with an Elite Pap pen (DBS, Pleasanton, CA, USA) and incubated in 3% H₂O₂ solution for 15 min to inhibit endogenous peroxidase activity. They were washed three times for 5 min with PBS and incubated with primary antibodies, TGF- β 3 (sc-82, Santa Cruz, CA, USA), Smad1, Smad2, Smad3 (sc-7960, Santa Cruz, CA, USA), IL-1 β (sc-7884, Santa Cruz, CA, USA), and TNF- α (sc-7317, Santa Cruz, CA, USA) for 18 h. Next, the sections were incubated with biotinylated IgG (supplied ready to use) for 30 min, followed by three washes in

PBS and then with streptavidin-peroxidase conjugate (supplied ready to use) for 30 min (KP-500, Universal Phosphatase Kit, Diagnostic BioSystems, Pleasanton, CA, USA) and washed with PBS three times. They were then incubated with a solution containing diaminobenzidine (DAB, 1718096, Roche, Mannheim, Germany) 50 μ L for each section for 5 min to visualize immunolabeling, and after rinsing with distilled water, counterstained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany). The sections were dehydrated with 80% and 95% alcohol and immersed in xylene and covered with mounting media (H701, CC/Mount, Universal Phosphatase Kit, Diagnostic BioSystems, Pleasanton, CA, USA). The negative controls received the same treatment as described above, but were incubated with rabbit IgG or mouse IgG instead of the primary antibodies.

Immunolabeling intensity was graded independently by two observers blinded to the experimental conditions with the following scale: mild (+), moderate (++) and strong (+++).

Data Analysis

The software SPSS 10.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical evaluation. All values are expressed as mean \pm SEM. Differences in MAP among groups were statistically analyzed with one-way ANOVA. A p value of <0.05 was considered as significant.

RESULTS

No mortality was encountered in any of the groups 8 h after CLP. There was no difference in MAP during the study period in all groups (Fig. 1); these data were taken from our previously published study.^[14] Immunohistochemical staining results are summarized in Table 1.

CLP caused mild increase in IL-1 β immunostaining in both C and D groups (Fig. 2). Immunoreactivity of TNF- α was mildly increased in both S and C groups (Fig. 3). TGF- β 3 immunostaining was mild in Groups S, C and D, whereas it was found moderately increased in Group L (Fig. 4). Smad1, Smad2 and Smad3 immunostaining was found moderate only in Group L (Fig. 5).

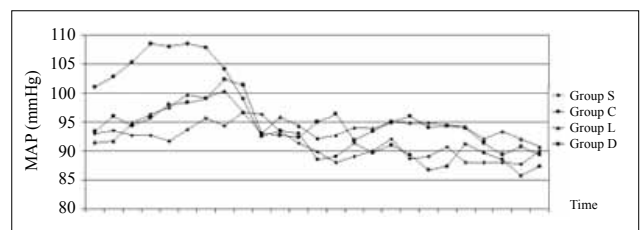


Fig. 1. Mean arterial pressures. No significant difference was seen between the groups.

Table 1. Immunolabeling intensity of IL-1 β , TNF- α , Smad1, Smad2, Smad3 and TGF- β 3

| | Group S (Sham) | Group C (Control) | Group D (Dobutamine) | Group L (Levosimendan) |
|----------------|-------------------|----------------------|-------------------------|---------------------------|
| IL-1 β | - | + | -/+ | - |
| TNF- α | -/+ | + | - | - |
| Smad 1/2/3 | - | - | - | ++ |
| TGF- β 3 | + | + | + | ++ |

Mild (+), moderate (++) and strong (+++).

DISCUSSION

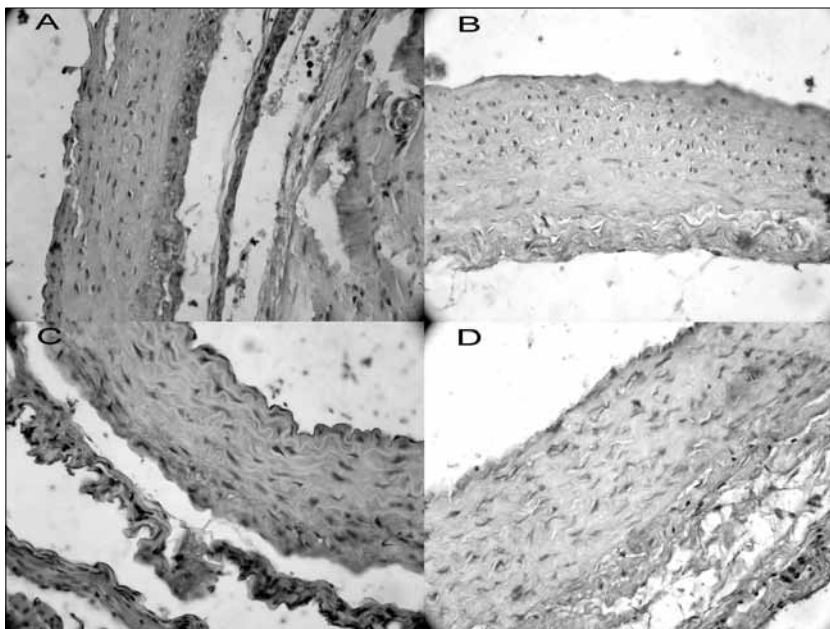
The effects of levosimendan on transforming TGF- β 3 and R-Smads in the aorta during the early stages of sepsis in rats were investigated in the present study. The ability of levosimendan to reduce pro-inflammatory cytokines and in addition to up-regulate TGF- β 3 and R-Smads was demonstrated, while dobutamine had no beneficial effect.

We used a peritonitis model with CLP that produces vasodilatation and increased cardiac output similar to early stages of human sepsis,^[16] which mimics the clinical situation. The early hyperdynamic state of septic patients corresponds well with the hemodynamic situation seen in rats after initiation of CLP.^[15,17,18] In contrast, lipopolysaccharide (LPS) models often generate a situation with a profound hypodynamic state.^[19] In the present study, during the first eight hours of CLP, hemodynamic variables did not change as severe as in the LPS-induced sepsis models and the macro-circulation was comparable between all of the treated groups. This enabled the model to test the effects of dobutamine and levosimendan on tissue injury by eliminating the detrimental effects of arterial hypo-

tension and myocardial depression induced by endotoxemia and the specific effects of these drugs on the myocardium and vascular tonus.

Abdominal hypertension worsens physiologic parameters including hemodynamics, abdominal perfusion, pulmonary functions, and survival.^[12,13] Secondary ACS is an important risk for patients with sepsis who are treated with large volumes of infused resuscitation fluids, which leads to decreased abdominal wall compliance, capillary leakage and bowel edema. Rezende-Neto et al.^[20] showed that intraabdominal hypertension stimulated the production of pro-inflammatory agents, and even after abdominal decompression, neutrophil accumulation and inflammatory infiltration continued. In the present study, we added abdominal hypertension to a well-known model of peritonitis in order to simulate the clinical picture of patients having intra-abdominal hypertension in addition to sepsis.

Case reports and observational studies suggest beneficial effects of levosimendan in septic patients,^[4,7] and preemptive use of levosimendan before the induction of endotoxemia in pigs prevented the development of hypodynamic endotoxic shock.^[21] Levosimendan

**Fig. 2.** IL-1 β immunostaining.

A: Group S (DAB x 40), B: Group C (DAB x 100), C: Group L (DAB x 40), D: Group D (DAB x 100).

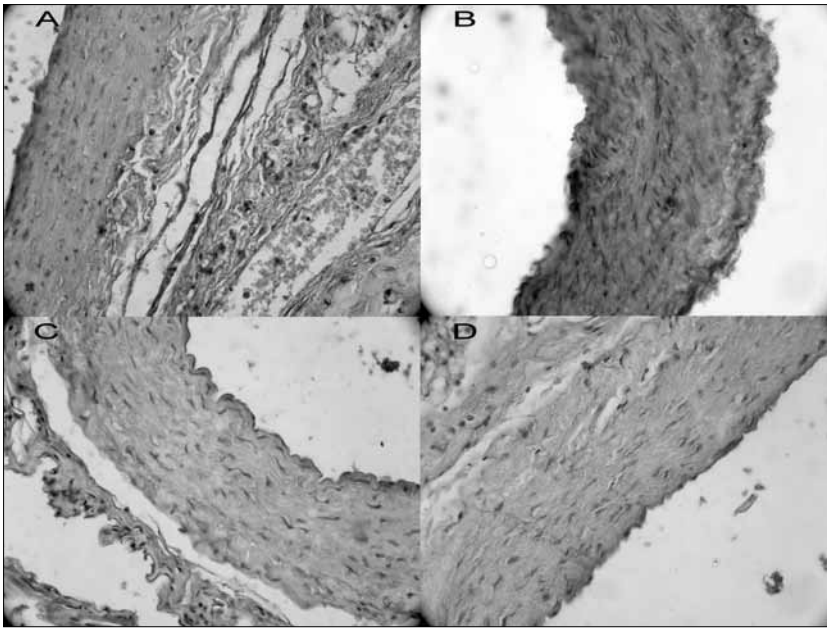


Fig. 3. TNF- α immunostaining.
A: Group S (DAB x 100), B: Group C (DAB x 100), C: Group L (DAB x 100), D: Group D (DAB x 100).

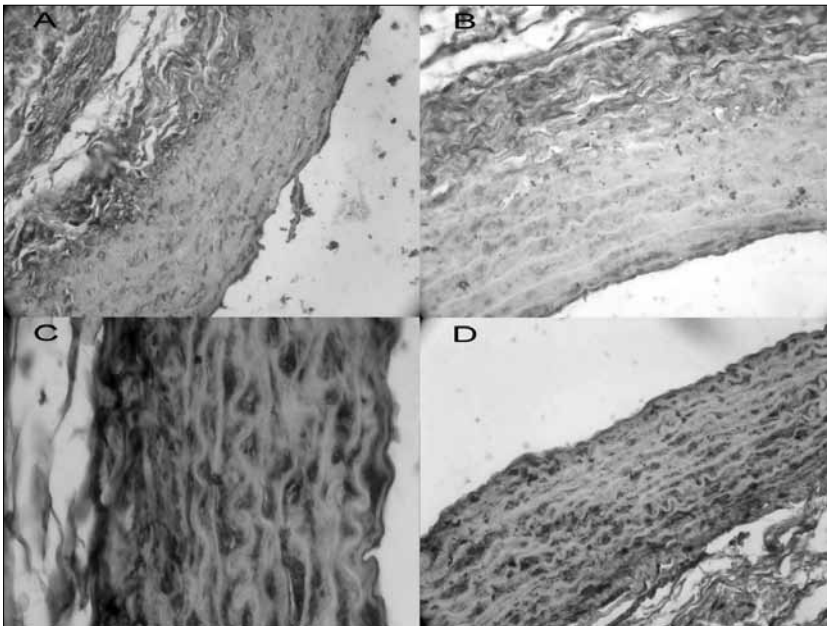


Fig. 4. TGF- β 3 immunostaining.
A: Group S (DAB x 40), B: Group C (DAB x 100), C: Group L (DAB x 100), D: Group D (DAB x 40).

improved hemodynamics, global oxygen transport, pulmonary circulation, and right ventricular function in septic shock patients with acute respiratory distress syndrome.^[8] Data from the available clinical studies suggest that rational use of levosimendan is a safe and effective alternative to increasing dobutamine doses in treating the failing heart of septic shock patients.^[7,8] Levosimendan exerts its positive inotropic effect by increasing contractile myofilament sensitivity to Ca^{++} ions. Besides its potency on Ca^{++} ion sensitivity, we speculated whether it would influence inflammatory cytokine production. Thus, we previously investigated the proinflammatory cytokine (TNF- α , IL-1 β) production in dobutamine- and levosimendan-treated

rats during the early phase of CLP-induced sepsis, and we found that levosimendan could diminish the proinflammatory cytokine production in lung tissue.^[14] In the present study, we aimed to investigate whether TGF- β 3 and R-Smads would play a role in this anti-inflammatory process.

TGF- β 3 is a multifunctional cytokine with a broad spectrum of effects on cell differentiation, proliferation, and activation of immune cells, and it is implicated in immune abnormalities linked to cancer, autoimmunity, opportunistic infections, and fibrotic complications.^[22]

TGF- β appears to play a key regulatory role in lim-

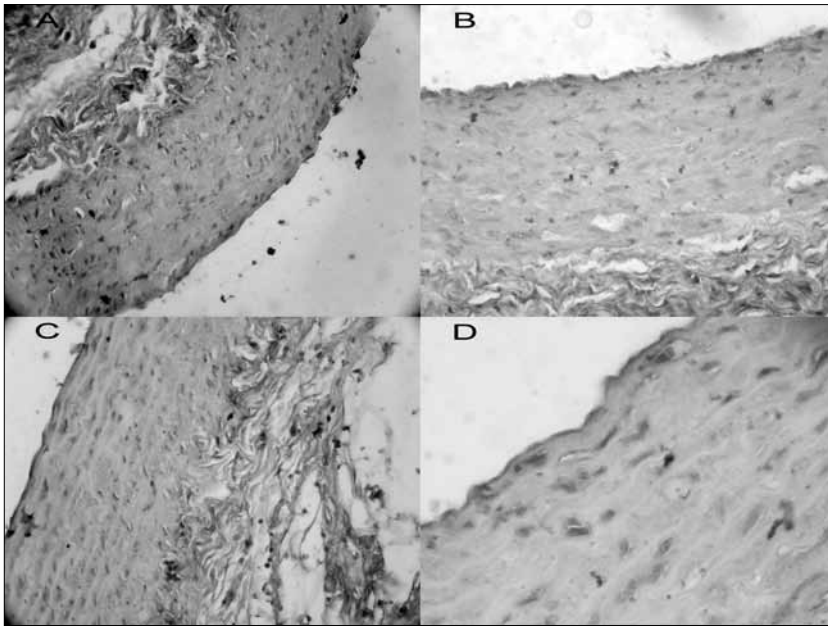


Fig. 5. Smad1, Smad2 and Smad3 immunostaining.
A: Group S (DAB x 40), B: Group C (DAB x 100), C: Group L (DAB x 100), D: Group D (DAB x 100).

iting inflammatory responses. TGF- β should ameliorate the adverse effects of proinflammatory cytokines on myocardial cells,^[23,24] and attenuate or eliminate the depressant activity of TNF- α and IL-1 β . The addition of TGF- β to human whole blood attenuated the ability of endotoxin to stimulate cytokine production.^[25] TGF- β may counteract the excess immune response that finally leads to sepsis syndrome and septic shock.^[26] It directly inhibits T-cell proliferation^[27] and regulates lymphocyte homeostasis by preventing the subsequent apoptosis of energized cells.^[28,29]

TGF- β -triggered signals are transduced by Smads, a family of proteins that serves as substrates for TGF- β receptors.^[30] With phosphorylation of Smad2 and Smad3, the phosphorylated intermediate associates with a co-Smad in the cytoplasm, then moving to the nucleus where transcriptional regulation occurs via direct DNA binding by the Smads complex.^[31]

It has been reported that TGF- β or Smad3 null mutant mice died by intraperitoneal injection of a non-lethal dose of LPS to wild type mice associated with increased proinflammatory cytokines.^[32]

Smad3 null mast cells showed enhanced production of proinflammatory cytokines upon LPS stimulation.^[33] The targeted disruption of Smad3 caused inflammation at the mucosal surfaces.^[30]

In our study, we observed that levosimendan treatment in the early stage of sepsis up-regulated the expressions of TGF- β 3 and Smad1, Smad2 and Smad3 in the aorta. We did not find IL-1 β and TNF- α immunoreactivity in the levosimendan-treated group, and diminished proinflammatory cytokine production should thus be attributed to antiinflammatory activity of TGF- β 3 and R-Smads. The results of the pres-

ent study suggested that dobutamine slightly reduced TGF- β 3 expression and did not affect Smad1, Smad2 and Smad3 production.

It has been recently demonstrated that TGF- β 1 blocks in vitro cardiac myocyte depression induced by TNF- α and IL-1 β .^[34] Improved expressions of TGF- β 3 and Smad1, Smad2 and Smad3 should play a role in the beneficial effects of levosimendan on cardiac performance during sepsis. The antiinflammatory effects of levosimendan should be investigated in larger series of experimental and clinical trials.

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