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Oxidative stress implication in a new *ex-vivo* cardiac concordant xenotransplantation model

JEAN-CHRISTOPHE CHARNIOT[1], DOMINIQUE BONNEFONT-ROUSSELOT[2,3], JEAN-PAUL ALBERTINI[1], KHALED ZERHOUNI[1], SYLVIE DEVER[2], ISABELLE RICHARD[3], PATRICK NATAF[4], ALAIN PAVIE[4], JEAN-JACQUES MONSUEZ[1], JACQUES DELATTRE[2], & JEAN-YVES ARTIGOU[1]

[1]*AP-HP, Hospital Avicenne, Department of Cardiology, Université de Paris XIII, Faculté de medicine de Bobigny, Paris XIII, France,* [2]*Federation of Biochemistry, GH Pitié-Salpêtrière (AP-HP), Paris, France,* [3]*Department of Biochemistry (EA 3617), Faculty of Pharmacy, Paris 5, France, and* [4]*Department of Cardiac Surgery, GH Pitié-Salpêtrière (AP-HP) Hospital, Paris, France*

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Abstract

Xenotransplantation (XT) reveals a growing interest for the treatment of cardiomyopathy. The major barrier is an acute vascular rejection due to an acute humoral rejection. This pathogenesis is a difficult issue and in order to elaborate means for its prevention, we analysed the implication of oxidative stress (OS) on hearts from mini-pigs followed by reperfusion with either autologous or human blood in an attempt to simulate xenotransplantation.

About 14 hearts were studied after a Langendorff blood reperfusion: allografts with autologous blood ($n = 7$) or xenografts with human blood ($n = 7$). Blood samples were drawn from the coronary sinus to assess ischemia and OS.

In xenografts, arrhythmias occurred more frequently ($p < 0.01$, left ventricular systolic pressure decreased more significantly ($p < 0.05$), thiobarbituric acid-reactive substances concentrations increased at 30 min (0.7 ± 0.1 vs. 2.4 ± 0.3 mmol/l; $p < 0.05$) while vitamin A levels decreased ($p < 0.05$).

XT was associated with a significant increase in ischemic injury and OS production. OS might play an eminent role in hyperacute humoral rejection.

Keywords: *Transplantation, reperfusion injury, reactive oxygen species (ROS), ischemia reperfusion*

Introduction

Although reperfusion reduces mortality among patients with acute myocardial infarction, its benefit is partly outweight by the subsequent accelerated myocardial damages induced by the restored blood flow a phenomenon known as the "reperfusion injury" [1–3]. A broad spectrum of mechanisms involved in reperfusion injury has been well-established [4]. It includes the generation of free radicals, reactive oxygen species (ROS) related damage, white blood cell activation, changes in myocyte calcium overload, impairment of microvascular blood flow and sympathetic activation [1,5,6].

Ischemia–reperfusion injury also occurs after cardiac transplantation [7]. Moreover, cardiac transplantation is further limited by donor organ shortage and other limitations related to the condition of heart preservation.

Conversely, xenotransplantation is not limited by such a lack of supply organs and, as a result, may provide an alternative approach for organ transplant.

Correspondence: J.-C. Charniot, Avicenne Hospital-Cardiology, 125 rue de Stalingrad, Bobigny 93009, France. Tel: 33 1 48 95 53 22. Fax: 33 1 48 95 53 88. E-mail: jean-christophe.charniot@avc.aphp.fr

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Unfortunately, severe obstacles compromise XT in clinical practice, including miscellaneous zoonoses, prominent immunological reactions, and finally, subsequent ethical consideration [8]. Cardiac grafts obtained from pigs are the favorite candidate organs for human heart transplantation. However, the immunological characteristics of both species, pigs and humans, whose phylogenic filiation separated in their evolution about 90 million years ago, are quite different, leading to unpresentable rejection reactions [9].

Vascular rejection is one of the most challenging issue in organ xenotransplantation (XT) [10]. Hyperacute and acute vascular mechanisms are involved. Hyperacute rejection is triggering by xenogenic natural antibodies, such as those directed against the antigens of the major histocompatibility complex or blood group antigens [11,12]. Their fixation on the graft antigens activates the recipient complement cascade which, in turn, leads to irreversible graft injuries [13]. Acute vascular rejection is characterized by focal ischemia, endothelial swelling, and intra-vascular coagulation. It occurs within minutes (10–30 min) to several days [14,15]. In addition, other factors may promote vascular disease in organ grafts. Ischemia–reperfusion injury begins soon after transplantation, leading to the recruitment of inflammatory cells, platelet stimulation by activating endothelial cells [16–18] and activation of the complement system through both the classical and the alternative pathways [19].

In this study, myocardial injuries and the involvement of oxidative stress (OS) are compared in discordant XT (human-pig) and allograft in pigs during hyperacute rejection. Circulating levels of several anti-oxidant compounds such as vitamin A, vitamin E, beta carotene were assayed. Indexes of lipid peroxidation and hemodynamic changes (left ventricular pressure, arrhythmias, biological markers of ischemia) were measured together with a pathological analysis during heart transplantation.

Material and methods

This investigation was performed in agreement with the 1996 Guide for the care and use of laboratory and with the Animal Welfare Act.

Experimental procedure

Fourteen mini-pigs (mean weight 30 ± 5 kg) were anesthetized with diazepam (Valium: 10 mg) and Nesdonal (15 mg/kg) and monitored. After tracheal intubation, the anesthesia was maintained by inhalation of 1–2% halothane. An external iliac artery catheter was placed for monitoring pressure and blood sampling. A median sternotomy was performed and the heart was exposed. Baseline graft function was assessed *in situ* before cardiectomy with assays as described below. An intravenous Heparin infusion (300 IU/kg) was administered in the superior vena cava.

Cardiac arrest was obtained after a timed cardioplegic infusion was initiated using an aortic canula at an aortic root pressure 65 mmHg, with the heart vented through the superior vena cava and the right pulmonary veins. Protective cardioplegic solutions at 4°C (Plegisol; Abbott laboratories, North Chicago, IL, USA) were used and composed as follows: sodium 110 mmol/l, potassium 16 mmol/l, chlorite 160 mmol/l, magnesium 16 mmol/l, calcium 1.2 mmol/l. The osmolarity was 260 mOsm/l. Cannulas for the perfusion circuit were inserted into the aortic root and the left atrium. A cannula placed in the pulmonary artery provided direct measurement of the coronary sinus flow. After cardiac arrest, hearts were rapidly excised using a standard procedure and weighed.

Myocardial preservation

To reduce ischemic injury, excised hearts were statically stored for 8 h in a profound hypothermia (4°C), using a B21 solution (Ringer Hartman solution) composed as follows: sodium 130 mmol/l, calcium 1.2 mmol/l, chlorite 160 mmol/l, lactates 28 mmol/l. At low temperatures, the heart metabolism decreases and as the solution does not diffuse into the coronary arteries, the implication of high concentration of potassium (in cardioplegia solution) decreases.

Reperfusion with organetic CCU7 instrument

After 8 h of myocardial preservation, hearts were placed into an organetic CCU7 apparatus (Figure 1). Two groups of mini-pigs were distinguished, depend-

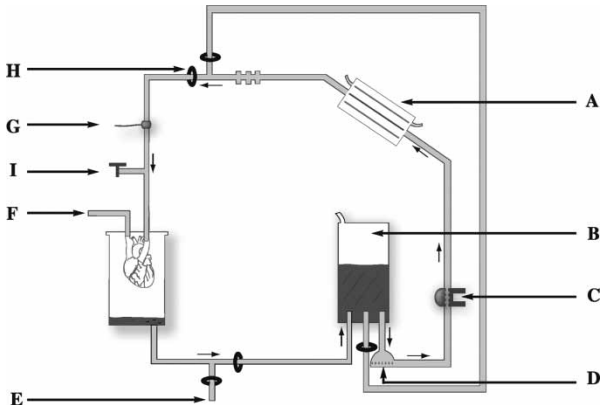

Figure 1. Organetic CCU7 apparatus. A, Oxygenator SCIMED 0.6 pediatric; B, phosphate–dextrose transfusion bags; C, temperature control (DIDECO); D, Biomedicus pump; E, peripheral blood taking; F, catheter in sinus coronary; G, pressure control; H, clamp; I, blood sampling.

Table I. Usual values in human and mini-pigs blood.

	Group I mini-pigs blood	Group II human blood
TBARS (μmol/l)	0.7 ± 0.2	0.9 ± 0.3
Erythrocyte GPx (U/l)	100 ± 20	30 ± 6
Plasma GPx (U/l)	2600 ± 400	470 ± 70
SOD (U/g Hb)	400–700	359–671
Vitamin E (μmol/l)	0.4–1.4	20–37
Vitamin A (μmol/l)	0.3 ± 0.7	1.5–2.6
Creatine kinase (U/l)	1200–1700	25–160
Lactate (mmol/l)	3–6	1–1.8

TBARS, plasma thiobarbituric acid-reactive substances; GPx, Glutathione peroxidase activity; SOD, Superoxide dismutase.

ing on the blood used for reperfusion purpose:

- Group I ($N = 7$): autologous oxygenated blood at 37°C. Blood (1 l) was collected in citrate phosphate–dextrose transfusion bags containing 2 ml of heparin solution, immediately after cardioplegia that induces cardiac arrest.
- Group II ($N = 7$): human oxygenated blood at 37°C. Blood samples were obtained by the blood transfusion center from a single patient suffering from Vaquez disease who was treated with iterative punctions (2 units/sample). In one patient, OS variations of basal values were lesser than in a group of subjects. Moreover this patient was not a smoker, nor diabetic nor received a treatment with any statin or angiotensin converting enzyme inhibitor which may interfere with the OS status. An informed consent was obtained from the patient. A normal hematocrit value was obtained after dilution in a salt solution (NaCl 0.9%).

Before starting reperfusion, electrolyte concentrations were corrected according to physiological normal values of the blood perfusate and repeatedly controlled. Partial pressures of oxygen (95–100 mmHg) and carbon dioxide (25–35 mmHg) were maintained using a membrane oxygenator/heat exchanger SCIMED 0.6 pediatric ventilated with a 95–5% oxygen–CO_2 mixture. Aortic root pressure was maintained at 65 mmHg using a Biomedicus pump (Figure 1).

Myocardial function was repeatedly assessed every 15 min during 3 h. Coronary sinus perfusates were sampled at 5, 30, 60, 120 and 180 min after reperfusion of the hearts placed into the CCU7 organetic instrument.

Oxidative stress evaluation

Blood samples were collected into the coronary sinus OS markers were determined as previously described [20]. Briefly, plasma thiobarbituric acid-reactive substances (TBARS) were assayed using a spectrofluorimetric method after condensation with thiobarbituric acid [21]. Plasma α-tocopherol, vitamin A and β-carotene levels were determined by reverse phase high pressure liquid chromatography (HPLC) [22]. Glutathione peroxidase activity was determined in plasma and erythrocytes by spectrophotometry at 340 nm using the Beutler technique, with *t*-butylhydroperoxide as substrate [23]. Superoxide dismutases (SOD) activity was determined in plasma by spectrophotometry with the method reported by Fridovich et al. [24].

Biological parameters of ischemia

Heart ischemic injury was assessed by lactate levels using the Bergmeyer's technique [25]. Creatine kinase (CK) activity was determined to assess myocardial damage [26].

Hemodynamic parameters

Assessment of left ventricular pressure was performed using an intraventricular 9F Millar catheter placed directly through the apex during the collection of the heart from the donor and also inside a fluid-filled latex balloon (with physiologic serum) placed across the mitral valve during reperfusion. Systolic pressure was measured with a 40 ml balloon inflation, and the end-systolic pressure-volume relationship was determined with several balloon inflations of 10, 20 and 40 ml. Values during reperfusion were expressed as a percentage of the baseline. Similarly, diastolic left ventricular function was assessed with the end diastolic pressure to volume ratio. Data were continuously recorded with a Powerlab data-acquisition system (ADI instruments, INC, Colorado Springs, CO, USA) interfaced with the Millar catheter and the flowmeter (Transonic).

A direct measurement of cardiac output was performed by collecting the overflow from the reservoir. Coronary flow was evaluated from the coronary sinus effluent via the pulmonary artery.

Conduction abnormalities (atrio-ventricular block) were treated with cardiac stimulation (external pacemaker with electrode implanted in the wall of right ventricle). Cardiac arrhythmias (ventricular tachycardia

Table II. Development of hyperacute vascular rejection.

	Group I mini-pigs blood				Group II human blood			
Macroscopic analysis								
Petechial lesions		+				++++		
Edema		/				+++		
Heart weight		/				+++		
Immunohistochemical analysis	T_5	T_{60}	T_{120}	T_{160}	T_5	T_{60}	T_{120}	T_{160}
Complement fraction								
C_{3d}, C_4, C_5	/	/	/	/	++	+++	+++	+++
Microscopic analysis								
Vessels								
Thickening of endothelial cells	++	++	++	++	+	+	++	++
Interstitial tissue								
Fibrin thrombi	/	/	/	/	+	++	+++	+++
Edema	+	++	++	++	+++	+++	++++	++++
Hemorrhage	/	+	++	++	+++	+++	++++	++++
Leukocytes adhesion	/	+	+	+	++	++	++	++
Myocytes								
Perinuclear halo	/	+	+	+	++	++	+++	++++
Necrosis	/	/	/	+	+	++	++	+++

/, no abnormality; +, minor abnormality; ++++, major abnormality.

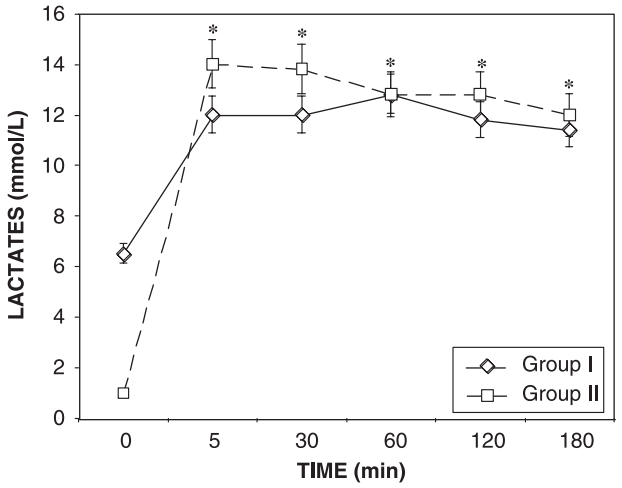

Figure 2. Lactate levels (mmol/l): [t_0 values: in the mini-pigs before anesthesia; in the patient, before blood sampling]. $\star p < 0.05$.

(VT), ventricular fibrillation (VF), atrial fibrillation (AF)) were treated by an electrical shock.

Histology

The hearts were weighed after reperfusion has been completed. For histological and immunohistochemical investigations, tissue samples were obtained from the right ventricle. Four cardiac biopsies were performed at 5, 60, 120 and 180 min after the heart was placed into the CCU7 Organetic. Muscle samples were fixed in 10% buffered formalin, embedded in paraffin, and splitted for conventional histochemical studies. Ten micro-m transverse sample sections were stained with hematoxylin and eosin and examined under a light microscopy by a specialized anatomopathologist.

Statistical analysis

For values with normal distribution, results are expressed as mean ± SD. Post operative hemodynamic and biochemical continuous and categorical variables were compared with one-way and two-way repeated measures ANOVA.

The Wilcoxson test for paired samples was used to compare data from both groups and between groups at corresponding experimental times with Bonferroni adjustements (SAS software).

A p value < 0.05 was considered a statically significant difference.

Results

We studied physiologic values of lactates, creatine kinase and OS parameters (Table I):

- In mini-pigs, samples were obtained from initially anesthezied animals before surgery,
- Usual values in human blood were obtained from 100 normolipidemic healthy 20–45 years subjects.

Development of hyperacute vascular rejection

We used a model system in which isolated mini-pigs hearts were revascularised with human blood to explore the sequence of events that may promote hyperacute vascular rejection of organ grafts. The development of hyperacute vascular rejection was characterized by a sequential thickening of endothelial cells, associated disruption of vascular walls leading to edema, hemorrhage, leukocyte adhesion and infiltration, fibrin formation thrombi, and extensive of fibrin deposits in cardiac xenografts (Table II).

Hemodynamic and metabolic function

Fourteen animals were studied. Initial ventricular fibrillation occurred in four hearts among which two rapidly evoluted into a "stone heart" after being placed into the organetic CCU7. A sinus rhythm restoration cannot be obtained after an electrical shock. Three of them had xenografts transplantation (group II), and only one belonged to group I. Arrhythmias and conduction disturbances were more frequent in xenografts than in controls (100 vs. 15%, respectively).

Cardiac output was stable in group I with a value of 300 ml/min and values progressively decreased after 75 min to 280 ml/mn. In the xenotransplantation group, cardiac output decreased significantly from 290–210 mL/mn between 5 and 75 min ($p < 0.01$), and after the values were constant. When comparing the two groups, a significant difference from 5 to 75 min ($p < 0.05$) was observed with a more marked alteration in the xenografts.

Five minutes after reperfusion, lactate levels significantly increased in each group ($p < 0.01$) (Figure 2). In group I, initial values were 6.1 ± 0.4 U/l and they increased to 11.9 ± 0.8 U/l. In group II, initial values were 1.8 ± 0.2 and they increased to 13.6 ± 0.7 U/l. After this period, lactates levels were stable in both groups but remained significantly different ($p < 0.05$) (Figure 2). Also, this difference was maintained from 15 to 90 min.

The comparison of the two groups showed a significant difference from 5 to 180 min ($p < 0.05$). This elevation was more important in group II than in group I.

In this two groups, no difference was observed at baseline in the end diastolic left ventricular pressure after 90 min of reperfusion. However, this pressure was reduced in the two groups and more significantly in xenografts ($p < 0.05$).

In xenotransplantation, a significant increase appeared in the heart wet weight from 200 ± 14 to 250 ± 25 g ($p < 0.05$), whereas no significant differ-

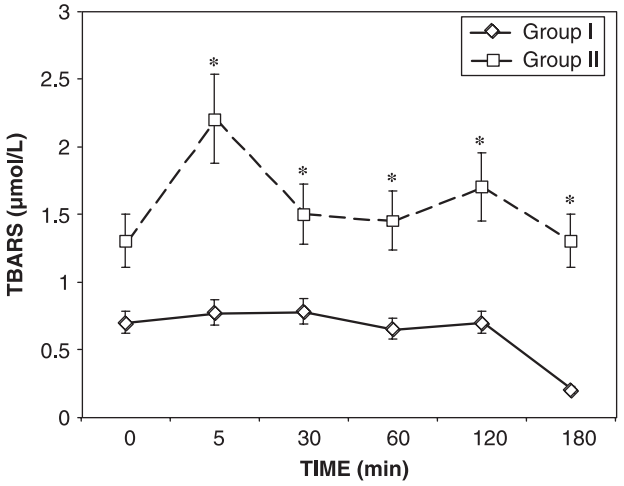

Figure 3. Oxidative stress parameters: TBARS levels (µmol/l) (t_0 values: in the mini-pigs before anesthesia; in the patient, before blood sampling). $*p < 0.05$.

ence was observed in controls (200 ± 15 vs. 205 ± 12 g).

Oxidative stress parameters

- *TBARS*. At 5 min, mean levels (TBARS) increased (+1.0 µmol/l) in the xenograft group, whereas these levels remained stable in the control group. After 30 min, TBARS levels were stable in both group. The analysis between both groups showed a significant difference at 5 min in group II ($p < 0.05$) and this difference was maintained at 30 and 180 min (Figure 3).
- *Alpha-tocopherol (vitamin E)*. In both group, a decrease of vitamin E concentration was observed.

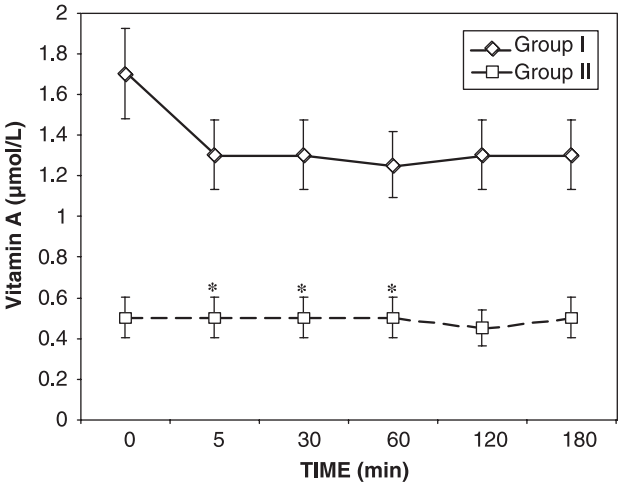

Figure 4. Oxidative stress parameters: vitamin A level (µmol/l) [t_0 values: in the mini-pigs before anesthesia; in the patient, before blood sampling]. $*p < 0.05$.

This difference was significant at 5 min. Analysis between groups did not show a significant difference.

- *Vitamin A*. In xenotransplantation, a decrease of vitamin A concentration was observed. This difference was significant from 5 to 30 min. Vitamin A levels were stable in controls (Figure 4). There was a significant difference between both groups from 5 to 60min ($p < 0.05$).
- *Glutathione peroxidase activity (GPx)*. Levels of erythrocyte GPx activity decreased at 5 min and remained stable after 15 min in each group. No significant difference between groups was observed. The interpretation of glutathione peroxidase activity in plasma could not be done as the hemolysis of red cells could overestimate this activity.
- *Superoxide dismutase (SOD)*. No significant difference in each group was observed during the time of the reperfusion. Analysis between groups did not show a significant difference.

Discussion

Transplanted organs between phylogenetically disparate species are frequently subject to hyperacute rejection which appears as a hallmark limiting XT.

In our study, we developed a simplified experimental model of XT consisting of excised mini-pig hearts perfused in Langendorff mode with either human or autologous blood. We compared the associated rejection phenomena in both settings simulating xenograft and allograft reperfusion respectively. The heart preparations were placed in a frozen (4°C) B21 solution for 8 h in accordance with previous studies reporting that such a moderate cold ischemia improves survival in concordant xenoheart transplants [27].

This present study demonstrates that ROS are generated during XT and may promote arrhythmias and impairment of left ventricular pressure during reperfusion.

Although similar studies assessing ROS production during XT remain still rare, many associated factors have been reported which may elicit vascular rejection and/or some degree of ROS implication:

1. Donor-specific antibodies may induce hyperacute graft rejection [28,29]. Complement fraction C4d deposits are consistently fixed on graft endothelial cells as a result of antibody-induced activation of the classical complement pathway which is considered as an independent marker of hyperacute rejection and subsequent long-term prognosis [30].
2. In addition to these humoral factors, recipient T-cells may interact with the graft endothelial cells to release pro inflammatory cytokines which, in

turn, further stimulate endothelial cells and induce cytotoxicity [31,32]. Disrupted and activated endothelial cells induce expression of tissue factor, adhesion molecules, chemokines and elicit ROS production [33,34].

3. This cascade is further enhanced by the monocytes/macrophage infiltrates which also secrete large amounts of pro-inflammatory cytokines such as TNF alpha and IL-1. TNF alpha and IL-1 also activate endothelial cells leading to further ROS production [35].
4. Activated platelets carry cell-surface bound cytokines, specially IL-1, that may directly target the endothelium to induce pro-coagulant and/or pro inflammatory changes leading to vascular or humoral rejection [18,36–38].

Several mechanisms have been proposed to explain ROS generation during xenotransplantation. A growing body of evidence has emerged to suggest that ROS produced during cardiac surgery result from an ischemia–reperfusion injury [2,4]. The subsequent activation of polymorphonuclear leukocytes and macrophages are directly implied in ROS production *in situ* and the resulting reperfusion injury [5,39].

Soon after transplantation, severe ischemia-reperfusion injury causes recruitment of inflammatory cells and activation of endothelium [15,39]. Ischemia–reperfusion injury stimulates platelets, which activate endothelial cells [17,18] and cause activation of the complement system through the classical and the alternative pathways [40,41].

Limitations of the study

TBARS assay constitutes a global evaluation of lipid peroxidation. As recently referred by Del Rio et al., plasma MDA or TBARS concentrations varied in a very wide range (from 0 up to 50 μmol/l) when obtained with methods developed from 1970 to 1995, thus suggesting a sample oxidation during analysis, as already asserted by various authors [42]. In the light of these results, biological significance of such an assay has often been criticized. However, in our study, plasma TBARS concentrations given as usual values were in the 1.00–1.50 μmol/l range, which is in agreement with a correct assessment of this marker. Nevertheless, according to the chemical conditions applied for the MDA–TBA reaction, values obtained from TBARS assays provide more information on the sample oxidizability, than of its oxidation. Therefore, in this experimental study, we evaluated oxidative stress on an isolated heart. Modifications of lactate and TBARS levels were only a reflect of heart metabolism.

As reported by Sies et al., among various intracellular antioxidants, glutathione (GSH) plays an important role, acting as a reductant in enzymatic reactions catalysed by peroxidase and thiol disulphide oxido-reductases [43]. In our study, red cells hemolysis limited the potential interpretation of plasma GPx activity.

Conclusion

Production of ROS and arrhythmias are frequently increased in a XT model than in control allograft model. OS might play an eminent role in hyperacute humoral rejection. The results of this study may be relevant in clinical practice, providing better understanding of the mechanisms of hyperacute vascular rejection and the related complications that may be lead to a specific therapeutic approaches.

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