



## An overview of chagasic cardiomyopathy: pathogenic importance of oxidative stress

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### ABSTRACT

There is growing evidence to suggest that chagasic myocardia are exposed to sustained oxidative stress-induced injuries that may contribute to disease progression. Pathogen invasion- and replication-mediated cellular injuries and immune-mediated cytotoxic reactions are the common source of reactive oxygen species (ROS) in infectious etiologies. However, our understanding of the source and role of oxidative stress in chagasic cardiomyopathy (CCM) remains incomplete. In this review, we discuss the evidence for increased oxidative stress in chagasic disease, with emphasis on mitochondrial abnormalities, electron transport chain dysfunction and its role in sustaining oxidative stress in myocardium. We discuss the literature reporting the consequences of sustained oxidative stress in CCM pathogenesis.

**Key words:** chagasic cardiomyopathy, reactive oxygen species, inflammation, mitochondria, oxidant/anti-oxidant status, oxidative damage.

### PARASITE, VECTOR AND TRANSMISSION

*Trypanosoma cruzi*, a parasitic protozoan of the ancient branch of eukaryotes (Kingdom Eukaryota, Order Kinetoplastida), is the etiological agent of Chagas disease in humans (Miles 2003). Currently, the World Health Organization estimates that 11–18 million individuals are infected worldwide (WHO 2002). Transmission of *T. cruzi* occurs predominantly via insect vectors of the subfamily *Triatoma*, family *Reduviidae*, referred to as “kissing bugs”. Residing in the peridomestic habitat of mud-thatch

houses in rural areas (Mott et al. 1978), *Triatoma infestans* in South America, *Rhodnius prolixus* in Venezuela, Colombia and Central America (Schofield and Dias 1999), and *Triatoma barberi* in Mexico (Guzman 2001), are the most common species responsible for transmission. Improvements in housing conditions and vector control measures instituted by the Southern Cone Initiative in 1991 have contributed to a decline in transmission in endemic countries (Schofield and Dias 1999). However, concern remains that reinfestation of homes by secondary sylvatic vectors, e.g. *Triatoma sordida*, will compromise the long-term efficacy of

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vector control measures in Brazil and other Southern American countries (Monteiro et al. 2001, WHO 2002). Blood transfusion and organ transplantation represent further routes of *T. cruzi* transmission. Although many countries in Latin America screen blood donations for *T. cruzi*, infection rates ranging from 0.1% to 24.4% are estimated to occur through transfusion (WHO 2002).

In the U.S., vector-transmitted human infections have been reported in the southern States (Ochs et al. 1996, Herwaldt et al. 2000). Several studies have shown the presence of the insect vector as well as infection of domestic dogs and wild animals in the US (Meurs et al. 1998, Bradley et al. 2000, Beard et al. 2003, Yabsley and Noblet 2002). Further, due to significant increases in immigration to the USA and Canada from endemic countries and perinatal transmission, it is estimated that ~ 100,000 people residing in USA may be infected with *T. cruzi*. Currently, the US does not screen blood donations, as no approved screening test is available (Dodd and Leiby 2004). These conditions, taken together, could contribute to the transmission of *T. cruzi* by blood-borne, congenital, and to a lesser extent, vector-borne routes, indicating the potential for the emergence of Chagas disease as a disease of public health importance in the USA (Leiby et al. 2000, Dodd and Leiby 2004).

#### CHAGASIC DISEASE

##### CLINICAL SYMPTOMS

Infection by *T. cruzi* elicits acute non-specific symptoms e.g. fever, malaise, edema and/or enlarged liver or spleen to the characteristic swelling at the site of entry- a *chagoma* (of the skin), or *Romana's sign* (of the conjunctiva or eyelid). In spite of the high blood parasitemia in the acute phase, clinical symptoms do not warrant hospital visit, and therefore, anti-parasitic treatment is often not initiated. In few (<5%) acute patients, sudden death due to congestive heart failure associated with myocarditis or meningoencephalitis may occur. The majority of patients enter an "indeter-

minate" phase that is defined by the presence of *T. cruzi* specific antibodies but the absence of clinical signs of cardiac abnormalities. Between 10–30 years after initial infection, an estimated 30–40% of "indeterminate" patients show recognizable signs and/or symptoms of a unique form of heart disease referred to as chagasic cardiomyopathy (CCM).

Generally, patients develop a complex constellation of symptoms and signs; their presence or absence and severity have been used to create a diagnostic scale for CCM (Rocha et al. 2003). The non-specific symptoms suggestive of heart problems include palpitations, dizziness, and syncope. Clinical manifestations of CCM are congestive heart failure, thromboembolism in brain, limbs or lungs, and ventricular fibrillation (Rassi et al. 2000). The principal defects in the conduction system present as a mixture of arrhythmias – e.g. tachycardia, bradycardia, ventricular fibrillation – and electrical impulse blockages – e.g. right bundle branch and left anterior fascicle block. These are identified by electrocardiography as well as via 24-hour Holter monitoring or stress (exercise) testing (Elizari 2002). Myocardial defects, that is, impairment in the architecture of the heart muscle, are mainly cardiomegaly with hypertrophy and dilation of the chambers; and aneurism, particularly in the apical portion of the left ventricle, which may rupture. These attributes can be identified via echocardiography and chest X-ray and are frequently observed at autopsy. Clinical manifestations of CCM may also be correlated with defective innervation and defective contraction within the myocardium. Pathogenic changes in the parasympathetic branch of the autonomic nervous system are sometimes observed at autopsy (Koberle 1968, Mott and Hagsstrom 1965, Oliveira 1985). Specifically, a reduction – or in some cases, a complete absence – of cells in the neuronal ganglia has been described and may be indicative of a role for impaired heart innervation in development of CCM (Marin-Neto 1998). Further, the basement membrane of the myocardial capillaries is abnormally thick, suggestive of microcirculation defects (Rossi et al. 2003). Summariz-

ing, the dilation and hypertrophy of the left ventricle combined with fibrosis at its apex are considered pathognomonic of CCM, while the distinctive clinical syndrome described above accounts for heart failure and subsequent death that occurs in chagasic patients.

#### PATHOLOGY

Several specific heart changes – at the gross and microscopic levels – are distinguishing in CCM. At autopsy, the most commonly observed abnormalities in the heart structure are global (biventricular) heart enlargement, apical aneurism, dilation/thinning and thrombi of the heart walls (Rossi et al. 2003). The histological examination of sections from both autopsy and biopsy specimens revealed tissue fibrosis, inflammation, and hypertrophy of cardiac fibers are the chief pathological consequences of chagasic heart disease. Fibrosis is most pronounced in the left ventricle and apex. This varies in severity and its location may be interstitial and/or diffuse. It corresponds to increased collagen fiber deposition around the muscle bundles that resembles reparative fibrosis resulting from microinfarction. These microscopic observations bear relationship to the gross pathological observations of heart enlargement of the hypertrophic and dilated form.

#### PATHOGENIC MECHANISMS (HISTORICAL PERSPECTIVE)

Extensive studies utilizing human biopsy and autopsy specimens- and experimental investigations have provided data on the clinical and pathological manifestations of chagasic disease. However, the mechanism(s) leading up to CCM remain uncertain. There is currently no universally accepted model to predict which individuals in the “indeterminate” phase will progress to chronic chagasic disease. Several fundamental enigmas remain unresolved. First, why does the parasite, which is shown to infect a wide variety of organs and cell types, primarily cause disease in the heart? Second, why does only a subset of infected (seropositive) individuals progress to CCM? Third, what accounts

for the long latency period between infection and appearance of heart disease? Nevertheless, a significant body of literature supports the hypothesis that immune responses, consistently triggered by parasite persistence or by the host response to self-antigens (autoimmunity) or both play a role in the development and/or propagation of pathological lesions. The concept of parasite persistence is that a few persisting parasites continually trigger immune responses, leading to chronic inflammation and cell death. Alternatively, the concept of autoimmunity is that the parasite has antigens that mimic the human host. In effort to control *T. cruzi* infection, the host produces antibodies and T cells that subsequently recognize self-antigens, and destroy the myocardial tissue.

#### PARASITE PERSISTENCE

Several lines of evidence establish that a low level of parasites remain in the blood and/or the heart tissue. First, direct detection in autopsy specimens and myocardial biopsies has been possible using new methods e.g. immunohistochemical and immunofluorescence techniques (Mortara et al. 1999, Higuchi et al. 2003) and PCR (Jones et al. 1993, Salomone et al. 2000), including *in situ* PCR (Jones et al. 1992). Second, in chronically infected individuals, the reactivation of acute disease, particularly meningoencephalitis, that occurs following immunosuppression due to AIDS (Rocha et al. 1994, Sartori et al. 1995) or drug therapy (Jardim and Takayanagui 1994, D’Almeida et al. 1996) illustrates that parasites persist in previously undiagnosed individuals for years after initial infection. Thirdly, transmission of *T. cruzi* can occur via blood transfusion and transplantation of infected organs obtained from asymptomatic individuals (Leiby et al. 2000). Finally, parasite persistence would explain the clinical benefit of anti-parasitic drug treatment in chronic infection, which has been observed in a limited number of studies (Andrade et al. 1992, Viotti et al. 1994). Altogether, these studies support the concept of parasite persistence and its likely pathologic role in CCM development.

## IMMUNE RESPONSES: PROTECTIVE OR PATHOLOGICAL

Sterilizing immunity does not appear to exist in *T. cruzi* infection. Nevertheless, the immune response that is mounted against *T. cruzi* after initial infection is capable of controlling the parasite, as evidenced by the eventual resolution of acute parasitemia. As mentioned above, these immune responses may contribute to pathology through either or a combination of two general mechanisms: i) a process of auto-immune destruction of host cardiac tissue (auto-immune hypothesis) or ii) through specific mediators of the immune response to *T. cruzi* itself or to its antigenic components that linger in the tissues (parasite persistence hypothesis).

*Experimental studies*

Assorted experimental approaches using animal and tissue culture models of *T. cruzi* infection and disease have provided a portrait of the immune response to *T. cruzi* infection. In addition, various knockout or immunocompromised animals have been utilized to dissect the relative contribution of different features of the immune response that mediate protection versus pathology. Briefly, in acutely infected animals (mice and rats), it is shown that macrophages (M $\phi$ ) and natural killer (NK) cells provide the first line of defense (Nogueira and Cohn 1978). *T. cruzi* elicits IFN- $\gamma$  production by NK cells, activating M $\phi$  (Torricco et al. 1991, Gazzinelli et al. 1992). In turn, M $\phi$  produce TNF- $\alpha$ , inducing nitric oxide (NO), which is toxic to parasites in vitro (Vespa et al. 1994, Martins et al. 1998). Interestingly, GPI-anchored macromolecules, abundantly expressed by the infective and intracellular stages of the parasite, have been characterized as the prime inducers of the proinflammatory cytokines (e.g. TNF- $\alpha$ , IL-1 and IL-6) and chemokines (e.g. IP-10, MCP-1, MIG, and RANTES) that may be key regulators of the innate and adaptive immune responses responsible for the control of acute infection. CD4<sup>+</sup> T cells assist in the control of *T. cruzi* through secretion of Th1 cytokines, amplification

of phagocytic activity of macrophages, and stimulation of B cell proliferation and antibody production (Aliberti et al. 1996, Brener and Gazzinelli 1997). CD8<sup>+</sup> T cells are shown to exhibit specific cytotoxicity in vitro in response to *T. cruzi*-expressed antigens (Garg et al. 1997, Wizel et al. 1997, Garg and Tarleton 2002) and are suggested to control *T. cruzi* either by cytolysis of the infected cells or by secretion of Th1 cytokines that induce trypanocidal activity. Altogether, these studies conclude that an efficient protective response to acute parasitemia is provided by Th1 cytokines, lytic antibodies and the concerted activities of macrophages, T helper cells and cytotoxic T lymphocytes.

With progression to indeterminate-chronic phase, parasite nests are not detectable by conventional histological analysis. However, a low level of inflammatory response constituted by macrophages and CD8<sup>+</sup> T lymphocytes persist in the heart. Although often they are not associated with amastigote nests or trypomastigotes, *T. cruzi* antigens may be present (Higuchi 1995, Reis et al. 1997). CD4<sup>+</sup> T cells, though less prominent in the chronic stage, are suggested to be associated with myocyte death and increased animal mortality (Soares et al. 2001) and appear to represent an autoreactive phenotype that contributes to tissue destruction (Soares and Santos 1999). At this later stage, continued production of IFN- $\gamma$  and IL-2 is believed to stimulate lytic antibody production by B cells (Brener and Gazzinelli 1997). Several investigators have utilized mice treated with antibody to immune mediators and murine models in which genes for various immune mediators have been deleted to evaluate the significance of inflammatory response in chagasic disease. These include mice deficient in inducible NO synthase (iNOS), IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\alpha$  receptor, and CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells (Minoprio et al. 1987, Tarleton 1990, Tarleton et al. 1992, Bachmaier et al. 1997, Martins et al. 1998, Aliberti et al. 2001, Chandra et al. 2002). The overall observation from these studies is that despite a general increase in parasite burden, the extent of cell death and tissue damage are diminished in mice deficient in inflam-

matory mediators compared to the wild-type controls, thus suggesting the pathological significance of persistent inflammation in chagasic disease.

#### *Human studies*

Many of the above experimental observations agree with the more limited number of human studies. In acute infection, hypergammaglobulinemia occurs and the continued presence of antibody is used to diagnose infection with *T. cruzi*. In human patients' cardiac biopsies, among the mononuclear cells, macrophages and CD8<sup>+</sup> T cells represent the majority of the infiltrate (Higuchi et al. 1997). IFN- $\gamma$  can be detected in tissues via *in situ* immunohistochemistry (Bahia-Oliveira et al. 1998, Correa-Oliveira et al. 1999) and appears to be correlated with the presence of CD8<sup>+</sup> T cells (Higuchi et al. 1997, Reis et al. 1997). In addition, *T. cruzi*-specific human CTL have been identified (Thomson et al. 1998, Wizek et al. 1998). While parasites are rarely seen in cardiac tissue, pseudocysts are associated with relatively sparse infiltrate of IL-2<sup>+</sup> and IL-4<sup>+</sup> lymphocytes (Higuchi et al. 1997, Reis et al. 1997). Again, CD8<sup>+</sup> CTL are suggested to be likely the major effectors of pathogenesis, contributing to the development of fibrosis in response to cytopathology (Brenner and Gazzinelli 1997).

To sum up, until recently, autoimmunity was believed to be the primary mechanism of chagasic disease development. A variety of autoantigens have been described and autoimmune-mediated processes have been demonstrated in experimental models (Leon et al. 2001, Pontes-de-Carvalho et al. 2002). Increasingly, with the demonstration of parasite antigens in the heart tissue, parasite persistence has gained favor among several investigators. We surmise that the contribution of parasite persistence, auto-antigens and chronic inflammation is not mutually exclusive and all these mechanisms may contribute, in part, to pathogenesis of CCM. The mechanistic studies identifying i) the mediators produced by cardiomyocytes in response to *T. cruzi* infection, that may trigger the migration of leukocytes and other cells to the heart; ii) the sig-

naling mechanisms regulated by the inflammatory cytokines (e.g. TNF- $\alpha$  and IL-1) that may evoke cell survival/cell growth or cell death responses in chagasic myocardium; and iii) the destructive effects of "oxidative burst" of activated inflammatory cells in CCM would be discussed elsewhere.

#### VASCULAR MEDIATORS IN ENDOTHELIUM

In addition to cardiomyocytes, *T. cruzi* infects endothelial cells in the heart. The role of vascular mediators e.g. endothelin-1 (ET-1) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) in CCM has recently been explored (Petkova et al. 2001). ET-1 is a vasoconstrictor produced by endothelial cells as well as cardiomyocytes and other cell types (Yanagisawa et al. 1988). Its synthesis by endothelial cells is increased during *in vitro* infection with *T. cruzi* (Wittner et al. 1995) and in the myocardium of infected mice (Petkova et al. 2000b). This appears to be regulated by the transcription factors, NF $\kappa$ B (Huang et al. 1999) and AP-1, in conjunction with the MAPK signal transduction pathway (Miyachi and Masaki 1999, Petkova et al. 2000a). *T. cruzi* infected endothelial cells have also been shown to induce cytokine gene expression (Tanowitz et al. 1992). This may contribute to CCM development, as cytokines stimulate ET-1 production (Kurihara et al. 1989, Martins et al. 1998). TXA<sub>2</sub> induces vasospasm and platelet aggregation, which is observed in CCM (Rossi and Ramos 1996). Thus, TXA<sub>2</sub> and ET-1 may potentiate CCM by promoting microvascular pathology, e.g., vasospasm, focal ischemia and microthrombi (Petkova et al. 2001). Vascular mediators, in conjunction with cytokines induced by *T. cruzi* infection, may be significant to the development of microcirculatory abnormalities that are well documented in CCM.

#### OXIDANTS, ANTIOXIDANTS AND OXIDATIVE STRESS (AN OVERVIEW)

##### REACTIVE OXYGEN SPECIES (ROS) AND REACTIVE NITROGEN SPECIES (RNS)

Broadly defined, ROS are derivatives of molecular oxygen (O<sub>2</sub>) (Nordberg and Arner 2001), e.g. su-

peroxide ( $O_2^{\cdot-}$ ), hydroxyl radical ( $HO^{\cdot}$ ), and hydrogen peroxide ( $H_2O_2$ ). ROS are produced through the action of specific oxidases and oxygenases, the Fenton reaction and are also by-products of the electron transport chain (Turrens 2003, 2004). RNS include  $\cdot NO$  and its derivatives.  $\cdot NO$  is produced by the enzymatic activity of NOS. Different NOS isoforms have been identified in the cytoplasm (iNOS), in mitochondria (mtNOS), or in specific cell types e.g. endothelial NOS (eNOS) (Andrew and Mayer 1999). ROS are unstable and react rapidly with other free radicals and macromolecules in chain reactions to generate increasingly harmful oxidants (Kirkinezos and Moraes 2001). The toxic effects of ROS are believed to vary in proportion to the quantity and their oxidant strength e.g.  $HO^{\cdot} \gg O_2^{\cdot-} > H_2O_2$ . While  $H_2O_2$  is not excessively reactive, it is highly diffusible and is a precursor of  $HO^{\cdot}$ .  $HO^{\cdot}$  is highly reactive at its site of production.  $H_2O_2$  and  $\cdot NO$  readily cross membranes and thus, are capable of affecting distant cellular targets. Interaction of  $O_2^{\cdot-}$  and  $\cdot NO$  results in highly toxic, stable peroxynitrite ( $O=NOO^-$ ) (Figure 1).

#### SOURCES OF ROS

##### *Oxidases and oxygenases*

Numerous oxidases and oxygenases expressed in different cell types and locations within the cell contribute to the formation of ROS. By definition, oxidases reduce  $O_2$  whereas oxygenases (oxidoreductases) transfer  $O_2$  to substrates. ROS production, termed the "oxidative burst" of activated phagocytic cells e.g. macrophages and neutrophils, results from NADPH oxidase and/or by myeloperoxidase activity (Halliwell 1991, Eiserich et al. 2002). This ROS production is critical to anti-microbial function, contributing either directly or indirectly to the killing of intracellular organisms. Myeloperoxidase, produced by neutrophils, converts  $H_2O_2$  and chloride ions into hypochlorous (HOCl) acid (Winterbourn et al. 2000). NADPH oxidase, produced by many types of phagocytes, reduces  $O_2$  to  $O_2^{\cdot-}$  (Cross et al. 1994, Babior 1999). Subsequently,  $O_2^{\cdot-}$  and HOCl further can react to form  $HO^{\cdot}$  (Candeias et al. 1993).

Other oxidases are more generally expressed in mammalian cells but differ in their basal level of expression and subcellular locations. Monoamine oxidase, present in the outer mitochondria membrane, converts  $O_2$  to  $H_2O_2$  on the cytoplasmic face (Hauptmann et al. 1996). Both xanthine oxidase and xanthine dehydrogenase, derived from xanthine oxidoreductase, produce  $H_2O_2$  and  $O_2^{\cdot-}$  in the process of degrading the purine hypoxanthine to uric acid (Berry and Hare 2004). In rats and some lower eukaryotes, xanthine oxidase is demonstrated to be both cytoplasmic and peroxisomal.  $O_2^{\cdot-}$  may also be produced by lipoxygenase, cyclooxygenase (McIntyre et al. 1999) and cytochrome P450-dependent oxygenases (Coon et al. 1992).

##### *Fenton chemical reaction*

Besides production by oxidases and oxygenases, the Fenton reaction is another mechanism of ROS formation. This reaction results in the  $Fe^{+2}$ - or  $Cu^{+}$ -mediated conversion of  $H_2O_2$  to  $HO^{\cdot}$  (Goldstein et al. 1993).

##### *Electron transport chain (ETC)*

Mitochondria are considered a major source of ROS production in heart (Loschen et al. 1971). In the mitochondria, the partial reduction of  $O_2$  occurs as a result of leakage of electrons from the ETC, contributing one, two or three electrons to form  $O_2^{\cdot-}$ ,  $H_2O_2$ , or  $HO^{\cdot}$ , respectively. Electron leakage can arise at a number of points in the ETC, producing  $O_2^{\cdot-}$  (Turrens 2004). As much as 2–4% of the reducing equivalents escape the respiratory chain, leading to  $O_2^{\cdot-}$  formation.  $O_2^{\cdot-}$  is dismutated by MnSOD to  $H_2O_2$  that may then be converted to highly reactive and harmful  $HO^{\cdot}$  radicals. Generally, the leakage of electrons at CI flavoprotein generates  $O_2^{\cdot-}$  in mitochondrial matrix while CIII ubisemiquinones ( $UQ^{\cdot-}$ ) generated at  $Q_1$  ( $UQ^{-1\cdot}$ ) and  $Q_o$  ( $UQ^{-o\cdot}$ ) sites release  $O_2^{\cdot-}$  in the matrix and intermembrane space of the mitochondria, respectively (Han et al. 2001, 2003).



SOD converts  $O_2^{\cdot-}$  to  $H_2O_2$  (Fridovich 1974). MnSOD, the mitochondrial isoform makes up  $\sim 70\%$  of the SOD activity in heart, and 90% in cardiomyocytes. The remaining fraction consists primarily of cytoplasmic CuZnSOD with  $<1\%$  extracellular SOD (ECSOD). The importance of MnSOD in regulating  $O_2^{\cdot-}$  in myocardium is demonstrated by the fact that MnSOD<sup>-/-</sup> mice die soon after birth with dilated cardiomyopathy (Li et al. 1995). GPx (isoforms GPx1 – GPx5), using glutathione (GSH), reduces  $H_2O_2$  or ROOH to  $H_2O$  or alcohols (ROH), respectively. GPx1 and GPx3 are the most abundant intracellular isoforms and GPx4 is a mitochondrial isoform. Unlike MnSOD, mice deficient in GPx develop normally and show no marked pathologic changes under normal physiologic conditions, and exhibit a pronounced susceptibility to myocardial ischemia-reperfusion injury (Ho et al. 1997). CAT, located in peroxisomes, is highest in the liver and erythrocytes, and converts  $H_2O_2$  to  $H_2O$  and  $O_2$ . Prx reduces peroxides, including  $H_2O_2$  and alkyl hydroperoxides (ROOH).

#### *Non-enzymatic antioxidants*

The role of GSH in maintaining cellular redox state is complex. GSH cooperates with GPx in the detoxification of  $H_2O_2$  to  $2H_2O$ . In addition, GSH participates in reactions with glutathione S-transferase (GST) to bind ROS e.g. attachment of  $\cdot NO$  to form S-nitrosoglutathione adducts. Glutathione reductase (GR) functions to regenerate antioxidant capacity, converting from glutathione disulfide (GSSG) to GSH. Vitamins and other chemical antioxidants play an important role in the control of ROS cascades. Vitamin E ( $\alpha$ -tocopherol) is active in membranes where it functions to reduce ROS and lipid peroxy radicals. Vitamin C (ascorbate) serves predominantly as an antioxidant in plasma due to its water solubility. It functions by reducing  $\alpha$ -tocopherol-lipid peroxide radicals to normal form (Nordberg and Arner 2001). Uric acid, found in extracellular fluids, detoxifies  $HO\cdot$ . Metal ions ( $Fe^{+2}$  or  $Cu^+$ ) contribute to ROS-mediated peroxidation of lipids via the Fenton reaction that produces  $H_2O_2$ . The se-

questration of these ions in protein-bound form, e.g. as iron-transferrin or copper-ceruloplasmin bound complexes, also adds indirectly to the antioxidant capacity of cells (Turrens 2004).

#### OXIDATIVE STRESS

When produced transiently in limited quantities, ROS and RNS play critical roles in normal developmental processes; ROS and RNS, control signal transduction mechanisms that regulate cell proliferation, differentiation and death (Droge 2002, Finkel 2003). However, when produced in excess or for sustained periods, or when the antioxidant system is compromised, cells are unable to efficiently scavenge free radicals, leading to ROS accumulation. ROS can rapidly oxidize proteins, lipids and DNA (Butterfield et al. 1998, Marnett 2000), thereby resulting in dysfunction of physiological processes and cellular damage leading to cell/tissue death. Results of a variety of studies on the mammalian system have shown that oxidative stress can be reliably measured by oxidative-damage biomarkers, such as lipid peroxides, protein carbonyls, and oxidative DNA modifications.

#### *Lipid peroxidation (LPO)*

Lipid peroxidation is the major biochemical consequence of oxidative deterioration of polyunsaturated lipids in cell membranes and causes damage to membrane integrity and loss of membrane protein function. Peroxidation, in general, is initiated by oxidative attack-mediated removal of a  $H\cdot$  atom resulting in carbon centered radical, that in aerobic cells undergoes molecular rearrangement following exposure to  $O_2$  to give a peroxy radical. Peroxyl radicals can combine with each other, attack membrane proteins, or abstract  $H\cdot$  from adjacent fatty acids side chains in a membrane thereby propagating a chain reaction of lipid peroxidation. The ETC and membrane phospholipids such as those in the mitochondrial membrane are particularly susceptible to LPO (Halliwell 1991, Cardoso et al. 1999). Among a wide range of aldehydic compounds, 4-hydroxy-2-nonenal (4-HNE) and malonylaldehyde (MDA) are



the most common reactive products of the peroxidation of membrane phospholipids (Zarkovic 2003). These aldehydic products are relatively stable compounds, and are able to diffuse and attack targets in the near vicinity as well as those distant from their site of origin (Esterbauer 1982).

#### *Protein oxidative modifications*

Many different types of protein oxidative modifications may be induced by direct or indirect attack by ROS/RNS and secondary by-products of oxidative stress (Dalle-Donne et al. 2003). These modifications result in protein carbonyl (PCO) derivatives (aldehydes and ketones), nitrative adducts (e.g. 3-nitrotyrosine) and Michael-adducts formation. Cys, His or Lys amino acids are the prime target of 4-HNE, a highly reactive  $\alpha\beta$  unsaturated aldehyde, resulting in Michael adducts formation and irreversible alkylation and introduction of carbonyl groups into proteins (Uchida and Stadtman 1992). Arg, Lys, Pro, and Thr residues may be directly derivatized by ROS leading to formation of protein carbonyls (Butterfield et al. 1998, Chevion et al. 2000). Carbonyl groups can also be introduced into proteins by oxidative reaction of sugar derivatives (ketoamines, ketoaldehydes and deoxyosones) with Lys residue in a process called glycation or glyoxidation. As a consequence of oxidative modifications, functional impairment of proteins occurs and furthermore, leads to protein turnover e.g. degradation by proteases via the proteasome (Floyd et al. 2001).

#### *DNA damage*

DNA can be oxidized by a variety of mechanisms, resulting in nucleotide damage e.g. formation of 8-oxoguanine lesions. As a result, DNA replication may be inaccurate leading to mutations and transcription errors. While mechanisms exist to repair these DNA lesions, the level of DNA damage may exceed the capacity of the cellular repair mechanisms. Furthermore, mtDNA is believed to be particularly susceptible to sustained damage, since mitochondria may lack appropriate DNA repair

mechanisms (Evans and Cooke 2004).

#### **MITOCHONDRIAL ABNORMALITIES AND OXIDATIVE STRESS IN CCM**

Oxidative stress is generally viewed as a protective defense mechanism employed by the host to control parasitic infection. There is, however, growing evidence to suggest that sustained oxidative stress may contribute to CCM pathology. Pathogen invasion- and replication-mediated cellular injuries and immune-mediated cytotoxic reactions are the common source of ROS in infectious etiologies. As mentioned in section "Immune responses: Protective or pathological", experimental studies have shown that *T. cruzi* infection elicits inflammatory cytokines, NO production, and oxidative burst, all of which, though essential for controlling acute parasitemia, may have toxic effects on host cellular components and be of pathological significance in CCM.

Mitochondria represent 30% of the total volume of cardiomyocytes and provide ~90% of the cellular energy through the oxidative phosphorylation pathway. As discussed in section "Electron transport chain (ETC)", the CI and CIII complexes of the respiratory chain are the prime site for electron leakage to oxygen, and free radical production in mitochondria. The rate of mitochondrial ROS release is inversely proportional to the rate of electron transport, exponentially increasing when CI or CIII complexes of ETC function at a sub-optimal level (Ide et al. 1999, Wallace 2000, Lesnfsky et al. 2001, Chen et al. 2003). Interestingly, mitochondria are targets to a variety of endogenous and exogenous insults that may affect ETC function. Further, CI and CIII are redox sensitive, as they contain  $Fe_4S_4$  clusters which when oxidized, release one iron atom, resulting in the inactivation of important functional Fe-S centers and enzyme activity. The released ferrous ions, when participating in the Fenton reaction, produce highly reactive  $HO^{\cdot}$  radicals. Consequently, ROS as a by product of respiratory chain is considered the major source of free radicals in the heart and mitochondrial dysfunction-mediated oxidative stress has

been shown in many cardiac pathologies (Sawyer et al. 2002). The importance of these findings as it relates to chagasic disease is that an early and consistent repression of CI and/or CIII activities associated with sustained oxidative stress is shown in mitochondria isolated from the myocardium of mice infected by *T. cruzi* (Vyatkina et al. 2004). A consistent decline in MnSOD activity, the major oxygen radical scavenger in the mitochondrial matrix, during progression of infection and disease in chagasic myocardium was also shown (Wen et al. 2004). These studies have led to the suggestion that a catastrophic cycle of mitochondrial functional decline and ROS generation, coupled with an inability to efficiently scavenge the mitochondrial ROS (due to MnSOD deficiency), predisposes the chagasic hearts to sustained oxidative stress during infection and disease development. We discuss the literature related to role of mitochondrial dysfunction and inadequate antioxidant defenses in sustaining the oxidative stress in CCM.

#### MITOCHONDRIAL DYSFUNCTION IN CCM

The early insights suggesting mitochondrial alterations in CCM were provided by electron microscopic analysis of biopsies from cardiac tissue of chagasic patients (Carrasco Guerra et al. 1987, Palacios-Pru et al. 1989, Parada et al. 1997) and experimental models (Uyemura et al. 1995, Garg et al. 2003). Microscopic examination of biopsy samples from the patients showed that degenerative myocardial changes occur very early during the indeterminate phase and become more pronounced with increased severity of clinical disease. In experimental models of chagasic disease, the ultrastructural evaluation of the morphological alterations in the myocardium has illustrated an accumulation of large, irregular nuclei, swollen and displaced mitochondria, and myofibrillar degeneration, that progress in parallel with the severity of disease (Garg et al. 2003). These studies have revealed two important observations. First, nuclear and mitochondrial structural damage occurs much earlier than do the clinical symptoms of disease. Second, the severity of these

aberrations increase with the evolution of chronic disease. Jointly, these observations imply that a correlation exists between the extent of specific organelle abnormalities and clinical severity of chagasic disease.

Recent molecular studies have profiled the changes in mitochondrial function-related gene expression in experimental models of *T. cruzi* infection and disease development (Garg et al. 2003, Mukherjee et al. 2003, Garg et al. 2004). These studies utilized global and custom-designed arrays and confirmed the array data by traditional and real-time RT-PCR and Northern blotting approaches. The overall picture that emerged from these studies was that the myocardial transcripts encoding metabolic enzymes involved in fatty acid  $\beta$ -oxidation were up-regulated, while the mRNAs for a majority of the subunits of the complexes of the ETC pathway were repressed in response to infection. It is important to note that the mtDNA-encoded transcripts for the subunits of the ETC complexes were repressed in response to infection (Vyatkina et al. 2004), before the alterations in nDNA-encoded transcripts were detected. The expression level of mtDNA-encoded ETC components that were examined (9 of 13) as substantially reduced (up to 80%) with progression to chronic disease phase. A loss in mtDNA-encoded transcripts (and presumably proteins) below the threshold level is likely to result in a deficiency of respiratory complexes in chagasic hearts.

We and others have demonstrated a substantial decline in respiratory chain complexes CI, CIII, and CV activities in the myocardium of infected experimental animals (Uyemura et al. 1995, Vyatkina et al. 2004). Along with a decline in total specific activity of respiratory complexes (determined by spectrophotometry assays), inactivation of the assembled complexes (determined by catalytic staining on blue-native gels) was also noted, suggesting that multiple mechanisms are likely to be involved in inhibition of the respiratory complexes in the chagasic myocardium. Interestingly, the finding of a loss in CI activity early in response

to parasite infection led to a suggestion that CI might be the potential site for ROS generation in acute murine myocardium. CIII deficiencies were consistent in cardiac mitochondria, but were not observed in skeletal muscle mitochondria at all stages of infection and disease progression, leading to an implication that CIII is the likely site for sustained ROS generation in chagasic myocardium (Vyatkina et al. 2004). Future studies would, hopefully, address the pathophysiological significance of mtROS in CCM.

In human patients, direct demonstration of mitochondrial dysfunction awaits further investigation. However, biochemical analysis have provided indirect evidence to suggest mitochondrial abnormalities in chagasic patients. For example, Carrasco et al. showed a reduction in the activities of succinate dehydrogenase and myosine ATPase by histochemical staining of small endomyocardial biopsies obtained from the chagasic patients (Carrasco Guerra et al. 1987). The histochemical alteration index, while increased in seropositive patients in the so-called "indeterminate" phase, was highest in chronic patients. In another study of chagasic patients, changes in the serum pattern of metabolic enzymes was documented (Alarcon-Corredor et al. 2002). The main finding in this study was a substantial increase in glutamate-oxaloacetate transaminase (GOT) and 3-hydroxy butyrate dehydrogenase (HBDH) activity. Importantly, high serum levels of GOT and HBDH were detected in indeterminate patients, and remained consistently high in patients advancing to clinical cardiac dysfunction. Considering the site (coronary sinus) and the extent of release of GOT and HBDH in indeterminate-to-chronic patients, it was surmised that mitochondrial and cell membrane injuries are the earliest events in chagasic disease, and the degenerative mitochondrial and cellular events persist with advanced disease. The detection of inorganic phosphorus and isocitrate dehydrogenase molecules in the serum of indeterminate patients, followed by a positive coronary sinus-femoral artery inorganic phosphate gradient with advancement of chronic disease further supports the

hypothesis of very early and progressive manifestations of mitochondrial metabolic abnormalities in chagasic myocarditis (Carrasco et al. 1997). Collectively, these studies support the hypothesis that mitochondrial metabolic abnormalities are manifested very early in infection and exacerbated during CCM progression.

#### FACTORS CONTRIBUTING TO MITOCHONDRIAL DYSFUNCTION

*T. cruzi* infection elicits substantial biochemical, molecular and immunological insults all of which may adversely affect mitochondria in chagasic myocardium. Invasion by parasite elicits transient elevations in intracellular  $Ca^{+2}$  (Burleigh and Andrews 1995) followed by release of the parasite from the parasitophorous vacuole. The acute phase of infection is then marked by massive replication of the parasite in host cell cytoplasm and activation of potent inflammatory reactions (section "Immune responses: Protective or pathological"). In mammalian cells,  $Ca^{+2}$  overload is known to induce the opening of mitochondrial permeability transition pores, leading to dissipation of the proton gradient (Korge et al. 2001, Kanno et al. 2002). Given, that maintenance of mitochondrial membrane potential and ion transport is essential for oxidative phosphorylation it is likely that *T. cruzi*-induced  $Ca^{+2}$  overload might be the primary signal in mitochondrial dysfunction. This notion is supported by others, who indicate that the elevated levels of  $Ca^{+2}$  contribute to impairment of mitochondrial respiratory enzyme activity (Medrano and Fox 1994, Liang and Molkentin 2002).

Following initial injuries, it is likely that mitochondria may be damaged by ROS originated via inflammatory mechanisms or respiratory chain impairment. The first evidence of oxidative damage to mitochondria was reported utilizing an experimental model of infection and chronic disease (Wen and Garg 2004). This study demonstrated a substantial increase in LPO and PCO derivatives in cardiac mitochondria of infected mice, compared to controls. The LPO derivatives of mitochondrial membranes

were detectable as early as 3 days post-infection, and gradually increased by >2-fold during the course of disease development. The PCO content in cardiac mitochondria became evident during the acute infection phase and remained consistently enhanced throughout the chronic phase of disease progression. ROS-induced LPO and PCO derivatives deposition in mitochondrial membranes is shown to cause increased permeability and loss of mitochondrial membrane potential and protonmotive force (Piper et al. 1994, Vercesi et al. 1997, Cardoso et al. 1999). We anticipate future studies would delineate the mechanistics of mitochondrial oxidative modifications of membrane lipids and proteins in alterations of mitochondrial integrity, dissipation of the mitochondrial membrane potential and protonmotive force, and respiratory chain inefficiency in CCM.

Direct oxidative modification of specific subunits of respiratory complexes may be an underlying means of inactivation of assembled mitochondrial complexes in chagasic hearts (Wen and Garg 2004). Cardiac mitochondria from infected mice were subjected to two-dimensional blue-native gel electrophoresis to resolve the subunits of the respiratory complexes. Carbonylated subunits derivatized with dinitrophenylhydrazine were then detected by immunoblotting and identified by N-terminal Edman sequencing. On the basis of the identity of subunits that were oxidatively modified, different mechanisms were proposed to participate in inactivation of CI and CIII respiratory complexes in chagasic hearts. Of the >42 subunits of CI, carbonyl adducts were primarily detected with NDUFS1, NDUFS2, and NDUFV1, the core subunits of CI complex (Papa et al. 2002, Carroll et al. 2003). Considering that genetic mutations in genes encoding NDUFS1 (Benit et al. 2001), NDUFS2 (Loeffen et al. 2001), and NDUFV1 (Schuelke et al. 1999) and oxidation/nitration of NDUFS2 (Murray et al. 2003) are linked to CI deficiencies in human and bovine hearts, it was surmised that oxidatively modified structural subunits contribute to the inactivation of the assembled CI complex in chagasic

hearts. Among the 11 components of CIII, consistent carbonylation of core proteins (UQCRC1 and most likely UQCRC2), thought to be involved in the cleavage and processing of the targeting presequence of Reiske 2 Fe-2S protein (ISP) (Iwata et al. 1998), was noted in chagasic hearts. The inappropriate processing of ISP by oxidatively modified core proteins may result in incorporation of the misfolded ISP in CIII. Ultimately, the consequences would potentially be the mis-assembly of the catalytic site and inhibition of the enzymatic activity of complex. Further studies would confirm the mechanistics of oxidative stress-induced CI and CIII inactivation in CCM. Nevertheless, the observation of dose-dependent HNE-mediated inhibition of respiratory complexes in the same study supports the idea that oxidative modifications contribute to inactivation of respiratory complexes in chagasic myocardium.

In chronically infected murine hearts, a substantial depletion of mtDNA was demonstrated and in addition was associated with reduced levels of mtDNA-encoded transcripts. These observations imply that a limited biosynthesis of mitochondria-encoded protein subunits may contribute to reduced assembly of respiratory chain complexes in chagasic myocardium (Vyatkina et al. 2004). What may cause mtDNA depletion in chagasic myocardium is not known. Numerous studies strongly support reactive species as playing a prominent role in mtDNA deletions through oxidative damage. Under conditions of oxidative stress, accumulation of significantly higher levels of DNA oxidation product 8-hydroxydeoxyguanosine in mtDNA compared to nuclear DNA and increased degradation of the mutated mtDNA is shown in a variety of *in vitro* and *in vivo* conditions (Palmeira et al. 1997, Williams et al. 1998, Serrano et al. 1999). It is postulated that increased ROS production may lead to mutations and eventually degradation of oxidatively damaged mtDNA, thus accounting for decreased assembly and activity of respiratory complexes in chagasic myocardium.

Taken together, in the animal model of CCM,

mitochondria dysfunction was evidenced at RNA, protein, and possibly DNA levels using several experimental approaches while indirect evidence for mitochondrial dysfunction are provided in human patients. Increased LPO and PCO deposition in mitochondria with disease severity, and oxidative modifications of subunits of the mitochondrial complexes provide strong evidence in support of sustained oxidative stress of mitochondrial origin in chagasic hearts. Future studies would determine whether impaired mitochondrial tolerance due to oxidative stress result in increased vulnerability of mitochondrial DNA and energetics and thereby constitute a mechanism in myocardial dysfunction in CCM.

#### INADEQUATE ANTIOXIDANT RESPONSE AND OXIDATIVE DAMAGE IN CHAGASIC HEARTS

The data discussed so far provides evidence to support the idea that chagasic hearts are likely to be exposed to ROS of inflammatory and mitochondrial origin. The myocardium contains high concentrations of various non-enzymatic and enzymatic scavengers of ROS (Antioxidants) which protects from oxidative damage. However, ROS production may overwhelm the ability of the cell to detoxify these radicals, resulting in ROS-induced oxidative stress. The myocardial cells, when oxidatively stressed, may exhibit saturation of the antioxidant defenses, loss of intracellular redox homeostasis, alterations in cellular signaling, and induction of pathological processes (Hensley et al. 2000, Martindale and Holbrook 2002, Ueda et al. 2002).

A series of recent studies have addressed the oxidative status and antioxidant defense capabilities during the course of infection and progression of chagasic disease in human patients and experimental models. The demonstration of a selenium deficiency that increased with severity of chronic disease in chagasic patients (Rivera et al. 2002) was probably the first observation suggesting that antioxidant deficiencies may be related to the progression of disease pathology. Further studies in experimental CCM models showed that selenium-

depletion was associated with increased susceptibility, myocarditis severity, and heart damage (Gomez et al. 2002), leading to higher mortality rate (de Souza et al. 2002). The myocardial damage in infected mice was arrested or reversed upon dietary supplementation with low doses of selenium (de Souza et al. 2003). In other studies, the detection of inflammatory cytotoxic mediators (TNF- $\alpha$  and NO) along with a reduction in plasma levels of GPx and SOD in patients led to a suggestion that an oxidant/antioxidant imbalance might drive the chagasic disease pathology (Perez-Fuentes et al. 2003). We have shown in an animal model that when antioxidant defense responses (constituted by GPx, GR, and GSH) were of sufficient magnitude (e.g. in skeletal muscle), *T. cruzi*-induced oxidative stress and damage was controlled (Wen et al. 2004). However, myocardium appeared to be poorly equipped with antioxidant defenses. In response to *T. cruzi*, a transient increase in antioxidant enzyme activities (GPx, GR) and reductant (GSH) level was noted in the myocardium of acutely infected mice. However, these antioxidants were static (similar to control level) or decreased during disease development. Consequently, myocardium of infected animals sustained oxidative damage evidenced by consistent increase in oxidative stress biomarkers (LPO, PCO, GSSG) during the course of infection and chronic disease (Wen et al. 2004). It was concluded that the glutathione antioxidant reserve is not depleted in the myocardium, but is not adequate to limit oxidative stress-induced damage during CCM development.

Finally, a consistent decline in MnSOD activity with progression of infection and disease is shown in a murine model (Wen et al. 2004). MnSOD coupled with GPx (mitochondrial and cytosolic) and CAT (cytosolic) is important in minimizing  $O_2^{\cdot-}$  and  $H_2O_2$  levels in the heart. When produced in excess, or when MnSOD activity is not sufficient,  $O_2^{\cdot-}$  participates in iron-catalyzed pathways forming highly reactive and damaging  $\cdot OH$  for which no antioxidant enzyme system exists (Tokoro et al. 1996). Additionally,  $O_2^{\cdot-}$  enhances the toxicity of phagocyte-induced NO that is elicited for parasitic

control in an infected host, by forming peroxynitrite (ONOO<sup>-</sup>) (Sato et al. 1993). Both ONOO<sup>-</sup> and ·OH are known to induce substantial tissue injury and dysfunction by virtue of their ability to nitrosylate and/or oxidize biomolecules (Ide et al. 2001, Katsanos et al. 2002). Authors (Wen et al. 2004) deduced that repression of MnSOD's protective ability contributes to oxidative modifications and dysfunction of respiratory complexes which could lead to a vicious cycle of uncontrolled ROS production. This hypothesis is supported by the observations of a decrease in CI complex-mediated respiration, and an increase in oxidative damage, DNA fragmentation, and cytochrome c release in MnSOD<sup>-/+</sup> mice which exhibit a 50% reduction in MnSOD activity compared to normal controls (Williams et al. 1998, Van Remmen et al. 2001). In MnSOD<sup>-/-</sup> mice neonatal lethality associated with the development of dilated cardiomyopathy and mitochondrial dysfunction provides further evidence for the importance of MnSOD activity in maintaining the integrity of the mitochondrial enzymes susceptible to direct inactivation by O<sub>2</sub><sup>-</sup> (Li et al. 1995).

#### SUMMARY

Sustained ROS generation (of inflammatory and mitochondrial origin) coupled with inadequate antioxidant response resulting in inefficient scavenging of ROS in the heart leads to long-term oxidative stress and subsequently oxidative damage of the cardiac cellular components during chagasic disease. Future studies testing the usefulness of therapies capable of enhancing mitochondrial function, antioxidant efficiency, or ROS scavenging in combination with anti-parasite drugs will provide convincing evidence to link oxidative stress as a causative mechanism in the development of CCM.

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#### RESUMO

Há evidências que sugerem que as miocardites chagásicas são devidas aos danos induzidos pelo estresse oxidativo, podendo contribuir para a evolução da doença de Chagas. Em doenças infecciosas, a formação de espécies reativas do oxigênio (ROS) é, principalmente, derivada de danos celulares mediados pela invasão e replicação do patógeno e por reações citotóxicas mediadas pelo sistema imune. No entanto, como as ROS são formadas e sua função no estresse oxidativo na cardiomiopatia chagásica (CCM) não estão completamente elucidadas. Nesta revisão, nós discutimos as evidências para o aumento do estresse oxidativo na doença de Chagas, com ênfase nas anormalidades mitocondriais, na disfunção da cadeia de transporte de elétrons e seu papel na manutenção do estresse oxidativo no miocárdio. Discutimos ainda, os resultados da literatura que relatam as conseqüências da manutenção do estresse oxidativo na patogênese da CCM.

**Palavras-chave:** cardiomiopatia chagásica, espécies reativas do oxigênio, inflamação, mitocôndria, relação oxidante/antioxidante, danos oxidativos.

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