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ORIGINAL CONTRIBUTION

Anti-inflammatory effect of oxytocin in rat myocardial infarction

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Abstract While an increasing amount of evidence demonstrates the homeostatic functions of the cardiac oxytocin (OT) system, less is known about the role of this hormone in the injured heart. The current study examined the effect of OT infusion on cell apoptosis, expression of proliferating cell nuclear antigen (PCNA) and inflammation in the acute and subacute phases of myocardial infarction (MI). Prior MI male Sprague-Dawley rats were infused subcutaneously with OT 25 or 125 ng/(kg h) for 3 or 7 days. Saline-treated MI and sham-operated rats served as controls. Echocardiography and analysis of cardiac sections were used to disclose OT actions. Left ventricular fractional shortening, estimated to be 46.0 ± 1.8% in sham controls, declined to 21.1 ± 3.3% in vehicle-treated MI rats and was improved to 34.2 ± 2.1 and to 30.9 ± 2.5% after treatment with OT 25 and 125 ng/(kg h), respectively. OT infusion resulted in: (1) increase of cells expressing PCNA in the infarct zone, diminished cell apoptosis and fibrotic deposits in the remote myocardium; (2) suppression of inflammation by reduction of neutrophils, macrophages and T lymphocytes; (3) depression of the expression of proinflammatory cytokines tumor necrosis factor-alpha and interleukin-6 with promotion of transforming growth factor-beta. OT treatment reduced expression of atrial and brain natriuretic peptides in the infarcted ventricle, as well as the concentration of both peptides in the circulation. These results indicate that continuous OT delivery reduces inflammation and apoptosis in infarcted and remote myocardium, thus improving function in the injured heart.

Keywords Oxytocin · Myocardial infarction · Inflammation · Apoptosis

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Introduction

During the first few days after myocardial infarction (MI), the dominant pathological processes are inflammation and cell death. The inflammatory response after MI is integral to the healing process and contributes to left ventricular (LV) remodeling [1, 3, 5, 16, 44, 63]. However, no effective therapeutic strategy against cardiac inflammation has been established.

Oxytocin (OT), a neurohypophyseal hormone, is involved in uterine contraction, the inflammation-stimulated process [9]. Our interest in the cardiac OT system emerged from longitudinal investigations on the role of the brain in the regulation of cardio-renal homeostasis [21, 38]. OT plays an important role in cardiovascular homeostasis by regulating blood volume *via* atrial natriuretic peptide (ANP) release from cardiac atria [22, 23] and reducing heart contractility [41]. Further experiments led to the observation of OT and OT receptor (OTR) synthesis in the vasculature [32] and cardiomyocytes (CM) [31]. Finally, OT was shown as a potent, naturally occurring cardiomyogenic factor, which by OTR upregulation promotes the differentiation of stem cells to mature CM [10, 19, 30, 48].

The pathophysiological roles of OT action in the cardiovascular system are beginning to be understood. In wound healing and experimental sepsis, OT treatment has been shown to modulate immune and anti-inflammatory response [4, 29, 49, 50, 57]. The involvement of OT and

the OTR in immune reactions is consistent with their presence in regulatory T cells [14], which play a critical role in the suppression of autoimmune reactivity and in termination of the inflammatory response. On the other hand, the inflammatory process modulates the OTR gene *via* acute phase reactants and interleukins (IL) [52].

Considering possible OT actions on the heart, we hypothesized that treatment with the hormone will attenuate inflammation in a rat model of MI. In order to assess the putative anti-inflammatory effects of OT in MI, the current study examined immune cells, expression of proliferating cell nuclear antigen (PCNA), apoptosis and the expression of inflammatory cytokines. Furthermore, we investigated the effects of OT treatment on the expression of natriuretic peptides and echocardiographic parameters of cardiac work. Studies were performed in the proliferative phase of MI healing (72 h) and at scar maturation (day 7) [16].

Methods

Animals

Experiments were performed according to the guidelines for animal experimentation of the Canadian Council on Animal Care and approved by the Ethics Committee of the CHUM and with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The male Sprague-Dawley rats (weighing 280–370 g) were investigated. They were housed at room temperature and under a 12 h light/dark cycle and fed a standard chow diet with free access to tap water.

OT treatment

To establish the OT dose tolerated by treated rats, an initial dose-dependent evaluation of OT's cardiovascular effects was undertaken in isoflurane-anesthetized normal rats. We observed no significant hemodynamic effect at the dose level of 250 ng/(kg h) infused continuously and subcutaneously for 3 h. Moreover, the additional control experiments demonstrated that control conscious rats receiving OT at 250 ng/(kg h) slept throughout the day—this symptom was not observed when dose of OT was reduced. This effect is consistent with OT-mediated sedation. Thus, we decided to test OT on MI rats at two dose levels: 125 and 25 ng/(kg h), administered subcutaneously by Alzet pumps for 1 week after the MI.

MI induction

The animals were anesthetized by intraperitoneal injection of ketamine 25 mg/kg, xylazine 2.5 mg/kg and acepromazine 1.25 mg/kg. They were orally intubated with a 16-G catheter and ventilated with a mixture of oxygen and isoflurane from a rodent mechanical ventilator (Harvard Apparatus, Holliston, MA). Anesthesia was maintained by isoflurane at 0.5% from the vaporizer with mechanical ventilation at 70 cycles per min and a tidal volume of 1.5 ml/100 g body weight. After anesthesia, the rat was placed in prone surgical position. A 0.5 cm incision was made on the neck and subcutaneous tissue was bluntly separated. The Alzet pump with OT or saline was then installed in the subcutaneous tissue, and the skin incision was closed. Once the Alzet pump was installed, the rat was immediately placed in supine surgical position and coronary ligation or sham operation was performed. Briefly, left thoracotomy was performed at the fourth or fifth intercostal space. The pericardium was opened and the heart and beginning of the left anterior descending coronary artery (LADCA) were exposed. The LADCA was ligated 2–3 mm from the left atrium. Sham-operated control rats underwent an identical surgical procedure, but the sutures were only passed under the coronary artery and not tied. Bupivacaine 0.25% was instilled locally at the surgical site.

Echocardiography

Transthoracic echocardiography was performed in rats anesthetized with 1.5% isoflurane supplemented with O_2. Their hearts were investigated in a vivid GE ultrasound machine with a 10 MHz transducer that provided M-mode tracings recorded according to the American Society of Echocardiography Guidelines (http://www.asecho.org/Guidelines.php). M-mode images from both the short- and long-axis were analyzed to quantify LV end diastolic/systolic dimension and shortening fractions. The following formula was applied to calculate LV fractional shortening: FS% = (end diastolic diameter − end systolic diameter)/(end diastolic diameter) × 100. The ejection fraction (EF) was obtained from the short-axis view according to the following formula: diastolic area − systolic area/diastolic area. Diastolic measurements were taken at maximum LV cavity dimension, whereas systolic parameters were assessed during maximum anterior motion of the posterior wall. Images were analyzed by ImageJ software (http://rsb.info.nih.gov/ij/).

Histology

At the end of the experiment, the rats were killed by decapitation, and their hearts were rapidly removed, blotted dry and weighed. For measurement of infarct size, the hearts were cut into slices 2 mm thick, which were stained with tetrazolium chloride [62]. The slices were photographed, and myocardial infarct size was determined

planimetrically as the ratio of the total endocardial and epicardial lengths of the scar for all slices to the total endocardial and epicardial circumferences of the LV. The heart was fixed by perfusion with 4% formaldehyde and 0.1% picric acid in 0.1 M phosphate buffer, pH 7.4 (1–4 days), embedded in wax, and cut into 5-μm sections. Plasma was kept at −80°C in order to measure concentrations of ANP and brain natriuretic peptide (BNP) by radioimmunoassay [22].

Histological examinations were performed in sections from cardiac intraventricular septum by hematoxylin/eosin (HE), Masson and picro-sirius red staining (double refraction) allowing specific green color extraction of collagen birefringence in black background. Quantification of birefringence in collagen areas was analyzed with Image J software (National Institutes of Health, Bethesda, MD (http://www.nih), using threshold function. Panoramic cross-sectional digital images of Masson-stained hearts were prepared using Adobe® Photoshop® CS software (Adobe Systems Inc., San Jose, CA) to measure (in millimeters) septal wall and LV free wall. DNA apoptosis was investigated by DeadEnd™ Fluorometric TUNEL System (cat. No: G3250, Promega Corporation, Madison, WI). The photographs were taken with the inverted microscope OLYMPUS IX 51, Tokyo, Japan (http://www.olympus.com) equipped with Q Imaging QICAM-IR Fast Digital 1394 CCD camera and QCapture acquisition software. The measurements were performed in at least four samples per tested group and 7–10 microscopic fields in individual section.

Immunohistochemistry

For immunohistochemistry, the antigenic sites were revealed by immersing the sections in 0.1 M citrate buffer, pH 6.0, heated to 90°C for 20 min, and allowed to cool slowly to room temperature. Controls obtained by the omission of primary antibodies were negative, demonstrating the specificity of immunohistochemistry. To detect neutrophils, rabbit polyclonal anti-human granulocytic myeloperoxidase antibody (RB-373-R7, Lab Vision Corporation, Fermont, CA) was deployed. Mouse anti-rat CD68 diluted 1/200 served as primary antibody for macrophage detection (MCA341R, Cedarlane Laboratories, Toronto, Ontario, Canada). Mouse anti-CD3 antibody (MEM-57, Cedarlane Laboratories) was used for the detection of T cells. Mouse monoclonal antibody for cardiac troponin T was supplied by Abcam (Ab27217, Cambridge, MA). Rabbit antibodies for PCNA were obtained from Abcam (Ab18197) and for caspase-3 from Pharmingen (551150, San Diego, Ca).The rabbit anti-eNOS (1:4,000, sc-654) were provided by Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibodies complexed to antigens were bound to biotin-labeled anti-rabbit immunoglobulin G (IgG) or biotin-conjugated anti-mouse IgG, and the signal was revealed with the Histostain-plus kit from Zymed Laboratories (San Francisco, CA, Cat No 85-9243). The slides were counter-stained with Mayer's hematoxylin. For each sample ($n = 4$–5), seven fields were counted for total number of cells versus cells positively stained in brown. The % of positive cells was calculated as follows: (number of positive cells)/(total number of cells in the field) × 100.

Real-time polymerase chain reaction

Gene expression was measured in total RNA as previously reported [10]. The primer sets that were purchased from Invitrogen Life Technologies Inc. are presented in Table 1. Optical data were collected during the annealing step of each cycle. To ensure that only one polymerase chain reaction (PCR) product was amplified, the melting curve

Table 1 Primers used for real-time PCR

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	Accession no.
IL-1β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC	M98820
IL-6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC	E02522
IL-10	TGCAACAGCTCAGCGCA	GTCACAGCTTTCGAGAGACTGGAA	NM012854
TGF-β1	GGTGGACCGCAACAACGCAATCTA	GGGTGGCCATGAGGAGCAGGAA	X52498
TNF-α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC	X66539
ANP	GGACCCAAGTTTCCTCGAGTAA	GGATCCCAAGCAGCGTCTT	NM008713
BNP	CCTTCAGGTATGCGGTATTTGG	AGGCTCCCAGGTGAGACAGTT	NM012611
OTR	GTCAATGCGCCCAAGGAAG	GATGCAAACCAATAGACACC	NM012871
GAPDH	TTCAATGGCACAGTCAAGGC	TCACCCCATTTGATGTTAGCG	NM017008

IL Interleukin, *TGF-β1* transforming growth factor β1, *TNF-α* tumor necrosis α, *ANP* atrial natriuretic peptide, *BNP* brain natriuretic peptide, *OTR* oxytocin receptor, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

was analyzed. The relative expression of the PCR products was determined by the $\Delta\Delta Ct$ method and GAPDH served as housekeeping standard.

Western blot

Equal concentrations of proteins were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes, blocked with 5%-milk-TBST (Tris base 10 mM, NaCl 100 mM, Tween 0.1%, pH 7.5) and probed overnight with rabbit primary anti-OTR antibodies (N-19, sc-8103, from Santa Cruz Biotechnology, Santa Cruz, CA) and peroxidase-conjugated secondary antibodies [goat anti-rabbit IgG (No. 111-035-144 from Jackson Immuno-Research Laboratories, West Grove, PA]. Blots were incubated with ECL chemiluminescent reagent and visualized on film The Amersham™ ECL Plus Western Blotting Detection System (RPN2132) was from GE Healthcare (Piscataway, NJ), and Progene® autoradiography films (No. 39-20810) were from Ultident Scientific (St. Laurent, Canada).

Statistical analysis

The data are presented as mean ± standard error of the means. Means were compared by one-way analysis of variance (ANOVA), followed by Tukey's post-tests. Two-way ANOVA of the data on OT treatment in sham-operated and MI rats was undertaken during the experiments (3 and 7 days) to evaluate independent variables, hormone concentration, cell number, cytokines expression and receptor levels as dependent variables. A P value of <0.05 was considered to be statistically significant.

Results

OT improves cardiac function in rat MI

As reported in Table 2, OT treatment for 7 days did not influence body weight, heart weight and heart-body weight ratios in sham-operated and MI rats. Infarct size was modestly reduced in MI rats given OT and a statistically significant difference versus controls treated with vehicle was found in rats receiving 125 ng/(kg h). Similarly, the infarct size registered after 3 days of treatment was not significantly different in MI rats receiving saline (39.40 ± 0.68%) and OT 25 ng/(kg h) (37.17 ± 1.01%), but smaller in rats treated with OT 125 ng/(kg h) (33.33 ± 1.38%, $P < 0.01$, $n = 6$ in all groups of rats). In sham rats, treatment with low (25 ng/(kg h)) and moderate (125 ng/(kg h)) doses of OT did not change LV end diastolic diameter (LVED) and LV end systolic diameter (LVES) and EF. On the other hand, OT treatment in MI resulted in a significant improvement in LVES and EF. As seen in Table 2, OT treatment resulted in changes of echographic parameters in the LV of injured rats. The EF dropped from 77.2 ± 4.3% in sham-operated animals to 49.8 ± 4.9% in MI rats ($P < 0.001$). OT treatment of 25 and 125 ng/(kg h) reverted the EF in MI rats to 65.1 ± 4.7% ($P < 0.01$) and 66.2 ± 4.4% ($P < 0.01$), respectively. Furthermore, assessments of cardiac geometries in rats on the seventh day after MI indicated a significant increase in LVES from 4.04 ± 0.27 mm in sham-operated controls to 6.41 ± 0.36 mm in vehicle-treated MI rats. OT 25 and 125 ng/(kg h) significantly reduced LVES to 5.5 ± 0.37 and 4.83 ± 0.3 mm, respectively ($P < 0.05$ vs. vehicle-treated MI controls).

As presented in Fig. 1, FS, estimated to be between 42.3–46.2% in all rats groups before experiment was not different after sham surgery and not changed by treatment

Table 2 Weight and echocardiographic parameters of SHAM and MI rats after 7 days of treatment with oxytocin

	SHAM + Vehicle $n = 8$	SHAM + OT 25 ng/(kg h) $n = 6$	SHAM + OT 125 ng/(kg h) $n = 6$	MI + Vehicle $n = 8$	MI + OT 25 ng/(kg h) $n = 9$	MI + OT 125 ng/(kg h) $n = 12$
Body weight (g)	316 ± 19	306 ± 16	318 ± 21	292 ± 27	307 ± 28	316 ± 9
Heart (g)	1.07 ± 0.11	1.06 ± 0.07	1.00 ± 0.06	1.02 ± 0.08	1.09 ± 0.08	1.00 ± 0.02
Heart/BW (mg/g)	3.4 ± 0.2	3.5 ± 0.1	3.2 ± 0.2	3.5 ± 0.3	3.5 ± 0.2	3.2 ± 0.2
Infarct size (%)				38.0 ± 2.0	34.3 ± 2.8	32.1 ± 2.9$^{\&}$
LVED diameter	7.65 ± 0.54	7.40 ± 0.52	7.63 ± 0.42	8.12 ± 0.22	7.72 ± 0.28	7.72 ± 0.32
LVES diameter	4.04 ± 0.27	4.17 ± 0.55	4.47 ± 0.32	6.41 ± 0.36*	5.50 ± 0.37*	4.83 ± 0.30$^{\&}$
EF (%)	77.2 ± 4.3	73.2 ± 6.7	71.5 ± 3.0	49.8 ± 4.9*	65.1 ± 4.7*$^{\&}$	66.2 ± 4.4*$^{\&}$

Results were analyzed by two-way ANOVA

LVED Left ventricular anterior wall thickness, *LVES* left ventricular posterior wall thickness, *EF* ejection fraction

* $P < 0.05$, effect of MI versus corresponding SHAM group, $^{\&}$ $P < 0.05$, effect of OT treatment

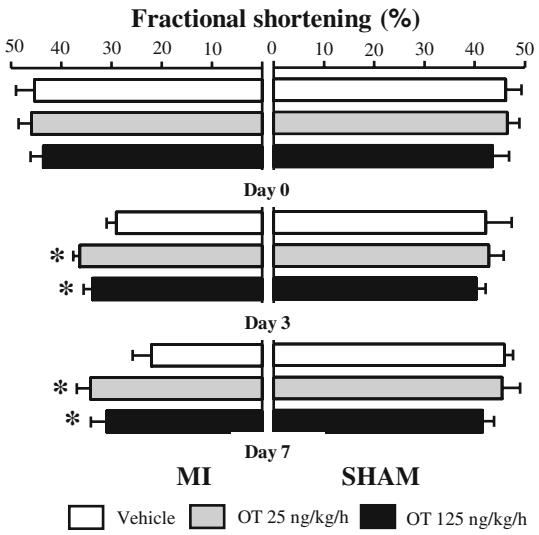

Fig. 1 Values of left ventricular fractional shortening in the sham-operated (*SHAM*) and infarcted (*MI*) rats treated with vehicle and oxytocin (*OT*) during 3 and 7 days. $n = 6$–12, $^*P < 0.05$, significant effect of OT treatment versus vehicle treatment

with both doses of OT during 3 and 7 days of investigation. On the other hand, the FS declined to 29.1 ± 1.9 or 21.1 ± 3.3% in vehicle-treated MI rats ($P < 0.01$) and was improved to 36.3 ± 1.8 or 34.2 ± 2.1% after treatment with OT 25 ng/(kg h) ($P < 0.01$) and to 33.8 ± 2.3 or 30.9 ± 2.5% after OT 125 ng/(kg h) ($P < 0.01$) in animals treated for 3 and 7 days, respectively.

OT infusion enhanced expression of troponin T in peri-infarct areas

To investigate whether OT treatment protects cardiac muscle in the infarct, we stained cardiac sections to detect troponin T, a CM marker. As illustrated in Fig. 2a, staining with troponin T disclosed rare tissue fragments in the peri-infarct area from MI rats receiving vehicle and abundant tissue fragments in the sections from MI rats exposed to OT (Fig. 2b, c). Moreover, some single cells in the infracted area were stained with troponin T antibody. As presented in Fig. 2d, quantitative analysis disclosed the presence of 6.5 ± 1.0% of these cells in MI controls treated with vehicle and a significantly higher number ($p < 0.05$) in scar tissue of MI rats administered OT 25 ng/(kg h) (19.8 ± 1.4%) or 125 ng/(kg h) (21.4 ± 4.4%).

In addition, analysis of the infarct border zone of OT-treated heart section indicated that troponin-containing cells were rarely stained with PCNA antibody, the marker of gross changes in DNA content or cell number (Fig. 2e). Intensive PCNA staining was found in the scar region usually dominated by fibroblasts and myofibroblasts (Fig. 2e.1, 2). PCNA staining was also observed in the vascular structures (Fig. 2e.3). In contrast, co-staining of troponin T and caspase-3 revealed the presence of apoptotic nuclei in CM and also in vascular and scar tissue cells (Fig. 2f.1–4). Furthermore, distribution of intensive PCNA staining in the infarct border zone corresponded to the distribution of cells stained by macrophage-specific marker CD68 (Fig. 2g).

OT treatment led to improved structure in remote myocardial areas 1-week post-MI

Myocardial infarction-induced thinning of the injured wall was partially reversed by treatment with OT (Fig. 3a). Moreover, this treatment resulted in several beneficial effects on the structure of noninfarcted remote myocardium. As illustrated in Fig. 3b, the significant increase of CM surface area after MI in rats receiving vehicle was reduced by OT treatment (25 ng/(kg h). Other structural changes in the remote cardiac region stimulated by MI—such as fibrotic deposits (Fig. 3c) and augmented CM apoptosis determined by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) methodology (Fig. 3d)—were also inhibited by OT infusion. Similarly, OT treatment diminished enhanced infiltration of macrophages observed in remote regions of the infarcted heart (Fig. 3e). These reported observations on rats treated with 25 ng/(kg h) OT were similarly revealed in the hearts of rats receiving 125 ng/(kg h) OT (data not shown).

OT infusion stimulated expression of the cell cycle regulatory protein PCNA and reduced apoptosis in the infarct region

As presented in Fig. 4a the LV free wall thinning in MI rats induced significant reduction of thickness ratio expressed as free wall thickness/septum thickness. Using this measurement, it was observed that OT infusion to MI rats significantly decreased free wall thinning. To investigate whether OT treatment can influence cell viability in the infarcted heart the expression PCNA and caspase-3 were studied in the cells present in the injured region. In MI rats, OT changed the expression profile of the cell cycle regulatory protein PCNA in cells present in the infarct region. As demonstrated in Fig. 4b PCNA-positive cells were more abundant at day 3 post-MI than at day 7 ($P = 0.0001$). OT increased the number of PCNA-positive cells both at days 3 and 7 ($P = 0.0001$). At day 7, 32.3 ± 2.6% of the cells expressed PCNA in the scar area in saline-treated MI rats (Fig. 4b1), a value which rose progressively to 45.7 ± 3.2% (Fig. 4b2) and 72.7 ± 3.9% (Fig. 4b3) after the administration of OT 25 and 125 ng/(kg h), respectively ($P = 0.0001$).

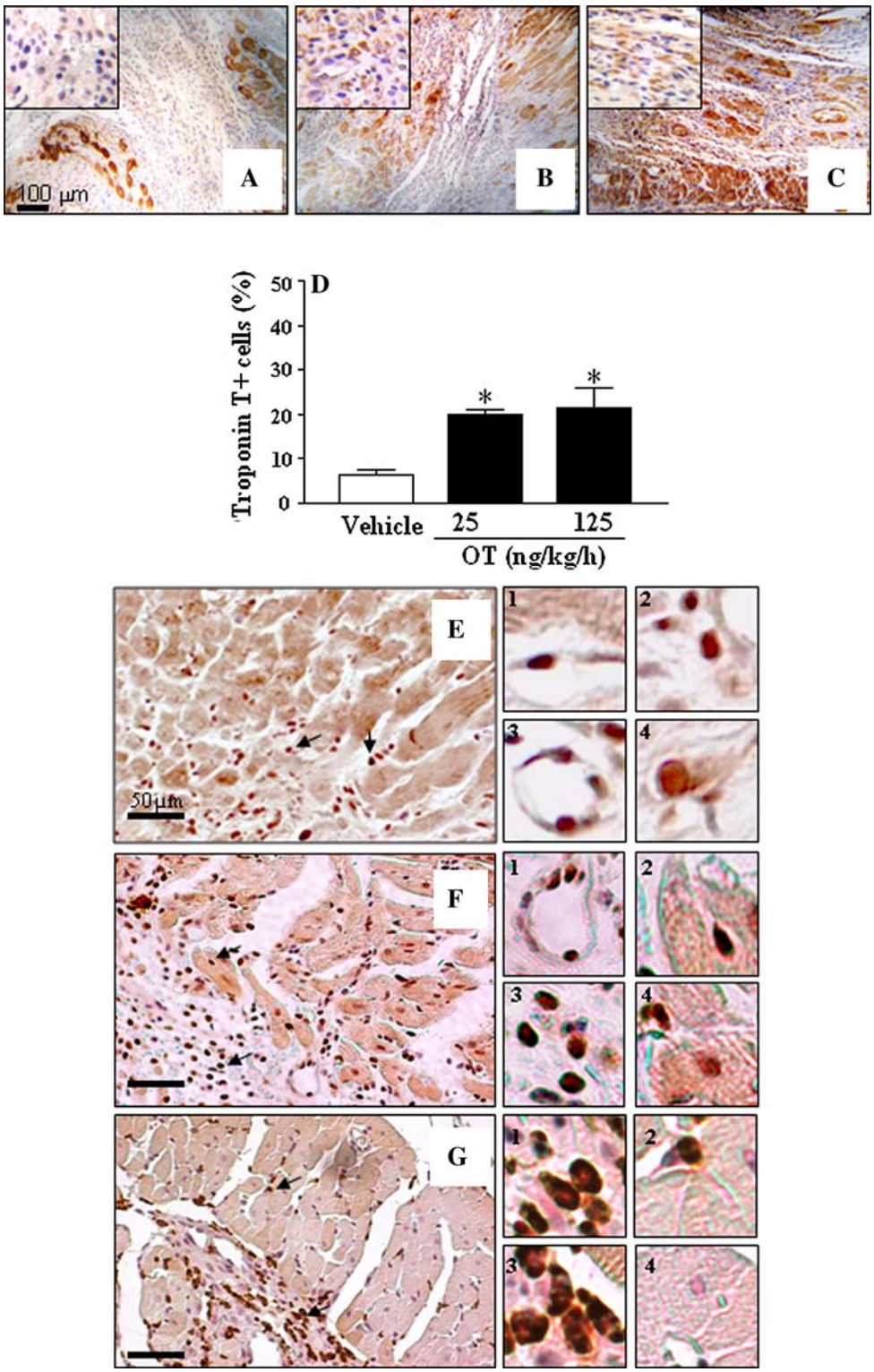

Fig. 2 Detection of troponin T by immunocytochemistry (**a**–**c**) and enumeration (**d**) of cells expressing this protein in the scars of MI rats treated for 7 days with vehicle (**a**), MI rats treated with 25 ng/(kg h) OT (**b**), and MI rats treated with 125 ng/(kg h) OT (**c**). *Bars* represent mean ± SEM as a percentage of positively stained cells, $n = 4$, $^*P < 0.05$, significant effect of OT treatment. Representative images of scar borderline in the heart section treated with 25 ng/(kg h) OT and stained with antibodies against troponin T (*brown*). **e** Co-staining with PCNA, *red color*. Small windows demonstrate frequent single staining of PCNA in the scar cells (*1*, *2*), microvessels (*3*) and rare troponin T and PCNA co-staining in small cells of the scar (*4*). **f** Co-staining with caspase-3 antibody (*dark brown*). Single staining of caspase-3 has been found in vascular (*1*) and scar cells (*3*) and double staining in CM (*2*, *4*)

Staining with caspase-3 antibody was undertaken to detect accumulation of apoptotic cells in the infarct. Experiment duration did not demonstrate a significant impact on the quantification of cells expressing caspase-3 (Fig. 4b, $P = 0.20$). On the other hand, OT significantly reduced the accumulation of caspase-3-positive cells in the infarcted area ($P < 0.0001$). This effect was dose-dependent in samples collected at days 3 and 7. For example, at day 7, in sections from rats treated with vehicle (Fig. 4b1), 40.2 ± 2.0% of the cells were stained with caspase-3, whereas in animals treated with OT 25 or 125 ng/(kg h), this number declined to 20.4 ± 1.9 and 14.0 ± 2.1%, respectively (Fig. 4b2, b3).

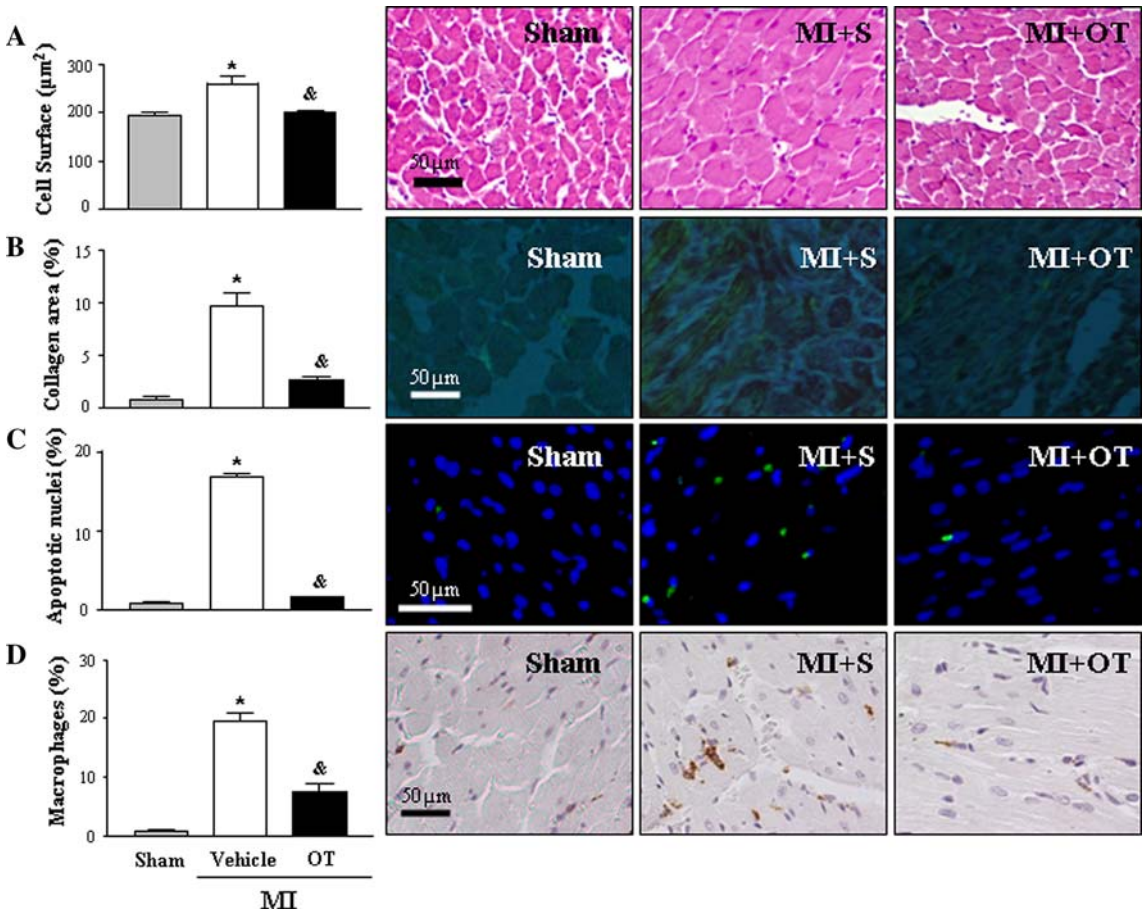

Fig. 3 Alterations of the LV structures in MI in the presence and absence of OT (25 ng/(kg h)) after 7 days of treatment. Changes are illustrated by images of samples from sham-operated (*Sham*), vehicle-treated MI heart (MI + S) and MI heart treated with oxytocin (MI + OT). **a** Thinning of injured wall in infarcted heart sections. Remote noninfarcted LV, MI resulted in: an increase of cardiomyocyte surface as measured under microscope in HPS-stained sections (**b**), an accumulation of collagen deposits measured by refractometry (**c**), an enhancement of TUNEL-positive cardiomyocytes (**d**) and augmented infiltration of CD68-positive cells. These alterations were partly reversed by OT treatment. $n = 4$, $^{*}P < 0.05$, MI effect; $^{\&}P < 0.05$, effect of OT treatment in MI

OT reduced the immune cell infiltration of infarcts

Cells expressing macrophage CD68 marker in the heart of sham-operated rats were barely detectable (data not shown). OT treatment duration after MI had a significant effect on the accumulation of CD68-positive cells ($P = 0.004$). As shown in Fig. 5a, in the infarcts of rats receiving the vehicle, 21.8 ± 2% of cells expressed CD68 at day 3 and the percentage increased significantly by day 7 (26.8 ± 1.7%, $P < 0.05$). OT treatment for 7 days reduced the accumulation of $CD68^{+}$ cells in the infarct ($P < 0.0001$), and at doses of 25 and 125 ng/(kg h), CD68 was detected in 15.5 ± 1.2 and 15.1 ± 1.3% of total cells counted, respectively (Fig. 5a2, a3).

As reported in Fig. 5b, the most spectacular impact of OT was a reduction in neutrophils ($P < 0.0001$), although treatment duration did not influence this result ($P = 0.94$). As illustrated in Fig. 5b1–b3, after 7 days of OT exposure, the number of neutrophils decreased from 7.3 ± 0.6% in vehicle-treated controls to 2.0 ± 0.3 and 1.1 ± 0.3% in rats given OT 25 or 125 ng/(kg h), respectively.

Because of the close interactions of monocytes/macrophages with T cells, we also stained infarcted LV sections with CD3 antibody in order to identify T cell receptors (Fig. 5c). Like $CD68^{+}$ cells, cells expressing the CD3 marker were more abundant on day 7 than on day 3 after MI ($P = 0.002$), and OT significantly reduced their number in the infarct ($P = 0.0002$). This effect was especially clear in sections collected at day 3.

OT modified cytokine and OTR expression in the injured heart

As presented on Fig. 6, Western blot demonstrated (Fig. 6a) that OTR protein decreases in injured LV after 3 and 7 days after surgery (~40–50%) and increases in rats given OT 25 ng/(kg h) (~2-fold). We found that MI significantly reduced OTR mRNA in infarcts compared to the

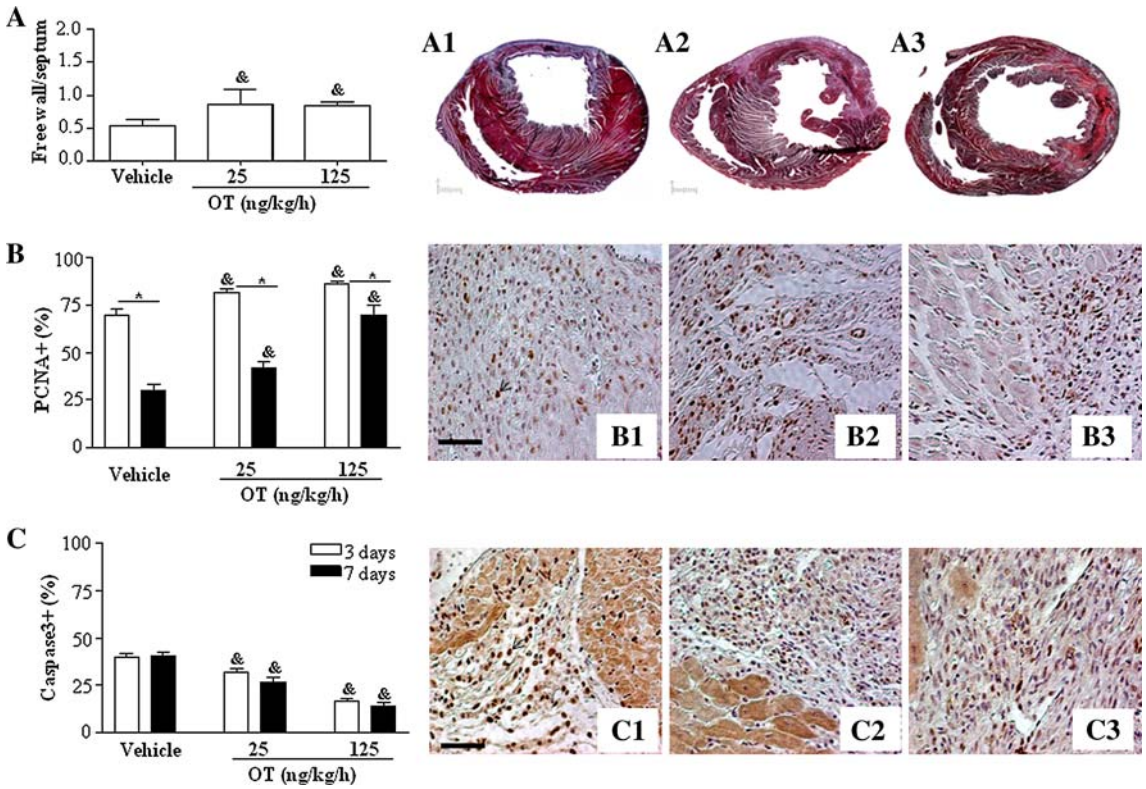

Fig. 4 *Bar graph* showing the thickness ratio (**a**) with free wall thickness/septum thickness in the hearts from MI rats. Ventricular transaxial plane showing representative Masson staining of a ventricular slices from MI rats treated with vehicle (**a1**), OT 25 ng/(kg h) (**a2**) and OT 125 ng/(kg h) (**a3**). In scar tissue, OT increases the number of cells expressing the cell cycle regulatory protein PCNA and reduces cells expressing the apoptotic marker caspase-3. Numerical representation of PCNA (**b**) and caspase-3-positive cells (**c**) in infarcts of rats treated with vehicle or OT for 3 or 7 days. **b1–b3** Representative photomicrographs of PCNA-positive cells stained on day 7. **c1–c3** Representative photomicrographs of caspase-3-positive cells stained on day 7. Two-way ANOVA of PCNA-positive cells indicated an interaction effect between sample collection time and OT treatment ($P < 0.001$). Similar analysis was negative for caspase-3 staining ($P = 0.38$). $n = 5$, $^{*}P < 0.05$, effect of sample collection time; $^{\&}P < 0.05$, effect of OT treatment

LV of sham-operated rats (Fig. 6b). OT significantly increased OTR expression in the infarcts and this effect was more evident after treatment with 25 ng/(kg h) OT than with 125 ng/(kg h) ($P < 0.05$).

We investigated whether OT treatment has an effect on the expression of inflammatory cytokines involved in scar formation. Specific mRNAs were analyzed by RT-PCR in samples collected in both injured and noninjured parts of the LV. The results demonstrated that mRNA expression of tumor necrosis factor-alpha (TNFα) and IL-6 was significantly higher in the infarct of MI rats than in the LV of sham-operated controls (Fig. 6c, d, respectively). Relative to vehicle-treated MI rats, exposure of injured animals to OT 25 or 125 ng/(kg h) reduced the transcripts of these proinflammatory cytokines to the level seen in sham controls. The expression of another proinflammatory cytokine, IL-1β (Fig. 6e) was also increased by MI and reduced by treatment with OT. Interestingly, the transcript of the anti-inflammatory cytokine transforming growth factor-beta (TGFβ), which was stimulated in the scar by OT in a dose-dependent manner, was significantly downregulated in the noninfarcted LV area (Fig. 6f). Opposite effects were observed in ventricular tissues collected outside the injury site, where TGFβ mRNA was downregulated by MI and not significantly changed by OT. In addition, both OT concentrations lowered IL-10 expression at the injury site (Fig. 6g).

OT infusion altered the expression of eNOS and natriuretic peptides

Immunocytochemistry disclosed eNOS protein in vascular structures of sham-operated (Fig. 7a), MI rats treated with vehicle (Fig. 7b), and treated with OT both at dose of 25 ng/(kg h) (Fig. 7c) as well as 125 ng/(kg h) (Fig. 7d). However, eNOS accumulation in CM localized in proximity of the scar was observed only in samples obtained from MI rats treated with OT but not those receiving vehicle. As seen also in Fig. 7, in sham rats, ANP was immunolocalized in the atria (Fig. 7e) but not in the LV (Fig. 7f). In the LV of MI rats treated with vehicle (Fig. 7g), OT 25 ng/(kg h) (Fig. 7h) or 125 ng/(kg h)

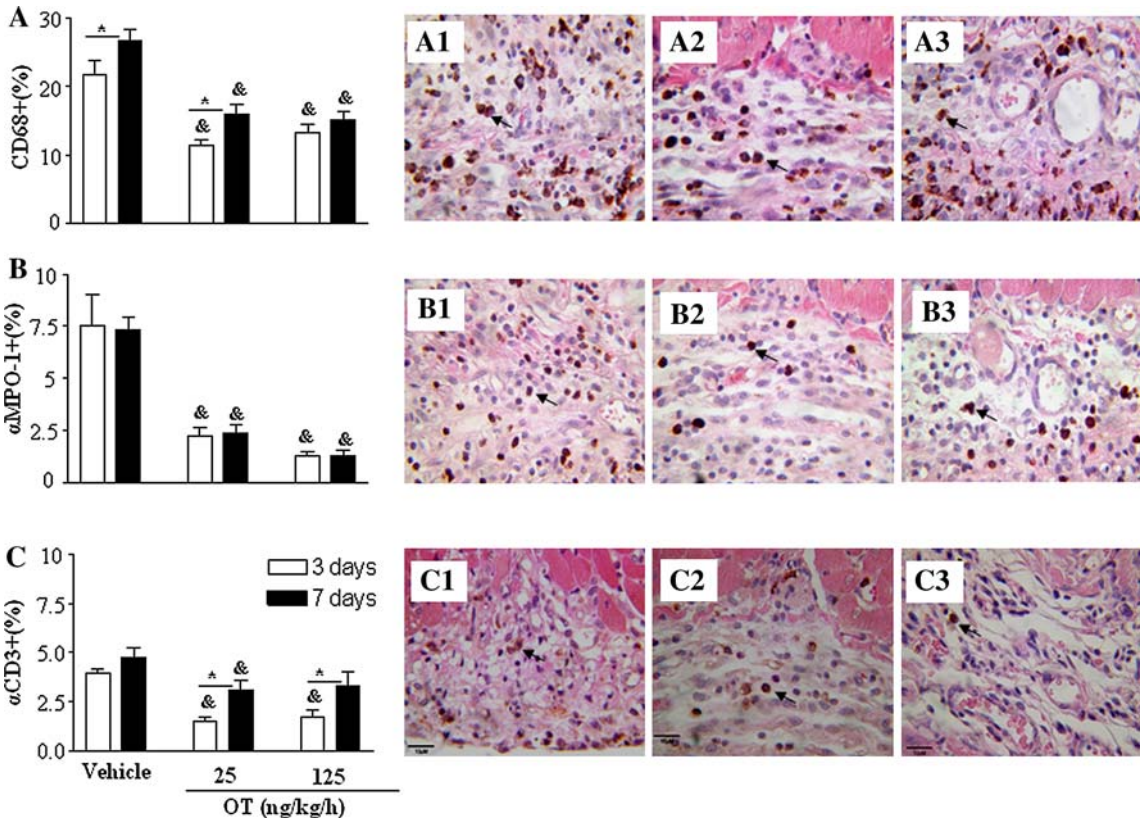

Fig. 5 OT reduces the number of immune cells infiltrating infarcted cardiac areas. *Bar* representation of the number of cells infiltrating rat heart in infarcted areas after treatment with OT or vehicle for 3 or 7 days post-MI: **a** expression of macrophage marker CD68; **b** positive for MPO-1, marker of neutrophils; **c** CD3 expression, a marker of T lymphocytes. The results are presented as percentage of total cells in the microscopic field. *Open bars* represent the percentage of cells in the infarcted site after 3 days of treatment with OT 25 ng/(kg h), OT 125 ng/(kg h), or vehicle, while *checkered bars* represent counts after 7 days of OT treatment ($n = 5$, for each condition). Compared to controls, there is a significant decrease in the number of immune cells infiltrating the infarcted region after 3 days as well as after 7 days of treatment, suggesting that OT has an anti-inflammatory effect. **a1**–**a3**, **b1**–**b3**, **c1**–**c3** Representative photomicrographs disclosing infiltration by macrophages, neutrophils and T cells, respectively, in the infarcted region after 7 days. **a1**, **b1**, **c1** Heart of a rat with myocardial injury treated with vehicle. **a2**, **b2**, **c2** Sections of infarcted hearts treated with OT 25 ng/(kg h). **a3**, **b3**, **c3** Sections of infarcted hearts treated with OT 125 ng/(kg h). $n = 5$, $^{*}P < 0.05$, effect of sample collection time; $^{\&}P < 0.05$, effect of OT treatment

(Fig. 7i), significant accumulation of ANP antigen was observed in the scar area. RT-PCR analysis of ANP and BNP mRNAs demonstrated significant elevation in the scars of 1-week post-MI rats, compared to the LV of sham-operated rats (Fig. 7j, k). The difference in ANP mRNA expression in the scar and noninjured LV was negative ($P = 0.15$). However, in OT-treated MI rats the ANP mRNA expression in the injured LV was significantly lower than in saline controls ($P < 0.001$). Plasma ANP level was higher in MI compared to sham rats ($P < 0.001$) and plasma ANP concentration decreased in OT-treated MI rats (Fig. 7l). As observed in Fig. 7k, the BNP mRNA reduction was noted in the LV scar area after treatment with OT. Plasma BNP (Fig. 7m) was higher in MI than in sham-operated controls ($P < 0.001$), but the effect of OT treatment on plasma BNP in MI rats was not significant ($P = 0.67$).

Discussion

The current study demonstrated that OT infusion over 3 and 7 days to rats with MI ameliorated echocardiographic parameters. The mechanism of action through which OT provides benefits to the injured heart is suggested by the following observed effects of OT treatment: (a) increased cell DNA content in cells present in the infarct zone; (b) increased cell population expressing a CM marker; (c) diminished cellular apoptosis, fibrosis and collagen deposits both in infarcted and noninfarcted zones of the injured heart; (d) reduced inflammatory cells migrating into the infarct (particularly neutrophils), and attenuated expression of proinflammatory cytokines in injured tissue.

An important aspect of this study is that cardiac OTR, initially downregulated in the infarcted heart, was subsequently activated in response to OT infusion. The structure

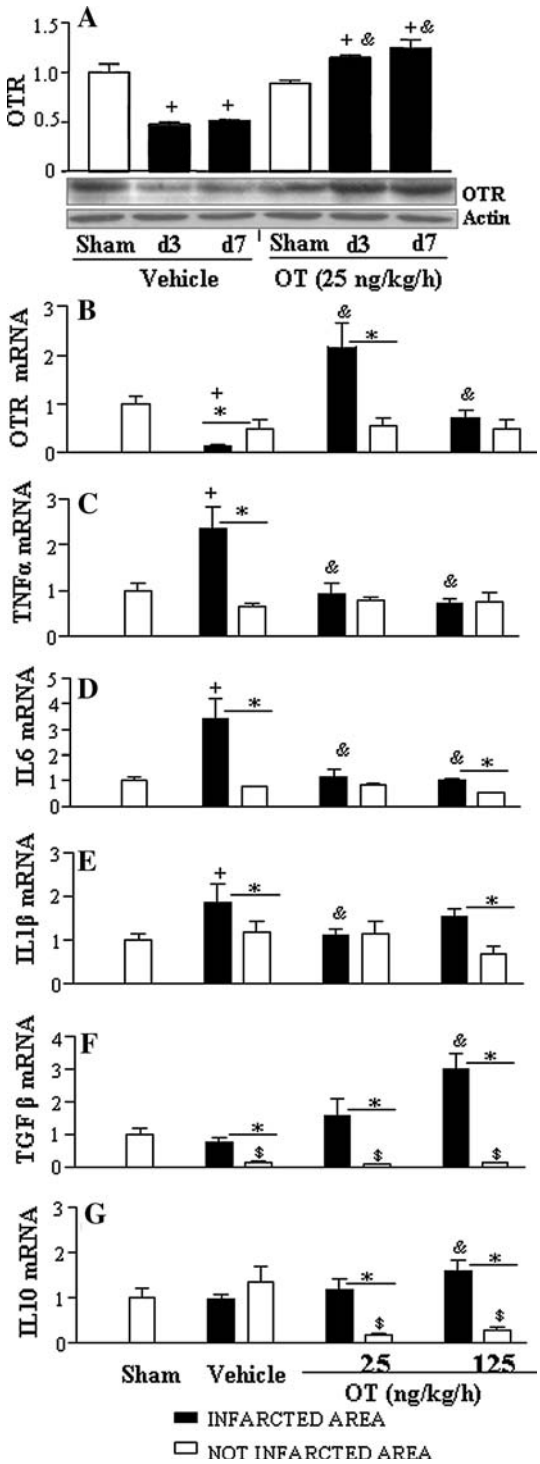

Fig. 6 Expression of OTR and RT-PCR measurement of cytokines in sham-operated and infarcted rat hearts. **a** Western blot of OTR was performed in samples of the infracted LV region from rats treated with vehicle and OT (25 ng/(kg h)) for 3 and 7 days. For mRNA analysis the heart tissue were obtained from rats subjected to treatment for 7 days with vehicle and with OT 25 and 125 ng/(kg h). Measurement was performed in infarcted and noninfarcted LV areas. Transcript measurement by real-time PCR is shown in **b** OTR, **c** TNFα, **d** IL-6, **e** IL-1β, **f** TGFβ, **g** IL-10. $n = 6$, $^{+}P<0.05$, difference between sham versus MI rats; $^{*}P < 0.05$, effect of sample collection in infarcted and noninfarcted area; $^{\&}P < 0.05$, effect of OT treatment in MI rats

of the OTR gene promoter region supports a potential role for proinflammatory cytokines as negative modulators of OTR gene transcription [52]. Consistent with this observation is the recent study associated reduced LV EF in mice subjected to ischemia/reperfusion injury with a lowered expression of OTR in the heart [28]. The results of our experiments show that OT treatment potently improved LV EF despite the presence of a significant infarct. The observed effects on the structural heart suggest that substantial recovery of cardiac function in OT-infused rats is due to beneficial effects on the remaining noninfarcted portion of the ventricle. OT treatment normalized CM hypertrophy, decreased fibrosis, collagen deposits and apoptosis in noninfarcted cardiac areas. We already reported that the fraction of cardiac fibroblasts contains OT [31] and express OTR (unpublished observations). The OT effect on proliferation of cardiac fibroblasts can be executed *via* biglycan, known as a target gene of Gq protein coupled receptors [59]. OT treatment lowered apoptosis both in injured and remote cardiac region. As recently reported the zone of apoptosis initially begins in the midmyocardium and extends into the subendocardium and subepicardium [47]. Then, in post-MI rats, CM apoptosis occurs continuously over an extended period of time and in the remote myocardium correlates with ventricular enlargement [46].

Limitation of myocardial infarct size provides important measure in the clinical settings of specific treatments [40]. However, some of the remodeling benefit may be result of a smaller initial injury in these hearts, rather than a direct effect of OT on the remote zone, especially that infarct measured after 3 and 7 days had similar size. Because myocardium starts to undergo irreversible injury within 20 min of ischemia, we initiated the OT infusion before production of MI. It is therefore possible that OT exposure in the short period before coronary ligation contributes to myocardial protection in rats. In this way, erythropoietin induced the phosphoinositide 3-kinases (P-I3-K)-dependent phosphorylation of Akt in the heart of Sham but not in post-MI treated rats [39]. Since OT stimulates ANP release from the heart atria into the circulation [22, 23], administration of OT before injury can also provide beneficial effects *via* the stimulation of ANP-related activation of protein kinase G in ventricular CM [61].

In the OT-treated, MI rats, numerous CM were disclosed in the infarct region as well as in the border zone. The possible explanation is that OT infusion protects CM from cell apoptosis in the infarcted heart. Some data suggest mechanisms of how OT mediates cell protection. The

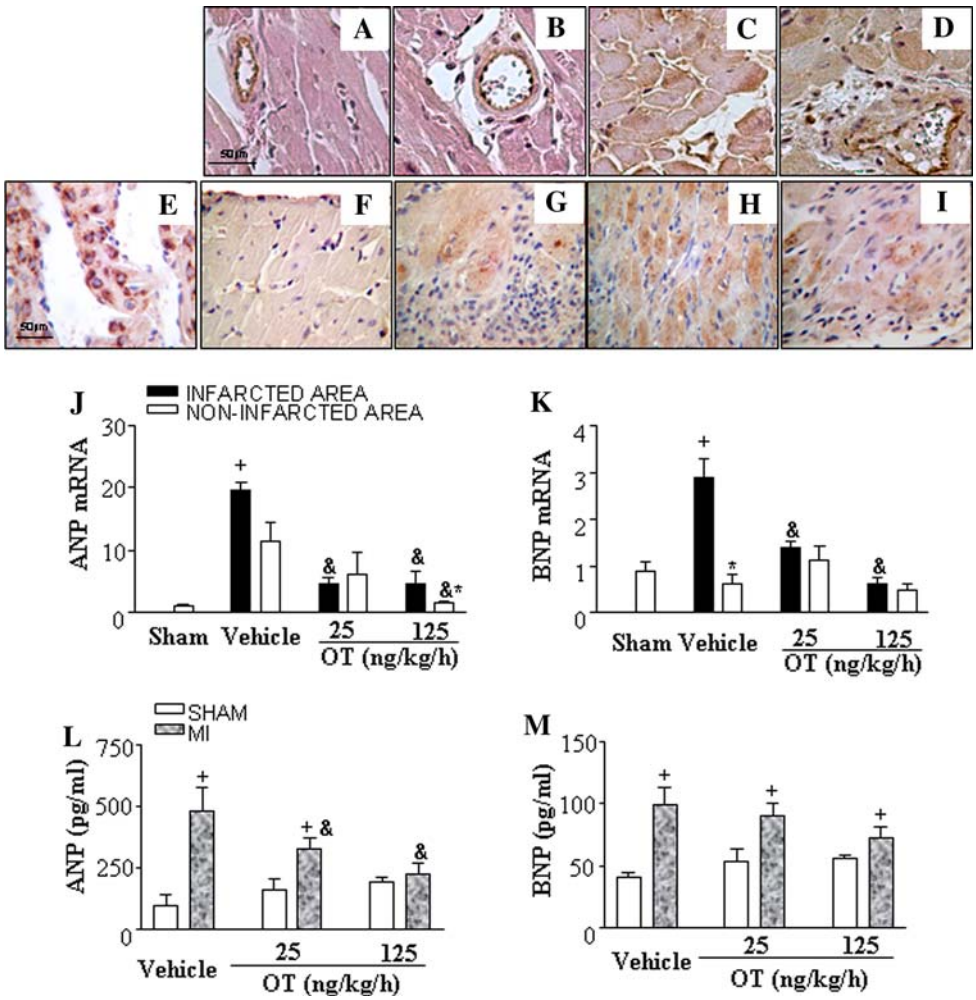

Fig. 7 Detection of eNOS and natriuretic peptides in the heart of sham-operated and MI rats. Immunocytochemistry of eNOS in the LV of sham-operated rats (**a**), LV of MI rats treated with vehicle (**b**), MI rats treated with OT 25 ng/(kg h) (**c**), and MI rats treated with OT 125 ng/(kg h) (**d**). In the infracted hearts images show cardiomyocytes in proximity to the infarction area. Immunocytochemistry of ANP in the atrium of sham-operated rats (**e**), LV of sham rats (**f**), LV scar of MI rats treated with vehicle (**g**), MI rats treated with OT 25 ng/(kg h) (**h**), and MI rats treated with OT 125 ng/(kg h) (**i**). ANP mRNA (**j**) and BNP mRNA (**k**) in injured and healthy parts of the LV of rats with MI and sham-operated controls. Plasma ANP (**l**) and BNP (**m**) levels in sham-operated and MI rats treated with vehicle or OT. Samples were collected after 7 days of exposure to vehicle or OT. $^{+}P < 0.05$, difference between sham versus MI rats; $n = 7$, $^{*}P < 0.05$, effect of sample collection in infarcted and noninfarcted areas; $^{\&}P < 0.05$, effect of OT treatment in MI rats

results indicate that OT treatment results in eNOS accumulation in the CM localized in proximity of infarction. In contrast, in the hearts isolated from sham-operated and MI rats treated with vehicle, the eNOS staining was limited to endothelial structures of coronary vessels. This observation suggests eNOS involvement in CM protection. In agreement with these data, we observed that OT increases glucose uptake in CM *via* the P-I3-K and potentiates glucose uptake effect of 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation [15]. P-I3-K pathways are considered beneficial during myocardial injuries [24, 25, 39, 42]. It was recently reported that in endothelial cells, the pro-angiogenic effect of OT involved activation of the PI-3-K/AKT/eNOS pathway [6]. We should also consider the phosphorylation of p38MAP kinase and extracellular signal-regulated kinase 2 (ERK 1/2) which may contribute to the proliferative activity of OT [11]. More recently, in a rabbit model of myocardial ischemia reperfusion, the OT treatment induced phosphorylation of ERK 1/2, AKT and eNOS in the cardiac tissue [35]. Therefore, OT, like other G-protein-coupled ligands, can act by activation of PI3K/Akt and project onto downstream kinases, such as glycogen synthase kinase 3β (GSK3β), targeting mitochondria in order to protect the cells [24, 25].

The calcium–calmodulin kinase kinase (Ca-CAMKK)-AMP-activated protein kinase (AMPK) pathways are also involved in OT-mediated glucose uptake in the skeletal muscles [36] and CM [15]. The activation of AMPK in the heart following ischemia and reperfusion is recognized as cardioprotective because AMPK limits apoptosis and cell damage [37, 51]. In this regard, Kewalramani et al. [34] have defined AMPK as a powerful cardiac anti-inflammatory protector against TNFα-triggered CM apoptosis through the phosphorylation of Bad protein and the subsequently suppressed mitochondrial apoptotic signaling. In addition, AMPK activation as well as CAMKK, stimulates the phosphorylation of various downstream kinases, including the eukaryotic elongation factor 2 kinase involved in the reduction of CM enlargement [26] and suppression of cardiac pathological hypertrophy [7].

In agreement with the observation that treatment with OT caused a decrease in heart rate and contractile function

in isolated, perfused heart [22], the negative chronotropic action of was recently associated with an attenuation of cardiac damage induced by ischemia reperfusion [45]. Therefore, OT, by activation of intrinsic cardiac cholinergic neurons *via* NO activation [41], can effectively inhibit cardiac sympathetic nerve activity and improve EF in rats subjected to MI. In addition, OT-mediated macrophage depletion can suppress the sympathetic hyperinnervation following MI [60] and reduce local inflammation associated with C-reactive protein and terminal complement complex [63]. Increased sympathetic activity documented in patients during acute MI is inversely related to EF and the site of infarction alone does not influence the magnitude of sympathetic nerve hyperactivity [20]. Further studies in ewes revealed no correlation between infarct size and any calculated index of the increase in cardiac sympathetic nerve activity post-MI [33].

The MI is recognized as a decisive factor amplifying an excessive and unnecessary inflammatory response [5]. OT treatment for 3 and 7 days after MI suppressed the accumulation of immune cells in infarcts, as identified by macrophage and T cell markers. Several subsets of T cells, like $CD4^+$ and $CD8^+$, expressed OTR mRNA, indicating an important role for the OT system in the response of these immune cells [43]. T cell infiltration is accompanied by monocyte/macrophage infiltration [27]. Therefore, by primarily affecting T cells, OT can also limit monocyte/macrophage infiltration, as observed in this study. On the other hand, recent studies indicate the presence OTR in monocytes and macrophages, suggesting that these cells are direct targets of this hormone in inflammation [43, 57].

At the cellular level, the dominant effect of OT was the inhibition of neutrophil infiltration in the infarct. A comparable decrease of neutrophils was seen after OT infusion for 3 and 7 days and this effect was dose-dependent, resulting in a 3.4–3.6-fold reduction by OT 25 ng/(kg h), and a 5.7–6.6-fold diminution by OT 125 ng/(kg h). In accordance with the present results, OT treatment alleviated oxidant renal injury in pyelonephritic rats both by its antioxidant actions and by preventing excessive infiltration of neutrophils [4].

During myocardial ischemia, the myocardial TNFα concentration is rapidly increased and contributes to the development of contractile dysfunction [12, 54]. Regulation of cytokines may be specific in the different parts of the infarcted ventricle and persist independently of the acute inflammatory response in infarcted hearts [9]. Inhibition of TNFα was reported to reduce infarct size in rabbits [3], however in porcine model it was observed that increased TNFα is causal for both contractile dysfunction and protection against infarction [55]. IL-1β and TNFα reduce ATP production probably due to the induction of oxidative stress and thereby myocardial oxygen consumption [13]. OT treatment can compensate ATP deficit by enhanced glucose uptake in CM [15]. Our data also suggest that improvement of cardiac contractile function in response to OT treatment is associated with reduced TNFα expression in the injured myocardium. These results are in agreement with studies in Sprague-Dawley rats [29] showing that OT attenuates the severity of colitis with concomitant reductions in the serum TNFα and inhibition of neutrophil infiltration into the injured colon.

Interleukin-6 synthesis is rapidly induced in mononuclear cells and CM of the ischemic myocardium [17]. The present study indicates an inhibitory effect of OT on IL-6 expression in the infarcted site of the rat heart. This observation is consistent with experiments demonstrating inhibition of IL-6 release by OT in pituitary cells [56] and most recently in macrophages and endothelial cells [57]. Furthermore, OT infusion resulted in stimulation of TGFβ expression in injured heart areas, pointing to improvement LV function [18] as well as beneficial effects such as reduced apoptosis and increased cell proliferation [16, 53]. Enhanced TGFβ can provoke CM differentiation from cardiac stem cells [2]. OT also stimulated anti-inflammatory IL-10 mRNA in LV scars. Because, the RNA from the infarct zone largely derived from nonmuscle cells invading the site of injury, it is possible that the changes to cytokines mRNA in the infarct is due to effects on the inflammatory cells in the region. Like in the pregnant uterus, OT can activate T helper type 2 cells stimulating IL-10, and suppress T helper type 1 cells responsible for the synthesis TNFα, the regulation of which is important for parturition induction [8]. Interestingly, the regulation of the appropriate cytokine genes can be executed *via* the cardioprotective calcineurin/NFAT pathway triggered by an elevated uterine OT system [58].

OT treatment significantly reduced ANP and BNP mRNA expression in the LV of MI rats and reduced the plasma concentration of these peptides. Because a high level of NP is a powerful marker of LV systolic dysfunction and poor prognosis after MI, these results support the conclusion that OT infusion has a beneficial effect on heart function after injury.

In summary, these novel findings suggest that OT may contribute to the protection of cardiac tissue in ischemic conditions. OT may exert an alternate role by regulating and maintaining a balance of anti-inflammatory and pro-inflammatory cytokines within the injured heart.

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