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José Pedro Oliveira Pinto

Haemodynamic and neuroendocrine effects of tezosentan in chronic experimental pulmonary hypertension

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E sob a Coorientação de:
Doutor Joaquim Adelino Correia Ferreira Leite Moreira

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Projeto de Opção do 6º ano – DECLARAÇÃO DE REPRODUÇÃO

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Abstract *Purpose:* Chronic pulmonary hypertension (PH) therapy is poorly investigated in intensive care. Our aim was to evaluate haemodynamic and neuroendocrine effects of the dual endothelin-1 (ET-1) blocker tezosentan in monocrotaline (MCT)-induced PH. *Methods:* Male Wistar rats (180–200 g, $n = 194$) randomly received 60 mg kg^{-1} MCT or vehicle, subcutaneously, and 2 days later, a subgroup of MCT-injected rats was gavaged with 300 mg kg^{-1} day^{-1} bosentan (MCT BOS, $n = 46$), while another (MCT, $n = 125$) and control rats (Ctrl, $n = 23$) received vehicle. At 25–30 days, 48 h after interrupting bosentan, rats randomly underwent either a dose–response evaluation (0.5–20 mg kg^{-1}, $n = 7$ each group) or a 4 h perfusion of tezosentan (20 mg kg^{-1} in 10 min + 10 mg g^{-1} h^{-1}) or vehicle ($n = 8$ per group, each). Haemodynamics, including blood gas analysis, were evaluated after thoracotomy under anaesthesia. After plasma, right ventricle (RV) and lung collection, plasma ET-1, cytokines, nitrate and 6-keto-PGF1α, and lung and right ventricular gene expression and cyclooxygenase (COX) and nitric oxide synthase (NOS) activities were quantified. *Results:* Monocrotaline resulted in PH, RV dilation and decreased cardiac output (CO) that were attenuated in MCT BOS. Pulmonary hypertension was attenuated by tezosentan without systemic hypotension. Tezosentan increased CO without changing ventilation-perfusion matching. Both bosentan and tezosentan reduced ET-1 and cytokine plasma levels and tissue expression, and inducible NOS and COX-2 RV activities. Bosentan increased nitrate plasma levels and non inducible NOS activities whereas tezosentan decreased circulating 6-keto-PGF1α but increased lung COX-1 activity. *Conclusions:* Tezosentan may be useful for haemodynamic handling and bosentan replacement in critically ill PH patients exerting important beneficial neuroendocrine and anti-inflammatory actions.

Keywords Right ventricle · Endothelin-1 antagonism · Cytokines · Nitric oxide · Cyclooxygenases · Pulmonary hypertension

Introduction

Pulmonary hypertension (PH), the most serious chronic disease of the pulmonary circulation, defined by mean pulmonary artery pressure greater than 25 mmHg, consists of an heterogeneous group of disorders characterized by vascular remodelling that leads to right ventricular (RV) failure. Currently, combined lung arteries vasodilators are the mainstay of treatment [1, 2]. Regrettably, acute therapy, which is of major importance to critical care practice, has been poorly investigated [3]. One of the most successful therapeutic approaches to PH, endothelin-1 (ET-1) blockade [4], is mostly restricted to chronic oral administration, and therefore is of limited utility. Tezosentan, a dual ET-1 antagonist, however, was developed for intravenous use and optimized to be rapidly-acting and short-lived, [5] and therefore is suited for fine dose adjustment to a desired haemodynamic effect. Moreover, it has advantages over the cumbersome inhaled therapies. Nevertheless, most lung vessel vasodilators are negative inotropes, either by direct myocardial actions, systemic hypotension, decreased coronary perfusion [6] or simply by reducing afterload [7]. Additionally, disturbances in ventilation-perfusion (VQ) matching are also usual, and could lead to hypoxia [8]. Endothelin-1 is a strong positive inotrope [9] but when chronically activated, as it happens in the failing heart, this supportive role is lost [10], suggesting ET-1 blockade could actually improve performance. As for VQ matching, acute ET-1 antagonism improved alveolar-arterial O_2 difference in lung injury [11] and did not disturb VQ matching in pulmonary thromboembolism [12]. Tezosentan has been found beneficial in acute experimental PH [13, 14] and also in an experimental model of left–right shunt in the newborn lamb [15], but a characterization of its pulmonary and myocardial effects in adult experimental chronic PH is lacking. Additionally, the myocardial and pulmonary vascular mechanisms involved in ET-1 blockade and whether tezosentan can be an effective replacement therapy for bosentan is also undefined. Our goal was to evaluate the haemodynamic, local myocardial and pulmonary, and systemic neuroendocrine effects of acute ET-1 antagonism with tezosentan in chronic PH induced by monocrotaline (MCT) in rats and to assess whether these were altered by previous chronic therapy with bosentan.

Methods

Animal model

Male Wistar rats, 180–200 g (Charles-River, Barcelona, Spain), randomly received 60 mg kg^{-1} MCT (Sigma Chemical, St Louis, MO) or vehicle subcutaneously administered. Randomly, 48 h later, some MCT-injected animals were gavaged 300 mg kg^{-1} day^{-1} bosentan (30 mg mL^{-1} in 5% gum Arabic; kindly provided by Actelion pharmaceuticals) (MCT BOS; $n = 46$), while others (MCT; $n = 125$) and controls (Ctrl; $n = 23$) received vehicle. Rats were housed five per cage under controlled environment (22°C, 12:12 h light–dark). Experiments conformed to the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Pub.No. 85-23, revised 1996).

Haemodynamics

After bosentan or vehicle interruption for 48 h, at days 25–30, rats were anaesthetized with sevoflurane (2.5–3% for maintenance) and 150 μg kg^{-1} intraperitoneal fentanyl, endotracheally intubated, mechanically ventilated (150 min^{-1}, 100% O_2, 14–16 cmH_2O inspiratory pressure, with tidal volume adjusted to animal weight, and 4 cmH_2O end-expiratory pressure; TOPO Small Animal Ventilator, Kent Scientific Inc.) and kept at 38°C on a heating pad. Warm Ringer's solution (30 mL kg^{-1} h^{-1}) was perfused through the femoral vein (Multi-Phaser™, NE-1000, New Era Pump Systems). After left thoracotomy, under surgical microdissection (Wilde M651, Leica microsystems), pressure–volume (PV) catheters were implanted through the apex along the left ventricle (LV) and RV (SPR-838 and PVR-1045, Millar Instruments, Houston, TX, respectively) long axes, a transit-time flow-probe was placed in the ascending aorta (200–367, Triton Technology), and a silk thread was passed around the inferior vena cava (IVC). Arterial blood gas (ABG) samples were collected (Stat Profile pHOx®, Nova Biomedical) from the femoral artery and ventilation was adjusted to achieve normocapnia. After a 15 min stabilization, recordings were done under constant haemodynamics and during transient IVC occlusion at sustained end-expiration. Parallel conductance and field inhomogeneity were estimated by 50 μL 10% saline injections and cardiac output (CO) measurement (Active Redirection Transit Time Flowmeter, System 6, Triton Technology), respectively. Data were continuously acquired (MPVS 300, Millar Instruments), digitally recorded at 1000 Hz (ML880 PowerLab 16/30, Millar Instruments), and analyzed (PVAN 3.5™, Millar Instruments). First, we performed a dose–response haemodynamic evaluation ($n = 7$ each group) of 0.5, 1, 5, 10, and 20 mg kg^{-1} intravenous tezosentan doses (kindly provided by Actelion pharmaceuticals; freshly dissolved in saline). Based on this assessment, an additional set of rats randomly received either an intravenously administered 20 mg kg^{-1} dose followed by 10 mg kg^{-1} h^{-1} tezosentan perfusion (TEZO; $n = 8$ per group), or saline (Vehicle; $n = 8$ per group). Haemodynamic recordings and ABG collection were repeated 1 h after stable effect.

A 2 mL venous sample was withdrawn, centrifuged and stored (−20°C) after 4 h. Animals were then euthanized by exsanguination under anaesthesia. Heparinised blood was used for volume calibration using a cuvette with standard wells. The heart, the RV, the LV and interventricular septum (IVS), and the lungs were weighed, snap frozen in liquid nitrogen and stored at −80°C. A flowchart with experimental methods can be found in Supplemental Fig. 5.

Gene expression

Total mRNA extracted from lung and RV free-wall underwent two-step real-time reverse transcription-polymerase chain reaction as described [16]. Results, normalized for β-actin, are presented relative to Ctrl Vehicle. Primers are given in Supplemental Table 1.

Enzymatic activity

Cyclooxygenase (COX) and nitric oxide synthase (NOS) activities (760151 and 760871, Cayman Chemical Company, respectively) were quantified in homogenates of equal amounts of each sample with and without specific COX-2, DuP-697 (70645, Cayman Chemical Company), and inducible NOS (iNOS), aminoguanidine (396494, Sigma-Aldrich), inhibitors. Background activity was assessed by heat-inactivation. Preliminary ultrafiltration was carried out in the NOS assay to concentrate proteins and eliminate tissue nitrates (Amicon® Ultra 30K, Millipore).

Plasma mediators

Circulating ET-1 (S-1171, Peninsula laboratories), interleukin-6 (IL-6; DE4845, Demeditec diagnostics GmbH) and tumor necrosis factor-α (TNF-α; 45-TNFRTU-E01, Alpco Diagnostics™) were quantified by quantitative enzyme immunoassay (UVM-340 monochromator based reader, ASYS Hitech GmbH). For ET-1 extraction was performed with Sep-Pak C_{18} columns (Waters). Nitrates and 6-keto-$PGF_{1\alpha}$, stable nitric oxide (NO) and prostacyclin (PGI_2) metabolites, respectively, were also quantified (Cat. No. 760871 and Cat. No. 515211, Cayman Chemical Company, respectively) after removal of large molecular weight proteins by ultrafiltration (Amicon® Ultra 30K, Millipore).

Statistical analysis

Analysis by two-way repeated-measures ANOVA for dose–response, two-way ANOVA for haemodynamics, ABG, gene expression, neuroendocrine and enzyme activities, and paired t test for perfusion, with Holm-Sidak's method for post hoc comparisons. ANOVA on ranks was used for non-normally distributed data. Chi-square was used to compare mortality. Two-tailed $P < 0.05$. Variables: mean ± SEM.

Results

Animal model

Mortality rates up to the point of haemodynamic evaluation were 77 and 43% ($P < 0.001$), in MCT and MCT BOS, respectively, and six and three additional rats were lost during haemodynamic evaluation. None of Ctrl animals died during follow-up or haemodynamic evaluation. Monocrotaline and MCT BOS presented lower body weights. MCT showed increased RV weight and RV to LV + IVS weight ratio which was attenuated in MCT BOS. No differences were observed between TEZO and Vehicle (Supplemental Table 2).

Dose–response evaluation

Monocrotaline and MCT BOS showed increased RV maximal pressures that were dose-dependently reduced by TEZO, while no effect was observed in Ctrl. Distinctly, TEZO only reduced LV pressure in Ctrl, not in MCT or MCT BOS. MCT however had lower LV maximal pressures compared with both Ctrl and MCT BOS (Supplemental Fig. 6). Higher doses elicited no additional effects (not shown).

Haemodynamic effects of tezosentan

Baseline and IVC occlusion derived haemodynamic parameters are given in Tables 1 and 2, respectively (representative PV loops presented in Supplemental Fig. 7). Compared with Ctrl, MCT showed not only PH and RV afterload (Table 1), as assessed by maximal RV pressure and arterial elastance (E_A), respectively, but also lower heart rate (HR), decreased CO (Fig. 1a), compromised RV ejection fraction (EF), disturbed relaxation, as assessed by τ (Table 1), and diastolic dysfunction, evidenced by an upward shift of the end-diastolic PV relationship (Table 2). Monocrotaline BOS showed an overall improvement compared with vehicle-treated MCT. Acute tezosentan perfusion also decreased maximal RV pressure and E_a both in MCT and MCT BOS, compared with vehicle perfusion, whereas CO (Fig. 1a), EF, τ and end-diastolic PV relationships were only improved in MCT and not in MCT BOS. The slope of end-systolic

Table 1 Haemodynamics before and after vehicle and tezosentan perfusion

	Before			After		
	Ctrl	MCT	MCT BOS	Ctrl	MCT	MCT BOS
Vehicle						
RV						
P_{max} (mmHg)	34.6 ± 1.5	56.8 ± 3.9*	43.5 ± 3.8*†	30.8 ± 1.6	55.8 ± 2.5*	43.7 ± 4.3*†
EDP (mmHg)	2.6 ± 0.6	4.4 ± 0.4*	3.4 ± 1.0	3.1 ± 1.2	3.8 ± 0.4	3.2 ± 0.8
EDV (μL)	229 ± 22	279 ± 32*	239 ± 39	226 ± 23	281 ± 29	259 ± 43
EF (%)	68 ± 5	31 ± 4*	43 ± 4*†	66 ± 5	30 ± 4*	45 ± 4*†
τ (ms)	8.88 ± 0.58	10.03 ± 0.77*	9.85 ± 0.79	9.76 ± 0.43	10.57 ± 0.60	9.65 ± 1.17
E_A (mmHg μL^{-1})	0.24 ± 0.03	0.82 ± 0.17*	0.54 ± 0.09*†	0.23 ± 0.03	1.02 ± 0.05*	0.44 ± 0.07*†
LV						
P_{max} (mmHg)	128.0 ± 2.4	98.0 ± 7.7*	122.3 ± 9.4†	122.9 ± 2.3*	98.5 ± 7.4*	121.4 ± 7.6†
EDP (mmHg)	4.5 ± 0.8	5.4 ± 0.9	4.2 ± 0.6	3.7 ± 0.6	5.7 ± 1.3	6.4 ± 0.9
EDV (μL)	244 ± 28	194 ± 31*	180 ± 19	232 ± 22	191 ± 24	209 ± 32
EF (%)	62 ± 3	51 ± 11*	53 ± 5	64 ± 5	47 ± 4*	46 ± 3*
τ (ms)	6.99 ± 0.22	8.90 ± 0.29*	7.86 ± 0.56†	7.44 ± 0.44	8.70 ± 0.61*	8.80 ± 0.84
E_A (mmHg μL^{-1})	0.93 ± 0.09	1.37 ± 0.36*	1.28 ± 0.17*	0.89 ± 0.09	1.28 ± 0.22*	1.35 ± 0.20*
HR (min^{-1})	429 ± 15	384 ± 23*	419 ± 11	419 ± 12	372 ± 16*	399 ± 17
Tezosentan						
RV						
P_{max} (mmHg)	33.4 ± 0.9	60.3 ± 4.2*	52.1 ± 5.4*†	28.6 ± 11.1‡	49.7 ± 2.5*‡	42.3 ± 2.6*‡
EDP (mmHg)	2.4 ± 0.4	4.2 ± 0.3*	3.9 ± 0.8	1.7 ± 0.4	3.1 ± 0.4‡	2.6 ± 0.7‡
EDV (μL)	239 ± 14	289 ± 11*	231 ± 30	248 ± 20	274 ± 40	256 ± 32
EF (%)	68 ± 3	32 ± 3*	48 ± 3*†	63 ± 4	48 ± 4*‡	45 ± 3*
τ (ms)	8.85 ± 0.47	11.32 ± 0.44*	9.78 ± 0.99	8.57 ± 0.50	9.76 ± 0.44‡	9.67 ± 1.17
E_A (mmHg μL^{-1})	0.21 ± 0.01	0.77 ± 0.10*	0.60 ± 0.10*†	0.20 ± 0.01	0.45 ± 0.05‡	0.40 ± 0.06‡
LV						
P_{max} (mmHg)	124.8 ± 2.6	98.7 ± 3.2*	125.7 ± 8.5†	110.9 ± 6.6‡	94.7 ± 3.3*	116.9 ± 7.5†
EDP (mmHg)	4.0 ± 0.7	5.1 ± 0.7	5.3 ± 0.8	4.6 ± 0.5	4.2 ± 0.5	4.4 ± 0.7
EDV (μL)	247 ± 17	189 ± 9*	213 ± 22	234 ± 24	202 ± 20	230 ± 30
EF (%)	64 ± 3	49 ± 5*	52 ± 6	66 ± 4	58 ± 3‡	52 ± 3
τ (ms)	7.38 ± 0.17	9.96 ± 0.78*	8.33 ± 0.53†	7.55 ± 0.39	9.35 ± 0.56*	8.59 ± 0.59
E_A (mmHg μL^{-1})	0.83 ± 0.04	1.16 ± 0.15*	1.25 ± 0.10*	0.69 ± 0.06‡	0.83 ± 0.10‡	1.01 ± 0.11‡
HR (min^{-1})	409 ± 13	354 ± 13*	399 ± 23	404 ± 16	338 ± 17*	383 ± 26

Right (RV) and left ventricular (LV) haemodynamic parameters in vehicle-injected (Ctrl), monocrotaline-injected (MCT) and monocrotaline-injected bosentan-treated rats (MCT BOS)

P_{max} maximal or systolic pressure, *EDP* end-diastolic pressure, *EDV* end-diastolic volume, *EF* ejection fraction, τ time constant of isovolumetric relaxation, E_A arterial elastance, *HR* heart rate

* $P < 0.01$ versus Ctrl and † $P < 0.05$ versus MCT on two-way ANOVA; ‡ $P < 0.01$ versus before on paired *t* test; $n = 8$ per group

PV relationship and preload recruitable stroke work (PRSW), load-independent indexes of contractility, were increased in MCT compared with Ctrl, and were neither attenuated by bosentan nor tezosentan. Since end-systolic PV relationship slope was preserved in both MCT BOS and MCT TEZO while E_A decreased compared with MCT, ventriculo-vascular coupling improved (Fig. 1b). As for the LV, MCT showed reduced maximal pressures, reduced end-diastolic volume and EF along with prolonged τ (Table 1) compared with Ctrl, while MCT BOS did not. Tezosentan perfusion also increased LV end-diastolic volumes and EF in MCT whereas in Ctrl it decreased maximal pressure. MCT showed upward-shifted LV end-diastolic PV relationships that were restored to normal both by chronic bosentan and by acute tezosentan therapy while PRSW was unchanged both in MCT compared with Ctrl or by chronic and acute ET-1 blockade (Table 2).

Respiratory effects of tezosentan

Animals were normoventilated and maintained acid–base balance throughout perfusion. Arterial O_2 pressure under stable ventilation was used as surrogate of oxygenation. Both MCT and MCT BOS showed decreased arterial O_2 pressures that were not altered by TEZO (Supplemental Table 3).

Effects of tezosentan on endothelin-1 and cytokine production

Monocrotaline showed increased plasma levels of ET-1, IL-6 and TNF-α (Fig. 2c), that were accompanied by increased local expression of TNF-α and ET-1 in the RV (Fig. 2b) and TNF-α and IL-6 in the lung (Fig. 2a). These were partly prevented by chronic ET-1 antagonism and

Table 2 Inferior vena cava occlusions before and after vehicle and tezosentan perfusion

	Before			After		
	Ctrl	MCT	MCT BOS	Ctrl	MCT	MCT BOS
Saline						
RV						
EDPVR (exponential)						
k_1	0.006 ± 0.001	0.015 ± 0.003*	0.011 ± 0.003	0.007 ± 0.001	0.016 ± 0.004*	0.010 ± 0.003
k_2	1.24 ± 0.29	0.25 ± 0.10	0.68 ± 0.21	0.92 ± 0.22	0.33 ± 0.20	0.90 ± 0.46
ESPVR (linear)						
Slope (E_{max}), (mmHg μL^{-1})	0.22 ± 0.04	0.50 ± 0.09*	0.50 ± 0.07*	0.24 ± 0.03	0.56 ± 0.12*	0.45 ± 0.09*
Intercept (μL)	−74.5 ± 34.8	71.5 ± 42.1*	33.2 ± 26.1*	7.0 ± 15.5	87.0 ± 37.9*	43.3 ± 11.47*
PRSW (mmHg)	20.8 ± 2.9	27.6 ± 3.3*	29.4 ± 3.4*	17.0 ± 2.4	28.6 ± 5.0*	29.4 ± 2.7*
LV						
EDPVR (exponential)						
k_1	0.015 ± 0.004	0.025 ± 0.005*	0.014 ± 0.003†	0.013 ± 0.003	0.036 ± 0.003*	0.011 ± 0.002†
k_2	0.92 ± 0.47	2.70 ± 1.02	1.07 ± 0.53	2.69 ± 1.31	1.72 ± 0.73	1.09 ± 0.48
ESPVR (linear)						
Slope (mmHg μL^{-1})	0.97 ± 0.23	2.42 ± 0.55*	1.89 ± 0.15	1.18 ± 0.24	2.52 ± 0.62*	1.76 ± 0.10
Intercept (μL)	−28.4 ± 52.2	41.4 ± 17.8	39.9 ± 4.8	−35.0 ± 21.4	14.7 ± 12.0	23.5 ± 4.5
PRSW (mmHg)	105.6 ± 22.2	126.5 ± 27.1	124.9 ± 11.7	106.8 ± 16.0	106.2 ± 7.6	144.4 ± 8.6
Tezosentan						
RV						
EDPVR (exponential)						
k_1	0.007 ± 0.002	0.015 ± 0.002*	0.013 ± 0.002	0.007 ± 0.001	0.012 ± 0.002‡	0.013 ± 0.002
k_2	0.84 ± 0.17	0.25 ± 0.06	0.90 ± 0.54	0.81 ± 0.20	0.60 ± 0.11‡	0.40 ± 0.22
ESPVR (linear)						
Slope (E_{max}) (mmHg μL^{-1})	0.21 ± 0.04	0.50 ± 0.06*	0.60 ± 0.03*	0.22 ± 0.04	0.52 ± 0.06*	0.47 ± 0.07*
Intercept (μL)	−58.2 ± 51.1	45.9 ± 20.2*	83.3 ± 17.1*	−13.2 ± 20.4	24.1 ± 9.6*	76.3 ± 35.7*
PRSW (mmHg)	17.6 ± 1.8	29.5 ± 2.6*	34.4 ± 1.4*	15.0 ± 2.0	33.6 ± 3.6*	34.5 ± 3.9*
LV						
EDPVR (exponential)						
k_1	0.012 ± 0.002	0.023 ± 0.002*	0.013 ± 0.004†	0.013 ± 0.002	0.012 ± 0.001‡	0.009 ± 0.003‡
k_2	0.85 ± 0.24	0.38 ± 0.32	0.88 ± 0.37	0.67 ± 0.28	1.15 ± 0.18‡	1.04 ± 0.37‡
ESPVR (linear)						
Slope (mmHg μL^{-1})	1.04 ± 0.14	2.11 ± 0.50*	1.56 ± 0.28	0.94 ± 0.12	2.25 ± 0.74*	1.70 ± 0.16
Intercept (μL)	−21.2 ± 33.8	3.9 ± 24.8	44.4 ± 39.2	−25.5 ± 25.3	9.6 ± 22.6	38.5 ± 12.7
PRSW (mmHg)	104.7 ± 17.8	121.3 ± 8.8	110.8 ± 17.7	130.9 ± 25.8	136.4 ± 17.5	121.3 ± 19.6

Right (RV) and left ventricular (LV) load-independent indexes derived from inferior vena cava occlusions in vehicle-injected (Ctrl), monocrotaline-injected (MCT) and monocrotaline-injected bosentan-treated rats (MCT BOS)

EDPVR end-diastolic pressure–volume relationship, k_1 and k_2, indexes of exponential function, *ESPVR* end-systolic pressure–volume relationship, E_{max} maximal elastance, *PRSW*, slope of preload recruitable stroke work

* $P < 0.01$ versus Ctrl and † $P < 0.05$ versus MCT on two-way ANOVA; ‡ $P < 0.01$ versus before on paired *t* test; $n = 8$ per group

markedly attenuated with acute antagonism in MCT BOS and MCT TEZO, respectively. Tezosentan also reduced plasma levels of TNF-α and IL-6 in MCT BOS, whereas it raised ET-1 RV expression and plasma levels in Ctrl compared with Vehicle.

Effects of tezosentan on prostaglandin and NO production

PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ was increased both in MCT and MCT BOS compared with Ctrl, which was attenuated after TEZO perfusion, compared with Vehicle (Fig. 3c). Contrastingly, TEZO increased 6-keto-$PGF_{1\alpha}$ in Ctrl. These plasma changes were accompanied by increased RV activities of both COX-1 and -2 and also by increased gene expression of COX-1 in MCT, that were attenuated by chronic and acute ET-1 antagonism in MCT BOS and MCT TEZO, respectively. While gene expression of COX-2, contrarily, was decreased in MCT with no changes after either BOS or TEZO (Fig. 3b). As for the lung, no changes were observed in either the expression or enzymatic activity of COX-2, whereas COX-1 showed increased gene expression in MCT, that was attenuated by either chronic or acute ET-1 antagonism, and markedly decreased activity both in MCT and MCT BOS, which was attenuated only by acute ET-1 antagonism in MCT TEZO (Fig. 3a). Plasma levels of nitrates were lower in MCT and restored to Ctrl values or higher in MCT BOS. Tezosentan had no effect compared with Vehicle (Fig. 4c). Plasma changes were paralleled by lower non-inducible NOS activity in the lung (Fig. 4a) and RV

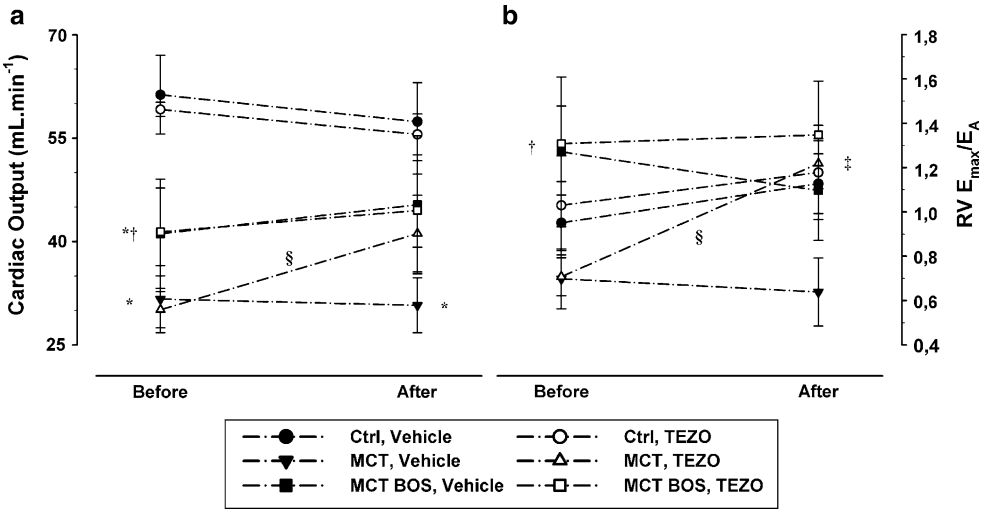

Fig. 1 Cardiac output (**a**) and right ventricular (RV) ventriculo-vascular coupling (**b**) before and after perfusion of saline (Vehicle) or tezosentan (TEZO) in vehicle-injected (Ctrl), monocrotaline-injected (MCT) and monocrotaline-injected bosentan-treated rats (MCT BOS); Ctrl (*circular* symbols), MCT (*triangular* symbols) and MCT BOS (*quadrangular* symbols) were given either a 20 mg kg^{-1} TEZO intravenous loading dose during 10 min, followed by a perfusion of 10 mg kg^{-1} h^{-1} (*white* symbols) or the corresponding volume of Vehicle (*black* symbols). E_{max} maximal elastance, E_A arterial elastance. $^{*}P < 0.01$ versus Ctrl, $^{\dagger}P < 0.05$ versus MCT and $^{\ddagger}P = 0.028$ versus Vehicle on two-way ANOVA; $^{\S}P < 0.001$ vs before on paired *t* test; $n = 8$ per group

(Fig. 4b) of MCT that was attenuated by chronic ET-1 antagonism in MCT BOS but only abrogated in the RV, not in the lung, by TEZO, whilst iNOS was overactive both in the lungs and RV of MCT. Tezosentan markedly attenuated both lung and RV activities whereas BOS only attenuated RV activity. As for gene expression, while in the RV no changes were observed in either eNOS or iNOS, in the lung eNOS was upregulated in MCT and MCT BOS, with no change after TEZO, and iNOS was downregulated in MCT, which was attenuated both by short-term and chronic ET-1 antagonism.

Discussion

We demonstrate that an acute intravenous infusion of the short acting dual ET-1 antagonist tezosentan attenuates PH, without compromising VQ matching or systemic pressure and even improving CO and ventriculo-vascular coupling, while concomitantly blunting inflammatory and vasoconstrictor mediator production in chronic experimental PH induced by MCT in rats. Part of these effects were also observed after previous therapy with bosentan.

Monocrotaline-induced PH is a well-established model with extensive neuroendocrine and inflammatory activation that rapidly progresses to RV failure [16]. In MCT we observed PH and increased afterload that were accompanied not only by RV hypertrophy but also by disturbed ventriculo-vascular coupling, RV dilation, decreased EF and CO, and compromised diastolic function. As for the LV, MCT showed decreased end-diastolic volumes, compromised diastolic function and lower LV maximal pressures, as expected by ventricular interaction [17]. Regarding gas exchange, under normoventilation MCT showed lower arterial O_2 pressures as expected [18]. Also as described, circulating levels of ET-1 and RV gene expression were increased [16], which was accompanied by marked inflammatory activation, as assessed by TNF-α and IL-6 gene expression and plasma concentrations [19]. Pulmonary hypertension is an inflammatory condition with endothelial dysfunction and loss of vascular control mechanisms. The imbalance between vasodilators and vasoconstrictors, namely an altered local ratio of thromboxane A_2 (TxA_2) to PGI_2, decreased expression of eNOS, and increased ET-1 plays an important role in its pathophysiology [20]. Inflammatory activation also compromises myocardial function. As reported, plasma NO levels and lung activity of eNOS were reduced in MCT-induced PH [21], while PGI_2 production was increased [22], which could be due to concomitant heart failure (HF), with platelet activation and systemic production [23], since it is normally decreased in human PH [24]. Nevertheless, prostanoid lung synthesis was compromised, since COX-1 activity was markedly reduced, as observed in hypoxic pigs that showed decreased PGI_2 to TxA_2 ratio [25]. On the other hand, in the RV of MCT, we observed high iNOS and inducible COX-2 activation. iNOS mediates the negative inotropic effects of cytokines, generating high NO quantities that profoundly depress myocardial function [26], while COX-2 produces high levels of prostanoids, mediating TNF-α responsiveness through TxA_2 production [27], and was shown to be upregulated in the failing heart

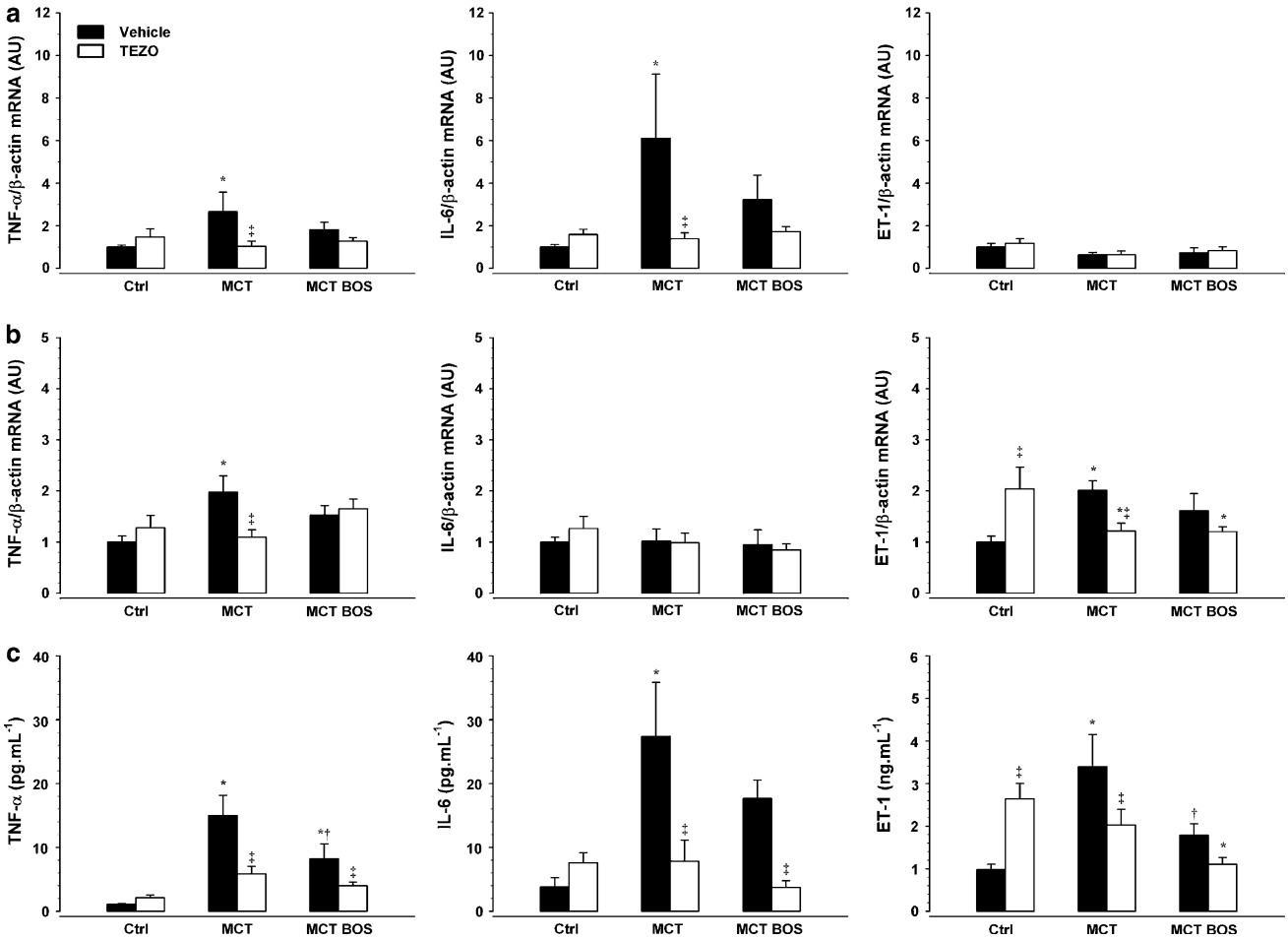

Fig. 2 Lung (**a**) and right ventricular myocardium gene expression (**b**) of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and endothelin-1 (ET-1) and corresponding plasma levels (**c**) in vehicle-injected (Ctrl), monocrotaline-injected (MCT) and monocrotaline-injected bosentan-treated rats (MCT BOS) undergoing either perfusion of vehicle (*black bars*) or tezosentan (TEZO; *white bars*); gene expression was normalized for β-actin and is presented in an arbitrary unit (AU), set as the average of Ctrl Vehicle. $^{*}P < 0.05$ versus Ctrl, $^{\dagger}P < 0.05$ versus MCT, and $^{\ddagger}P < 0.05$ versus vehicle; $n = 7$ per group

[28]. Additionally, in the failing MCT RV we also observed decreased eNOS and increased COX-1 activities. eNOS, the major NO source in the normal heart, is reduced in HF and restoration of its activity exerts protective actions [29], whereas COX-1 is typically increased by oxidative stress and its antagonism exerts beneficial effects [30].

Right ventricle disturbances were attenuated in MCT BOS and ventriculo-vascular coupling was improved, as reported [31]. Systemic hypotension, as assessed by LV maximal pressures, was not observed which could be not only due to restored LV preload and reduced ventricular interaction but also to improved myocardial function [16] and preserved CO. Chronic ET-1 antagonism did not alter overall VQ matching. Contrarily to earlier stages of disease [32], chronic ET-1 antagonism reduced plasma concentration and RV expression of ET-1 in MCT, probably due to attenuation of PH [16, 31] and not to ET-1 antagonism itself [5]. Indeed, plasma ET-1 levels increased in Ctrl after tezosentan. Alongside haemodynamic benefits and reduced ET-1 activity, MCT BOS also showed increased NO plasma levels and eNOS lung activity, as reported for bosentan-treated PH patients [33].

Nevertheless, our main goal was to evaluate the effects of the short-acting intravenous dual ET-1 antagonist tezosentan in chronic PH. Since perfusion doses were not previously established for this model we carried out a dose–response evaluation. Tezosentan dose-dependently reduced RV maximal pressures up to 20 mg kg^{-1}, doses higher than previously described [34], without significant reduction of LV pressure, not only in MCT but also in MCT BOS, suggesting an additional benefit of acute ET-1 blockade even after chronic bosentan. Based on previous works that have shown molecular changes as soon as 4 h after drug perfusion [35], we then evaluated haemodynamic, respiratory and neuroendocrine responses to

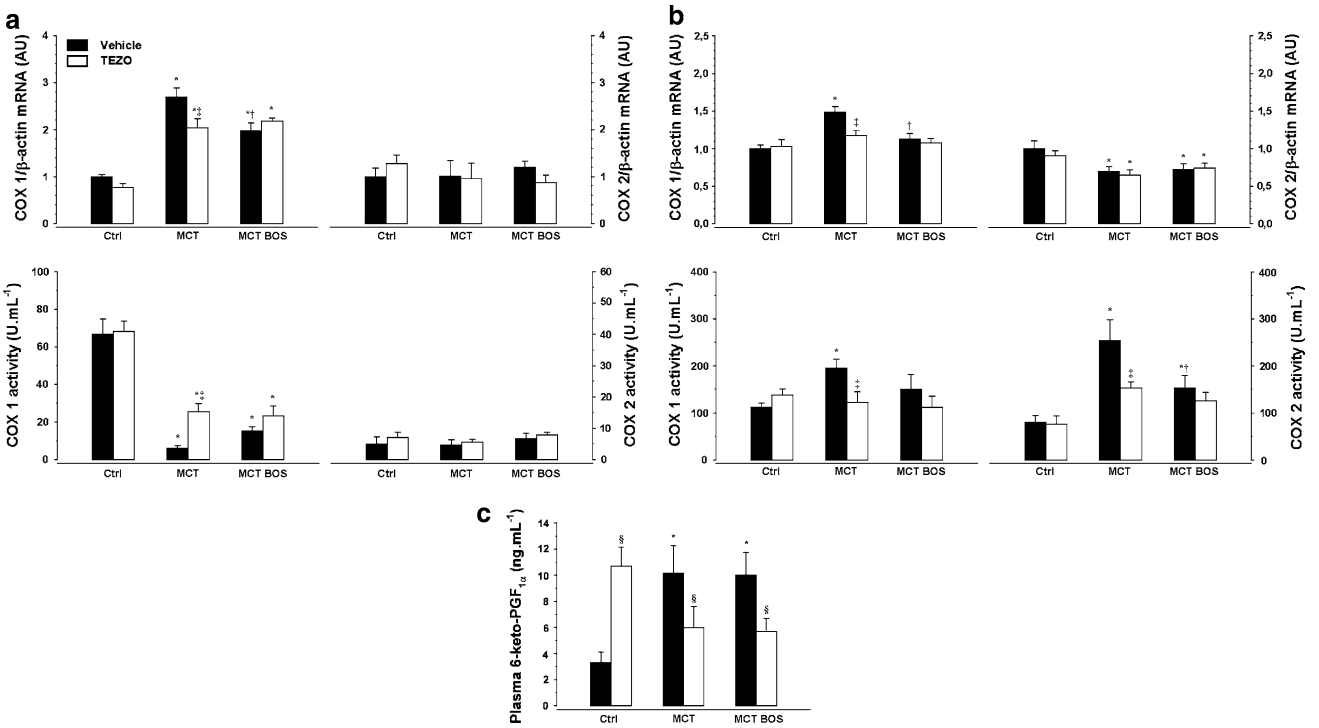

Fig. 3 Lung (**a**) and right ventricular myocardium (**b**) cyclooxygenase gene expression and enzymatic activity and 6-keto-$PGF_{1\alpha}$ plasma concentrations (**c**); gene expression and enzymatic activities of cyclooxygenase-1 (COX 1) and -2 (COX 2) and plasma concentrations of 6-keto-$PGF_{1\alpha}$ in vehicle-injected (Ctrl), monocrotaline-injected (MCT) and monocrotaline-injected bosentan-treated rats (MCT BOS) given vehicle (*black bars*) or tezosentan (TEZO, *white bars*). Gene expression was normalized for *β*-actin and is presented in an arbitrary unit (AU), set as the average of Ctrl Vehicle. $^{*}P < 0.05$ versus Ctrl, $^{\dagger}P < 0.05$ versus MCT, and $^{\ddagger}P < 0.05$ versus vehicle; $n = 7$ per group

tezosentan. While vehicle had no effects, tezosentan attenuated PH and RV afterload, both in MCT and MCT BOS. Ventriculo-vascular coupling, EF and CO, however, were only improved in MCT. Similar to chronic ET-1 antagonism, tezosentan did not disturb VQ matching. Moreover, tezosentan perfusion was also able to acutely reduce circulating levels and RV expression of ET-1 in MCT, as observed after chronic bosentan. Both chronic and acute dual ET-1 antagonism with bosentan and tezosentan, respectively, attenuated inflammation in MCT, namely TNF-α and IL-6 circulating concentrations and tissue expression, which could be due not only to direct ET-1 antagonism [36], but also to the beneficial haemodynamic effects and improved CO and thus better tissue perfusion. Additionally, it should be mentioned that the anti-inflammatory effects of ET-1 antagonism might have been partly responsible for improved haemodynamics and lower ET-1 activity after acute and chronic ET-1 antagonism, as cytokines also induce ET-1 production [37]. Indeed, we observed a marked attenuation of inflammation induced iNOS and COX-2 activation in the RV of MCT after either acute tezosentan perfusion or chronic bosentan therapy. The fast haemodynamic, neuroendocrine and anti-inflammatory effects observed with tezosentan are not surprising, since it has been successful in experimental septic shock and acute lung injury [11]. As additional effect, tezosentan increased COX-1 activity in the lungs of MCT. Although ET-1 induces the expression of COX-1 [38], we interpret this upregulation of COX-1 activity after ET-1 blockade as a consequence of decreased pulmonary vascular load and increased flow, as observed in cell culture [39]. Anti-inflammatory and COX-mediated effects have been recently described also for levosimendan in chronic experimental PH [40].

Concerning the effects of acute ET-1 antagonism in PH animals that had previously undergone chronic ET-1 blockade. We must stress that even though bosentan was withdrawn for 48 h its molecular effects surely will last longer and therefore tezosentan was not expected to exert major actions. Still, although we found no amelioration in CO, we did observe reduced PH, improved ventriculo-vascular coupling and further anti-inflammatory effects, which may support its use as a replacement drug.

To conclude, we have demonstrated in chronic experimental MCT-induced PH, that acute ET-1 antagonism with tezosentan attenuates Pulmonary hypertension and improves CO with concomitant reduction of ET-1 and inflammatory cytokine levels, and increased vasodilator production. Part of these beneficial effects were also observed after previous chronic ET-1 antagonism with

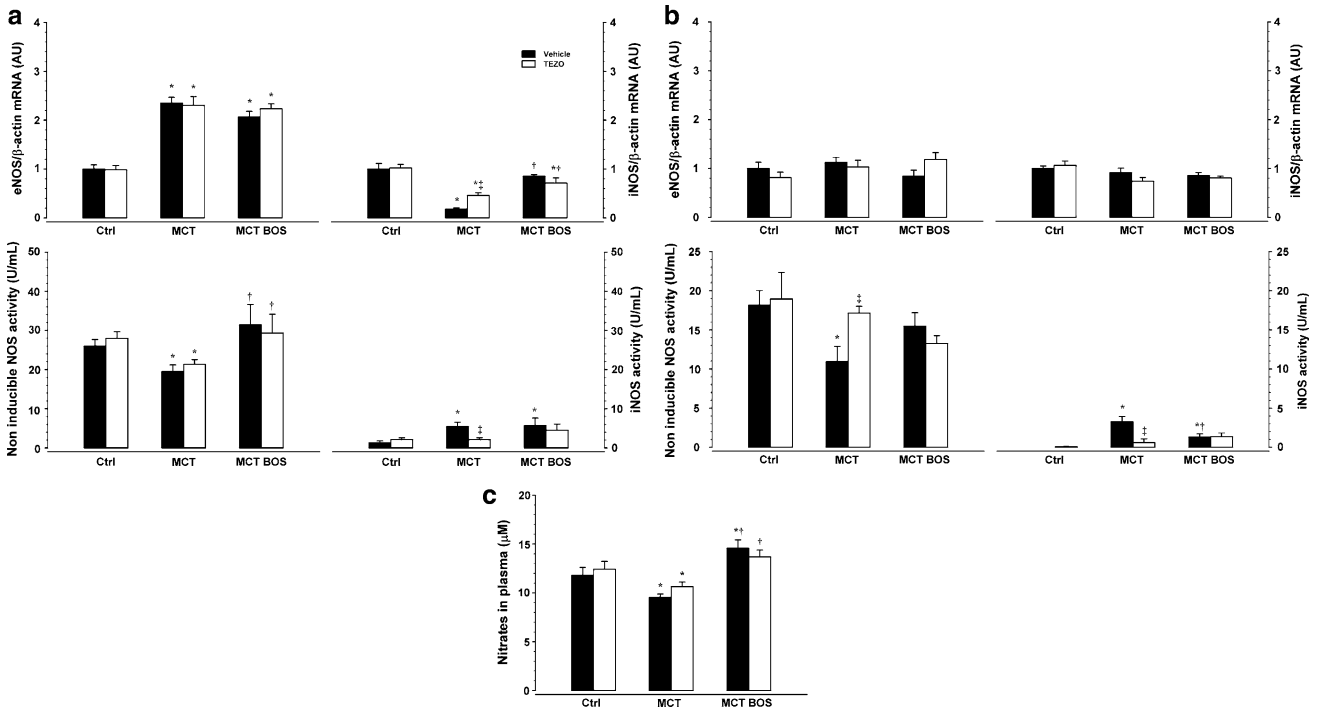

Fig. 4 Lung (**a**) and right ventricular myocardium (**b**) nitric oxide synthase gene expression and enzymatic activity and plasma concentrations of nitrates (**c**); gene expression of inducible (iNOS) and endothelial nitric oxide synthases (eNOS) and enzymatic activities of iNOS and non inducible NOS in vehicle-injected (Ctrl), monocrotaline-injected (MCT) and monocrotaline-injected bosentan-treated rats (MCT BOS) given vehicle (*black bars*) or tezosentan (TEZO, *white bars*). Gene expression was normalized for β-actin and is presented in an arbitrary unit (AU), set as the average of Ctrl Vehicle. $^{*}P < 0.05$ versus Ctrl, $^{\dagger}P < 0.05$ versus MCT, and $^{\ddagger}P < 0.05$ versus vehicle; $n = 7$ per group

bosentan. Nevertheless, though tezosentan further attenuated PH it did not additionally improve CO in MCT rats previously treated with bosentan. Results suggest tezosentan may be a good replacement drug when the enteric route of administration is not tolerated or a precise real-time control of haemodynamics is required.

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Anexo

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- The study represents the results of primary scientific research.
- Results reported have not been published elsewhere.
- Experiments, statistics, and other analyses are performed according to a high technical standard and are described in sufficient detail.
- Conclusions are presented in an appropriate fashion and are supported by the data.
- Your manuscript must be written in intelligible English.
- The research meets all applicable standards for the ethics of experimentation and research integrity.
- The article adheres to appropriate reporting guidelines and community standards for data availability.
- All conflicts of interest are clearly stated in the manuscript.

If you need any additional details on the following instructions or if you want to submit an outline, please contact the Intensive Care Medicine Head Office at journal.icm@sls.aphp.fr

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- Original papers must not exceed 3,000 words and should not include more than 6 illustrations and tables. Note that each separate part of a figure (a, b, etc ...) counts as an illustration. Up to 40 references are permitted. When reporting the results of a randomized controlled trial, author(s) should use the CONSORT statement as a guide in preparing the manuscript (http://www.consort-statement.org/). If the authors consider that their manuscript needs to be longer than the mentioned limit or contain more figures or tables, they can explain their reasons in the cover letter to the Editor-in-Chief. Complementary information can be published in electronic supplements. Authors of original papers are requested to provide the following information:

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Imaging in intensive care medicine

- For images to be accepted, they must be of high scientific quality and value as well as didactic and self-explanatory. They will need to be perceived as unique and must adhere to ethical standards (patient/relative approval when appropriate, protection of patient identity and privacy, IRB approval when appropriate). The accompanying text should not exceed 200 words.

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- Letters to the editor (Correspondence) address published articles or contain interesting case studies. They must not exceed 500 words, 5 references and 1 figure or table. Correspondence addressing a previously published article is usually sent to the authors for response. The final decision on publication of this correspondence is taken at the end of this process. Letters submitted may be subject to shortening and editorial revision.

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