Propranolol does not affect the oxidative burst of rat neutrophils or complement serum opsonizing capacity in *in vivo* and *in vitro* experiments

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Summary

Propranolol is a ß-adrenergic antagonist used for the treatment of a variety of cardiac conditions and has a palliative value when used in situations in which adrenergic signals and symptoms are involved. It is used as a co-adjuvant in hyperthyroidism to decrease heart rate and output, as well as the tremor. The aim of this work was to study the effect of propranolol treatment on the oxidative burst of rat peripheral blood neutrophils and on the complement system.

In the *in vivo* study, Wistar male rats were treated with propranolol by gavage for 16 days with 220 or 440 μg/animal/day. These doses are equivalent to 80 or 160 mg/adult human (~70 day/kg), respectively. Neutrophils were obtained and stimulated with opsonized immune complexes (opIC). The oxidative burst of control (from water treated rats) or propranolol-treated rat cells was measured by luminol- and lucigenin-dependent chemiluminescence (CL). The CL of treated rat neutrophils was not affected by any of the propranolol doses studied when compared to the control responses.

In the *in vitro* study whole blood and serum from Wistar male rats were incubated for 1 h at 37C° with propranolol at three different concentrations (17.5, 35 and 70 μg/mL). After incubation, neutrophils were isolated from whole blood and stimulated with opIC while treated sera were used to opsonize IC. IC opsonized with treated sera were used to stimulate neutrophils from normal rats. In both cases oxidative burst was measured by luminol- and lucigenin-dependent CL. No differences in responses or activities were detected between *in vitro* treated neutrophils or sera and their respective controls.

These results suggest that this drug, at the concentrations studied and with the experimental approach used, had no effect on the oxidative burst during phagocytosis of opIC or on the complement opsonization capacity of the sera.

Introduction

Phagocytes play a crucial role in host protection against infection by killing invading microorganisms. For this purpose, they are equipped with molecular machinery that works by a series of rapid and regulated responses. Upon activation, neutrophils are able to generate toxic oxygen derivatives

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essential for host defense. Intracellular oxidant production results mainly in the destruction of phagocytosed pathogens whereas the extracellular generation of reactive oxygen species (ROS) mediates inflammatory tissue damage.

Propranolol is a non-specific ß-adrenergic blocker used in the treatment of a variety of vascular and cardiac conditions. It has a palliative value when used in situations involving adrenergic signals and symptoms. This drug is used in the treatment of

hyperthyroidism to decrease the heart rate and output, as well as the tremor due the thyrotoxicosis. Various activities are attributed to propranolol regarding the neutrophils function such as stimulation of the normal and abnormal neutrophil's motility *(Gatner & Anderson, 1980)* and antioxidant and prooxidant properties *(Saleh et al., 2001)*. The literature results are very variable, depending on the stimulus used and on the mechanism of ROS generation.

In phagocytes propranolol inhibits the production of diglyceride from phosphatidic acid *(Koul and Hauser, 1987)*. However, studies on the influence of propranolol on the phagocytosis activity of neutrophils have reported opposite findings. Della Bianca et al. (1991) showed that phagocytosis and respiratory burst were unchanged in neutrophils treated with propranolol while Ohkuro et al. (1995) reported inhibition of CR3-mediated phagocytosis. We have observed enhanced oxidative burst of neutrophils from patients with hyperthyroid Graves disease *(Russo-Carbolante et al, 2001)*. We compared the chemiluminescense (CL) responses of neutrophil phagocytosis of IgG immune complexes when mediated by Fcγ and complement receptors, of these patients and from healthy donors. These patients were taking propranolol at the time of assessment (up to 160 mg/day). Due to the controversial and sometimes opposite results found in the literature depending on each experimental model, in this investigation we studied the effect of propranolol treatment on the oxidative burst of treated neutrophils (*in vivo* and *in vitro*) and on the opsonizing capacity of the complement system *(in vitro)* using serum opsonized immune complexes (opIC) as a cell stimulus. The aim of the study was to use an animal model to assess the oxidative burst mediated by complement receptors of neutrophils from rats subjected to propranolol, to rule out the possibility that the results found in the previous study were due to an action of the propranolol instead of the thyrotoxicosis.

Materials and Methods

Chemicals
Propranolol was purchased from Galena Química e Farmacêutica LTDA, Brazil, as propranolol hydrochloride salt. Histopaque® gradient (1077 mg/mL) was purchased from Sigma Chemical Co (St. Louis, MO, USA)

Animals
Outbred male Wistar rats weighing 180-200 g, 45 days of age, bred in the house-breeding program of the animal house of the University of São Paulo School of Medicine at Ribeirão Preto, were used in all experiments. The animals received water and standard rat chow (Nuvilab CR1 ratos – Nuvital Nutrients S.A., Colombo, PR, Brazil) ad libitum, and were maintained in a controlled temperature $(22 \pm 2 \text{ o C})$ and humidity $(55 \pm 2\%)$ room, with 12 hours of dark and 12 hours of light. No more than five animals/cage were kept in propylene cages of 41x34x16 cm dimensions. These animals supplied polymorphonuclear (PMN) neutrophils and serum for the functional tests.

The total number of animal used in the *in vivo* tests was: 24 treated with 220 μg, 24 treated with 440 μg propranolol/day and 28 controls receiving tap water. All animals were treated for 16 days. The number of animals used in the *in vitro* tests was: 7 treated and 9 controls for the 17.5 μg/mL propranolol concentration test, 10 treated and 9 controls for the 35 μg/mL concentration, and 11 treated and 7 controls for the 70 μg/mL concentration. There is a difference between the number of tests with luminol and lucigenin because sometimes the number of neutrophils obtained from whole blood was not enough to do both tests.

Propranolol treatment and neutrophil isolation

In vivo experiments
Three groups of animals were independently treated

with propranolol for 16 days. Two different doses were tested in each group. The treated rats received propranolol daily, by gavage, as a solution with the desired dose to be tested. Two drug doses were used: 220 and 440 μg/animal/day. The control animals received tap water with the same scheme of treatment. These doses are equivalent to 80 and 160 mg/adult/day (~70 kg), respectively, used by hyperthyroid human patients. After 16 days of treatment the animals were killed by decapitation and blood was collected into sodium-EDTA tubes. Rat neutrophils were isolated from whole blood using Histopaque® 1077 gradient as described elsewhere *(Russo-Carbolante et al., 2002)*, the number of cells was determined manually in a Neubauer chamber and adjusted to 2 x 10^6 cell/mL in Hank's balanced solution, pH 7.2, with 0.1% gelatine. Next, the cells were stimulated with opIC.

In vitro experiments
Normal untreated rats were bled by decapitation to supply PMN neutrophils and serum for the *in vitro* propranolol incubations or untreated PMN and serum (controls). Three series of independent experiments were carried out.

The three propranolol concentrations tested were 17.5, 35 and 70 μg/mL.

a-Cells: Each concentration of the drug was incubated for 1 hour in a water bath at 37ºC with anticoagulated whole blood. After the incubation period the PMN were isolated using a Histopaque® gradient and the number of cells was determined and adjusted to 2 x 10^6 cell/mL in Hank's balanced solution, pH 7.2, with 0.1% gelatine. Complement-opsonized IC were then used to stimulate the cells and the oxidative metabolism of the latter was assessed by chemiluminescence production.

b-Sera: To study the influence of this drug on serum complement we used the same concentrations as used in the cell study. The serum and drug mixture was incubated for 1 hour at 37ºC. The concentrations used were based on the mean of the normal rat hematocrit range *(Wolford et al, 1986)*. These sera were then used to opsonize IC that ulti-

mately worked as a stimulating agent of normal neutrophil oxidative metabolism.

Viability tests carried out with Trypan blue exclusion indicated no more than 9% of nonviable cells present in every cell suspension used in the experiments.

For each of these *in vitro* treatments there was a control (cell or serum) consisting of the cells or serum from the same animal as that used in the tests. The controls were submitted to the same incubation, dilution and processing using 0.15 mol/L NaCl instead of the drug solution.

Chemiluminescence assays
Chemiluminescence generation is a sensitive indicator of oxidative metabolism *(Allen et al., 1972)* and the present assays were performed as described by Cheung et al., (1983). Rat neutrophils (1x10^6 for the *in vivo* test or 5x10^5 for the *in vitro* test) diluted in Hanks' balanced salt solution, pH 7.2, were mixed with 60 μg of opIC in the presence of either luminol or lucigenin in a final volume of 1mL. The luminol- or lucigenin-dependent chemiluminescence (CL) responses were monitored at 37º C for 10 minutes with an Auto Lumat LB 953 luminometer from EG&G Berthold (Bad Wildbad, Germany). The results are expressed as the area under the curves of the CL profiles. The CL profile is illustrated in Figure 1 as cpm/minutes.

Statistical Analysis
Statistical analysis of the difference between control and *in vivo* treated animals was performed by one-way Anova with Dunnet's post test. For analysis of the *in vitro* tests, the Paired-*t* test was used. P<0.05 was considered significant.

Results

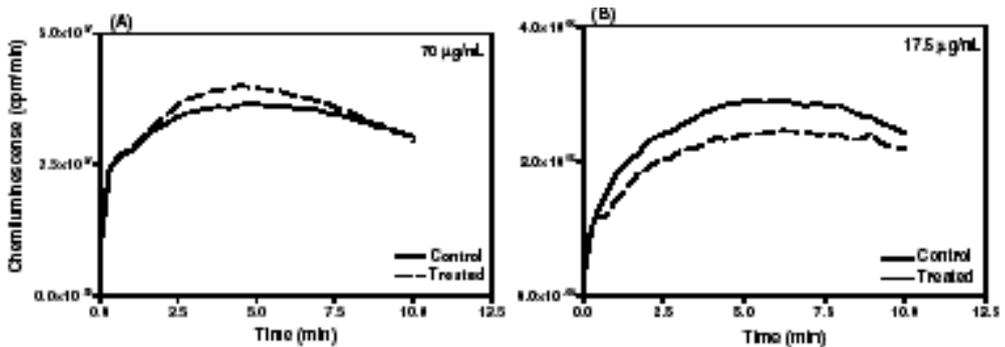*Kinetics of CL production by rat neutrophils treated in vitro or in vivo with propranolol*
The kinetics of CL production from each experimental protocol is presented in Figures 1 and 2 as cpm/minutes for each animal.

Figure 1 presents the CL profiles for treated and control neutrophils at both doses of *in vivo* propranolol treatment. The results indicate the same efficiency of stimulus binding to the cells to induce NADPH-oxidase activity. However, there could be differences in the complement receptor expression and/or function, even though the total CL produced were similar.

Figure 2 presents the kinetics of CL production from the *in vitro* tests as cpm/minutes, showing the CL profiles for treated and control neutrophils exposed *in vitro* to propranolol. The results indicate the same efficiency of stimulus binding to the different treated and control cells to induce NADPH-oxidase activity.
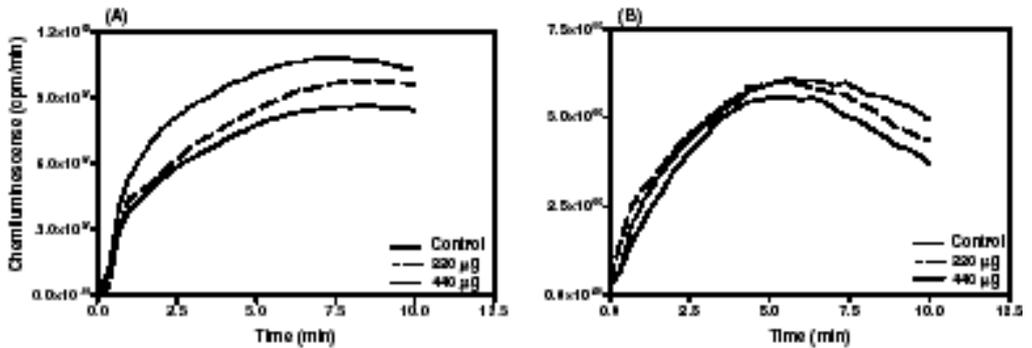
Figure 1. Representation of the kinetic profiles of CL production by rat neutrophils stimulated with opIC. The animals were treated with two different doses of propranolol for 16 days. Neutrophils (1×10^6) were incubated with 60 μg opIC in 1 mL Hanks' solution, pH 7.2, 0.1% gelatine and 10^{-4} M luminol (A) or lucigenin (B).

Figure 2. Representation of the kinetic profiles of CL production by rat neutrophils stimulated with opIC. Neutrophils (5×10^5) were exposed *in vitro* to different concentrations of propranolol. The cells were incubated with 60 μg opIC in 1 mL Hanks' solution, pH 7.2, 0.1% gelatine and 10^{-4} M luminol (A) or lucigenin (B). In A cells were incubated with 70 μg/mL while in B the drug concentration was 17.5 μg/mL.

Effect   of   propranolol   on   cellular chemiluminescence

CL values were calculated by the area under the curve (AUC) of the CL profile over a time period of 10 min. The absolute values of the AUC for control and *in vivo* treated cells with their means are presented in Figure 3. The values in Figure 4 are related to the *in vitro* treated cells and their controls.

The data show that propranolol had no significant effect on the oxidative burst of rat neutrophils during phagocytosis of complement-opIC at any of the *in vivo* (220 and 440 µg/animal/day) or *in vitro* (17.5, 35 and 70 µg/mL) concentrations studied (one-way Anova with Dunnet's post test and paired *t*-test, respectively).

Effect of propranolol on serum opsonization capacity

CL values were calculated by the area under the curve (AUC) of the CL profile over a time period of 10 min. The absolute values of the AUC for the normal PMN stimulated with IC opsonized by control and by *in vitro* treated sera, with their means, are presented in Figure 5.

Our data show that propranolol had no significant effect on the complement opsonizing activity at any of the *in vitro* concentrations studied (17.5, 35 and 70 µg/mL) as seen by the same capacity of the IC opsonized with the different sera to stimulate the oxidative burst of normal rat neutrophils (Paired *t*-test).

Discussion

One of the most important functions of neutrophils is the host defence against bacterial infection, a role based on functions such as phagocytosis, degranulation of proteases and generation of reactive oxygen species (ROS). Phagocytosis can be triggered by ligands that bind to receptors for the Fc region of IgG (FcγRII and FcγRIII) and to receptors for complement fragment C3b and its breakdown product iC3b (CR1 and CR3). These complement and Fc receptors have a synergistic action during the phagocytic response of human neutrophils *(Okuro*

et al., 1995; Sutterwala et al., 1996). Some ligands are able to trigger phagocytosis and/or to generate burst depending on which receptor they bind to. Ligands such as chemoattractants (lipids and peptides) induce chemotaxis and trigger the respiratory burst. Cytokines are proteins secreted by inflammatory cells, which prime neutrophils but require binding to an additional unrelated receptor to trigger the respiratory burst. Some phagocytic stimuli bind to CR1 and FcR, activating the oxidative burst concomitantly with the phagocytosis of the particles to which they are attached (McPhail et al., 1992). The mechanism of activation of the oxidative burst can be different for each stimulus. Some transduction pathways are mediated by protein G and are pertussis toxin sensitive and others are insensitive. Phospholipases A_2, C and D and tyrosine kinases are among the transduction pathways. CR1 and CR3 mediate phagocytosis but do not activate directly NADPH oxidase *(Ahearn and Fearon, 1989; Newman and Johnston, 1979)*.

Hyperthyroidism determines a net enhancement in the oxidant capacity of PMN *(Russo-Carbolante et al., 2001)*. The oxidative stress induced by thyrotoxicosis can aggravate free radical mediated tissue injury *(Asayama et al., 1987; Pereira et al., 1994)*. Propranolol is used in hyperthyroidism to decrease the heart rate and output, as well as the tremor due the thyrotoxicosis. In this investigation we studied the effect of propranolol treatment on the oxidative burst of neutrophils, in an animal model, to assess the oxidative burst mediated by complement receptors of neutrophils from rats subjected to propranolol. This approach can help to attribute the high CL responses found in those patients to the thyrotoxicosis state and rule out an action of propranolol in this system.

The CL produced by stimulated PMN in the presence of luminol or lucigenin depends on the formation of ROS by the cell. There are suggestions in the literature of both antioxidant or pro-oxidant properties of propranolol *(Saleh et al., 2001)*. In the present study, with the *in vitro* and *in vivo* experimental design described, we did not find a reduction or

enhancement of the oxidative burst of neutrophils treated with propranolol. Also, no change was observed in the capacity of opsonization of treated sera. Since ROS can be generated via different molecular mechanisms, the observed differences reported in the literature in response to propranolol may be dependent on the mechanism of ROS generation. Differences in drug concentration used, *in vivo* and *in vitro* experimental conditions, species variation and sensitivity among cell types, routes of administration, procedures and methods must be also considered.
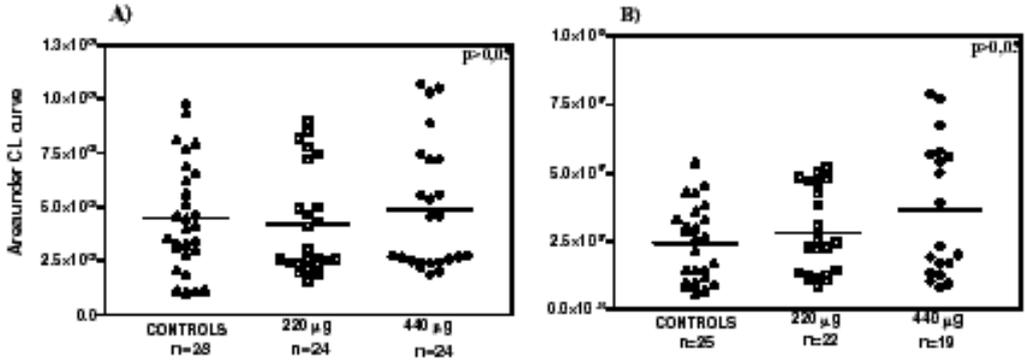
Figure 3. Effect of different concentrations of propranolol on cellular CL of rat neutrophils. Cells from 16 days treated rats (1 x 10^6) were stimulated with 60 μg opIC in 1 mL Hanks' solution, pH 7.2, 0,1% gelatine and 10^{-4} luminol or lucigenin at 37^0 C for 10 min. CL was calculated as AUC of the profile. Sera to opsonize IC were a pool of NRS diluted 1:2 in CFD/Mg+^{2}/gelatine: A) Luminol-dependent CL; B) lucigenin-dependent CL. No significant differences were observed. Statistics: Anova one way, Dunnett's post test.
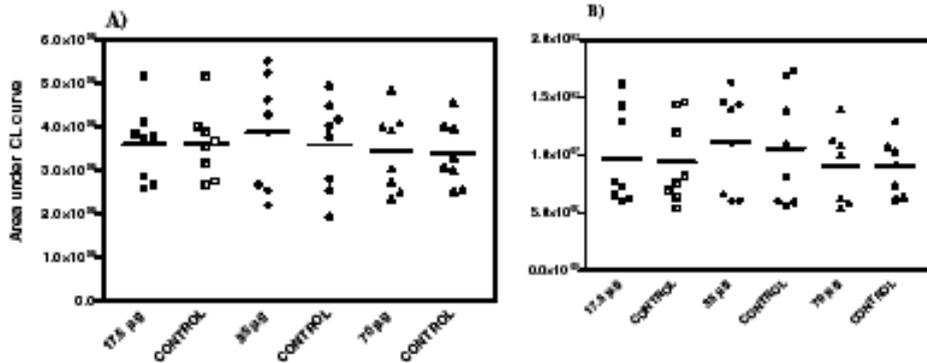
Figure 4. Control and treated cells (5 x 10^5) were stimulated with opIC in 2 mL Hanks' solution pH 7.2, 0.1% gelatine and 10^{-4} luminol or lucigenin at 37^0 C for 10 min. CL was calculated as AUC of the profile. The control cells were incubated with NaCl 0.15 M. Sera to opsonize IC were a pool of NRS 1:2 in CFD/Mg+^{2}/gelatine. A) Cells *in vitro* treated with propranolol; Luminol-dependent CL; B) Cells treated in vitro with propranolol; Lucigenin-dependent CL. No significant differences were observed. Statistics: Paired test.
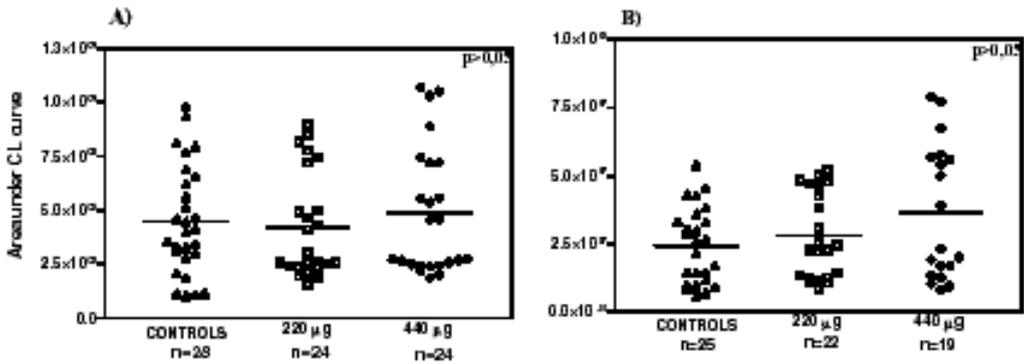
Figure 5. Normal cells (5 x 10^5) were stimulated with opIC in 2 mL Hanks' solution pH 7.2, 0.1% gelatine and 10-^{4} luminol or lucigenin at 37°C for 10 min. CL was calculated as AUC of the profile. Sera to opsonize IC were treated *in vitro* with propranolol in three different concentrations and diluted 1:2 in CFD/Mg^{+2}/gelatine: A) Serum *in vitro* treated with propranolol; Luminol-dependent CL; B) Serum *in vitro* treated with propranolol; lucigenin-dependent CL. The control sera were incubated with 0.15 M NaCl. No significant differences were observed. Statistics: Paired test.

Although at *in vitro* concentrations of 70 μg/mL the results were at the boundary of significance, this is a very high concentration that does not apply to normal pharmacological use of this drug.

In conclusion, the present results show that this drug at the studied concentrations and with the experimental approach used have no effect on the oxidative burst mediated by Fcγ and complement receptors during the neutrophil phagocytosis of complement-opIC when these cells are exposed to the drug *in vitro* or *in vivo*. Moreover, the drug had no effect on the opsonization capacity of the sera.

Acknowlegments

References

Ahearn JM & DT Fearon: Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21). Adv. Immunol. 1989, *46*, 183-219.

Allen RC, RL Stjernholm & RH Steele: Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. 1972, *47*, 679.

Asayama K, K Dabashi, H Hayashibe, Y Megata & K Kato: Lipid peroxidation and the free radical scavengers in thyroid dysfunction in the rat: A possible mechanism of injury to heart and skeletal muscle in hyperthyroidism. Endocrinology 1987, *121*, 2112-2118.

Cheung K, AC Archibald & MF Robinson: The origin of chemiluminescence produced by neutrophils stimulated by opsonized zymosan. J. Immunol. 1983, *130*, 2324-39.

Della Bianca V, M Grzeskowiak, D Lissandrini & F Rossi: Source and role of diacylglycerol formed during phagocytosis of opsonized yeast particles and associated respiratory burst in human neutrophils. Biochem Biophys Res Commun, 1991, *177*, 948-955.

Gatner EMS & R Anderson: An *in vitro* assessment of cellular and humoral immune function in pulmonary tuberculosis: Correction of defective neutrophil motility by ascorbate, levamizole, metoprolol and propranolol. Clin. Exp. Immunol. 1980, *40*, 327-336.

Koul O & G Hauser: Modulation of rat brain cytosolic phosphatidate phosphohydrolase: effect of cationic amphiphilic drugs and divalent cations. Arch. Bioch. Byophys. 1987, *253*, 456-61.

McPhail LC, SL Strum & PA Leone: The neutrophil respiratory burst mechanism. Immunol Ser 1992, *57*, 47-56.

Newman SL & RB Johnston Jr: Role of binding through C3b and IgG in polymorphonuclear neutrophil function: studies with tripsin-generated C3b. J Immunol. 1979, *123*, 1839-1846.

Ohkuro M, M Ogura-Masaki, K Kbayashi, M Sakai, K Takashi & S Nagasawa: Effect of iC3b binding to immune complexes upon the phagocytic response of human neutrophils: synergistic functions between FcgR and CR3, FEBS Lett. 1995, *373*, 189-192.

Pereira B, LF Rosa,DA Safi, EJ Bechara & R Curi: Control of superoxide dismutase, catalase and glutathione peroxidases activities in rat lymphoid organs by thyroid hormones. J Endocrinol, 1994, *140* 73-77.

Russo-Carbolante EMS , Polizello ACM, Azzolini AECS & Luciano-ValimYM: Assessement of the oxidative burst and the CR1, CR3, FcgRII, FcgRIII in emcocytes from Grave's disease patients. Molecular Immunology, 2001, *38* (2-3)120.

Russo-Carbolante EMS, AECS Azzolini, AMC Polizello & YM Lucisano-Valim: Comparative study of four isolation procedures to obtain rat neutrophils. Comparative Clinical Pathology 2002, *441* (11), 71-76.

Saleh S, HY Aboul-Enein, R Parhar, K Collison, & T Al-Mohan: Ca^{+2} - dependent production of reative oxygen metabolites by human neutrophils in response to fluorinated propranolol analogues. Biochem. Pharmacol. 2001, *61*, 517-525.

Sutterwala FS, LA Rosenthal & DM Mosser: Cooperation between CR1 (CD35) and CR3 (CD11b/CD18) in the binding of complement-opsonized particles. J. Leukocyte Biol. 1996, *59*, 883-890.

Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, Ruhren R: Reference range data base for serum chemistry and haematology values in laboratory animals. Journal of Toxicology and Environmental Health. 1986, *18*, 161-188.