ELSEVIER

International Immunopharmacology 3 (2003) 1439–1445

International Immunopharmacology

www.elsevier.com/locate/intimp

Digitalis inhibits and furosemide does not change the in vitro phagocytic function of neutrophils of healthy subjects

Maria Imaculada Muniz-Junqueira[a,*], Lícia Maria Mota[a], Rodrigo Barbosa Aires[a], Luiz Fernando Junqueira Jr.[b]

[a] *Laboratory of Cellular Immunology, Pathology Area, School of Medicine, University of Brasilia, 70.910-900 Brasilia, DF, Brazil*
[b] *Division of Cardiology, Brasilia University Hospital, University of Brasilia, Brasilia, DF, Brazil*

Received 25 February 2003; received in revised form 21 April 2003; accepted 21 May 2003

Abstract

This work evaluated the in vitro influence of digitalis and furosemide on the phagocytic function of neutrophils of healthy individuals. Phagocytosis of *Saccharomyces cerevisiae* by peripheral blood neutrophils of 20 healthy individuals was assessed in the absence or presence of deslanoside or furosemide. The Wilcoxon test was employed to compare the data expressed as median and extreme values. Digitalis reduced the number of yeasts ingested by neutrophils (2.23, 1.23–4.01 versus 1.89, 0.87–2.79; $p=0.019$). It did not influence the percentage of these cells engaged in phagocytosis, although there was a tendency for reduction (71%, 23–95% versus 57%, 8–93%; $p=0.11$), which resulted in decreasing the phagocytic index from 192 (30–381) to 125 (10–218) ($p=0.028$). Furosemide had no significant influence on the number of *S. cerevisiae* phagocytosed (2.23, 1.23–4.01 versus 1.96, 0.70–4.45; $p=0.89$), the percent of phagocytosing neutrophils (71%, 23–95% versus 73%, 9–96%; $p=0.86$) and the phagocytic index (192, 30–381 versus 152, 10–428; $p=0.95$). These findings indicate the inhibitory influence of digitalis on in vitro neutrophil phagocytic function of healthy subjects, and suggest that this effect might impair the innate immune defense response. On this basis, they could contribute to improve digitalis therapy and advise that this drug class should not be associated with other drugs that may also impair the immune function, or might be used with caution or even avoided in subjects with infections.

Keywords: Digitalis; Furosemide; Neutrophils; Phagocytosis; Heart failure

1. Introduction

Neutrophils play an important role in defense against extracellular pathogens by means of phagocytosis clearing these bacteria from blood stream and infected tissues [1]. Patients with deficiencies in number or function of phagocytes are prone to develop pyogenic infections that might be life-threatening [2]. Moreover, secondary immunodeficiencies caused by inadequate function of these cells may complicate several diseases [3].

The complex process of phagocytosis is triggered by the initial interaction between the phagocyte and the particle to be ingested. After this phagocyte–particle

* Corresponding author. Tel.: +55-61-2342159; fax: +55-61-2733907.
E-mail address: mimjunqueira@unb.br (M.I. Muniz-Junqueira).

1567-5769/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1567-5769(03)00142-5

contact, intracellular signals are triggered with cytoskeletal rearrangement, alterations in membrane trafficking, activation of microbial killing mechanisms, and production of pro- and anti-inflammatory cytokines and chemokines [4]. Initial interaction of the particle and the phagocyte occurs through several receptors, whose engagement can induce a complex series of intracellular events that may subsequently activate or inhibit biologic cell responses [4].

There are several data suggesting that digitalis and diuretics may influence immune function. It was observed that digoxin might impair mobilization of neutrophils during pneumococcal pneumonia in mice [5]. In addition, digitalis may stimulate calcium influx to cell [6], providing calcium intracellular for the contractile protein performs phagocytosis. The diuretic furosemide may decrease the production of the pro-inflammatory cytokines TNF-α, IL-6, IL-8 by peripheral blood mononuclear cells, and high concentration of this drug in culture may be cytotoxic for phagocytes [7]. It may also block Cl^- transport altering the ion transport through cell membrane [8]. These are essential steps on phagocytosis process, and alterations in these aspects may influence phagocyte functions.

Congestive heart failure is a very common clinical condition in which digitalis and furosemide drugs are often employed. This syndrome has long been observed to be associated with infections due to *Streptococcus pneumoniae* [9], and with increased frequency of extracellular bacteria pulmonary infections [10]. In addition, patients with congestive heart failure and/or in use of digitalis have more risk for community-acquired pneumonia according to some epidemiological surveys [11,12]. Several factors often associated with heart failure, such as pulmonary edema, hypoxemia and dehydration, can disrupt lung-host defense and enhance susceptibility to infections with bacterial pathogens [13]. However, it has been observed that such factors are frequently absent in patients with pneumococcal pneumonia [14], while the use of digitalis and furosemide appears to be usual among congestive heart failure patients with acute bronchus-pulmonary infection [14]. Although cardiac disease is recognized as a high-risk condition to secondary pulmonary infection, the mechanism by which it predisposes to infections it is not fully established [13].

It remains still unclear the influence of digitalis and furosemide on phagocytosis, which represents the first line of defense of organism against extracellular pathogens. Therefore, in the present work, we aimed to evaluate the in vitro influence of the digitalis drug class deslanoside and the furosemide on the phagocytic function of neutrophils of healthy adult individuals. Such evaluation may be important in understanding possible effects on phagocytes in patients treated with these drugs.

2. Material and methods

2.1. Individuals and blood samples

Peripheral venous blood samples were obtained from 20 healthy adult volunteer subjects, 13 men and 7 women, aged 18–45 years (24.6 ± 7.8 years), for in vitro evaluation of neutrophil phagocytic function. Possible influencing factors on the immunological functions were ruled out in the evaluated subjects and none was using any drug.

The ethical rules of the Helsinki Declaration and those from the Brazilian National Council of Health for experimentation in human beings were strictly followed throughout this work. The Human Research Ethical Committee of the School of Medicine of the University of Brasilia approved the experimental protocol, and each volunteer gave written informed consent for blood donation and participation. This study was exclusively designed and conducted with full autonomy by the authors, and no conflict of interest was present.

2.2. Phagocytosis evaluation and test of drugs

Phagocytosis of *Saccharomyces cerevisiae* was adapted from the technique previously described by Brandi [15]. Briefly, samples of 40 μl per area of whole heparinized peripheral blood obtained by means of venopuncture from each subject were placed on clean glass slides containing eight marked areas of 7-mm diameter each, in duplicate preparations, and incubated in a wet chamber for 45 min at 37 °C. The slides were then rinsed with 0.15 M phosphate-buffered saline (PBS), pH 7.2, at 37 °C, to remove nonadherent cells. Adhered cells ($12{,}534 \pm 5050$

cells; 93.5 ± 1.08% neutrophils) were or were not incubated for 30 min with therapeutic concentration of deslanoside (Sandoz) (2 μg/l) [16] or furosemide (Hipolabor) (8 mg/l) [17] in Hanks–Tris solution. After 30-min incubation, slides were washed, and treated or control neutrophils were incubated for 30 min with a suspension of 2.5×10^5 *S. cerevisiae* in 20 μl Hanks–Tris solution (Sigma, St. Louis, MO, USA), pH 7.2, with 10% human serum from the donor individual, and with the same previously described drug concentrations, in a wet chamber at 37 °C, to allow phagocytosis. Slides were rinsed with 0.15 M PBS at 37 °C to get rid of non-phagocytosed *S. cerevisiae* and the final washing was made with 30% serum in Hanks–Tris. The slides were fixed with absolute methanol and stained with 10% Giemsa solution. The number of *S. cerevisiae* bounded to or ingested by 200 neutrophils in individual preparations was assessed by optic microscopy. Microscopic fields distributed throughout the slide were randomly selected and all neutrophils in each particular field were examined. The phagocytic index was calculated as the average number of attached plus ingested *S. cerevisiae* per phagocytosing neutrophils multiplied by the percentage of these cells engaged in phagocytosis [18].

Digitalis drug was used in the concentration of 2 μg/l to test the effect on the phagocytic function of neutrophils, considering that pharmacokinetic studies had shown that the upper limit range of therapeutic concentration of the drug is between 1.7 and 2.5 μg/l [16], and that some subjects may present manifestations of toxicity when digitalis attains plasma level above 2 μg/l [16,19]. In addition, phagocytes were treated for 30 min with digitalis before adding *S. cerevisiae* to be phagocytosed, considering that after a single therapeutic dose administered to subjects with a variety of conditions, a peak level of about 2 μg/l of the drug remains for approximately 30 min, and after 1.5–2 h, the concentration of the drug decreases to a very small value of 10 times lower, which remains for 16–36 h [20]. Furthermore, mature neutrophils have a small span of life, remaining in blood stream for only a few hours (6–8 h) [21].

Furosemide also presents a very similar pharmacokinetic. After a single therapeutic dose administered to healthy individuals, a peak level of about 8 mg/l of the drug is attained, which remains for approximately 30 min. One hour after the administration, the serum level falls to lower than 1 mg/l, remaining at this level for a few hours (7–8 h) [22].

2.3. Yeast preparation

Baking yeast (*S. cerevisiae*) was prepared according to the technique of Lachmann and Hobart [23]. In short, 50 g of fresh live yeast (Fleischmann, Brazil) was suspended in 220 ml of PBS, pH 7.2, autoclaved at 120 °C for 30 min, washed in PBS until a clear supernatant was obtained, and the sediment was suspended in 28 ml of a 0.1 M 2-mercaptoethanol solution in PBS. After 2-h incubation with stirring, yeasts were washed again and suspended in 55 ml of 0.02 M iodoacetamide in PBS. After additional 2-h incubation at room temperature with stirring, they were washed three times, and suspended in 220 ml of PBS, pH 7.2. Yeasts were again autoclaved, washed and suspended in 110 ml of veronal-buffered saline, pH 7.2, containing sodium azide, and stored at 4 °C until use. For each experiment, yeast suspension were washed in PBS, quantified and sensitized with 10% fresh serum from the donor individual in Hanks–Tris solution for 30 min at 37 °C.

2.4. Statistical analysis

The variables expressing the phagocytic function were previously tested employing the Kolmogorov–Smirnov test for the normality of their distribution, before paired comparative analysis. Considering that some distributions were nonnormal, the Wilcoxon nonparametric statistic test was uniformly employed to compare pair-wise samples in the absence or presence of the drugs, and the statistics are expressed as median, quartiles and extreme values. The Spearman's rank correlation coefficient (r_s) was estimated to evaluate the correlation between the phagocytic index and age of the subjects. Differences with a two-tail value of $p < 0.05$ were considered statistically significant. The Prism® software package (GraphPad, USA, 1997) was employed for analysis and graphical design of the data.

3. Results

No significant correlation was found between the in vitro phagocytic index of neutrophils and the age of

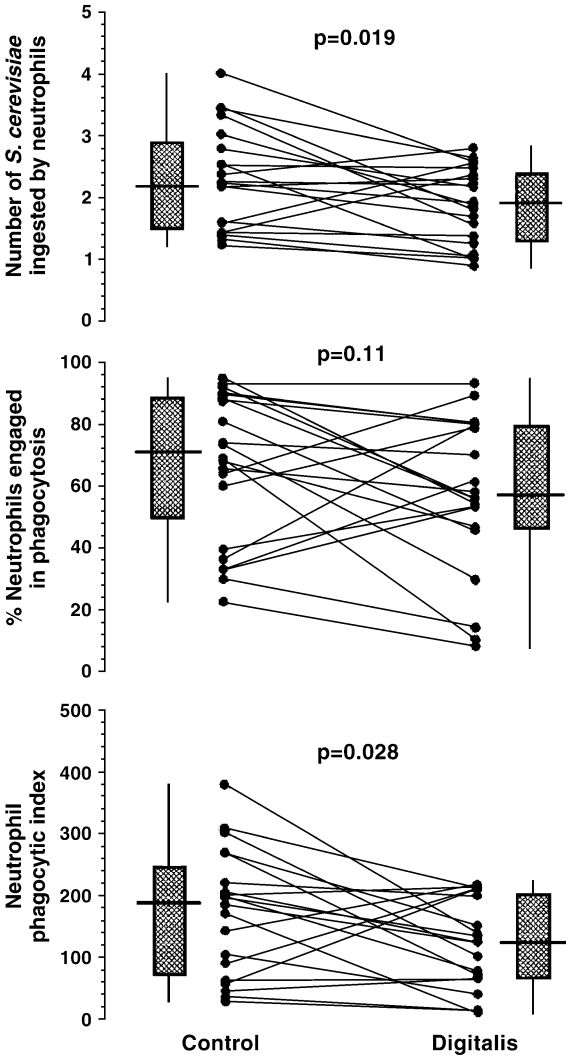

Fig. 1. In vitro influence of digitalis on phagocytic capacity of neutrophils of 20 healthy adult individuals. Individual values are shown for the number of *S. cerevisiae* (2.5×10^5 per marked area) ingested by phagocytes (top), proportion of phagocytes engaged in phagocytosis (middle), and the respective phagocytic index (bottom). Median, upper and lower quartiles and extreme values are also shown. The effects of the drug were tested by the Wilcoxon test.

the subjects from which the blood samples were obtained for control ($r_s = 0.03$, $p = 0.89$) and treated with digital ($r_s = 0.15$, $p = 0.53$) or furosemide ($r_s = -0.27$, $p = 0.25$) groups.

The phagocytic function of neutrophils observed comparatively in the absence or presence of digitalis is illustrated in Fig. 1. The median (extremes) of phagocytic index of neutrophils of healthy subjects significantly decreased from 192 (30–381) in the absence of digitalis to 125 (10–218) in the presence of therapeutic concentration of the drug ($p = 0.028$) (Fig. 1, bottom). This reduced phagocytic index was

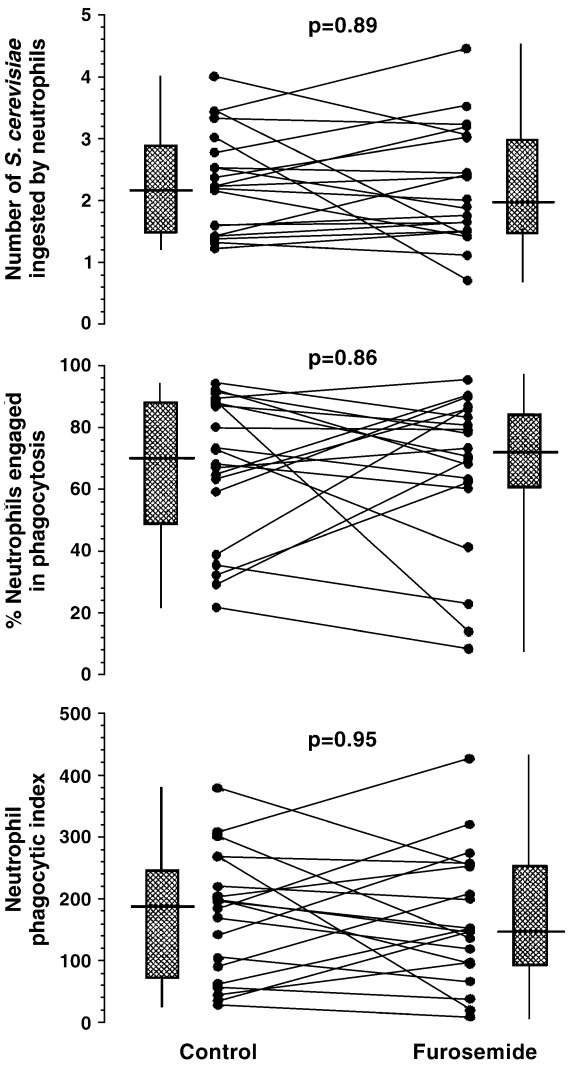

Fig. 2. In vitro influence of furosemide on phagocytic capacity of neutrophils of 20 healthy adult individuals. Individual values are shown for the number of *S. cerevisiae* (2.5×10^5 per marked area) ingested by phagocytes (top), proportion of phagocytes engaged in phagocytosis (middle), and the respective phagocytic index (bottom). Median, upper and lower quartiles and extreme values are also shown. The effects of the drug were tested by the Wilcoxon test.

due to the lower number of ingested *S. cerevisiae* that decreased from 2.23 (1.23–4.01) to 1.89 (0.87–2.79) ($p=0.019$) (Fig. 1, top). There was only a tendency for decrease, without statistical significance, of the number of neutrophils engaged on phagocytosis, from 71% (23–95%) to 57% (8–93%) ($p=0.11$) (Fig. 1, middle). Phagocytic function of neutrophils observed in the absence or presence of furosemide is comparatively illustrated in Fig. 2. Differently of digitalis, furosemide had no influence on median and extreme values of phagocytic index (192, 30–381 versus 152, 10–428) ($p=0.95$) (Fig. 2, bottom), percentage of neutrophils engaged in phagocytosis (71%, 23–95% versus 73%, 9–96%) ($p=0.86$) (Fig. 2, middle) and number of *S. cerevisiae* ingested by neutrophils (2.23, 1.23–4.01 versus 1.96, 0.71–4.45) ($p=0.89$) (Fig. 2, top).

4. Discussion

The objective of the present work was to evaluate the in vitro influence of the often-employed digitalis and furosemide drugs on the phagocytic function of neutrophils of healthy individuals in order to contribute to better understand the action of these therapies in different clinical settings. Our data showed that cardiac glycoside deslanoside in therapeutic concentration decreased the in vitro phagocytic capacity of neutrophils of healthy adult individuals by 35% after about 1-h incubation with these cells. Differently, furosemide had no influence on phagocytic function of neutrophils. There is only one work assessing the influence of digitalis on phagocytosis by neutrophils of healthy individuals, by Esposito et al. [24], and they were unable to demonstrate this effect of the cardiac glycoside on phagocytes, possibly due to the very small number of individuals (only three) evaluated.

The present findings suggest that unfavorable consequences, as predisposition to infections, might result in individuals under treatment with digitalis due to inadequate phagocytosis by neutrophils, adding another factor to those already influencing neutrophil function due to the clinical condition under treatment, as for example, the heart failure syndrome. Treatment with deslanoside might constrain neutrophils to cope with bacteria that depend on these phagocytes for clearing up the infection. In fact, it has been described in mice that digitalis may impair the host defense against *S. pneumoniae* [13]. Such findings may broaden the understanding of the increased susceptibility of patients with congestive heart failure to infections with *S. pneumoniae* [14], and the increased frequency of extracellular bacteria pulmonary infections in these patients [10]. The inhibitory influence of digitalis on phagocytes may concur to decrease the clearance of these bacteria from blood stream and pulmonary tissue by neutrophils. This fact suggests that, in addition to the disturbed pulmonary blood flow due to congestive heart failure, the associated phagocytic function deficiency might also contribute to impair pulmonary defense against bacteria, increasing the susceptibility to infections, as observed in these individuals. In addition, congestive heart failure may be associated with altered cytokine production [25,26], and the deregulated production of cytokines may alter phagocyte functions [27]. Thus, digitalis drugs may superimpose its inhibitory influence on phagocytes whose function has been previously altered in consequence of the subjacent clinical condition. Furthermore, several antibiotics currently used to treat infections in clinical medicine may influence phagocytosis, by decreasing or increasing this function [28]. Based on these observations, the inhibition of phagocytic function should be taken into consideration when it is necessary to prescribe therapy against infections in patients under treatment with cardiac glycoside to avoid possible summing undesirable side effects of different drugs, such as antibiotics and digital, in the same patient.

These considerations on the heart failure are only speculative, considering the findings of neutrophils derived from healthy subjects, since we did not study subjects with this syndrome to evaluate directly the possible effects of digitalis and furosemide on the phagocytic function of these immune cells. There are several concerns to study individuals with this syndrome in order to evaluate the influence of these drugs without bias. Different etiological agents or factors can cause heart failure, such as infectious (Chagas' disease, viruses), toxic, autoimmune, atherosclerotic, and others [10,29–31], able to differently influence immune function [32–34, Muniz-Junqueira et al., submitted for publication]. Furthermore, it has been observed that the presence of heart failure per se may

influence immune function, due possibly to a deregulated production of cytokines [25,26]. In addition, patients with heart failure are usually concomitantly treated with several other drugs [35].

Additionally, it should be pointed out that we chose to study young healthy adult individuals to evaluate the influence of digitalis and furosemide on phagocytic function in order to avoid possible age-related intervening factors able to interfere with phagocytosis by neutrophils. Although the higher prevalence of heart failure is observed in elderly individuals, there are several inconveniences in evaluating immune function in the elderly, due to the underlying pathological processes and nutritional alterations often present in these individuals [36], which may influence phagocytosis by neutrophils [37,38]. Furthermore, heart failure syndrome may occur in any age in dependence of its etiology. Those caused by congenital heart disease, acute and chronic rheumatic cardiopathy, Chagas' heart disease, different primary or secondary cardiomyopathy and virus myocarditis predominate in young individuals [10,39].

On the other hand, with respect to furosemide, we found that this diuretic drug had no in vitro influence on phagocytic response after incubation with neutrophils. Differently of digitalis, the phagocytes of healthy subjects treated with furosemide showed variable individual phagocytic response, resulting in no effect on median of the sample examined.

Concluding, our data showed that cardiac glycoside inhibits the in vitro phagocytic function of neutrophils of healthy subjects. This fact needs to be considered when prescribing this drug, particularly in patients with congestive heart failure, because it may impair the most important innate immune response against bacteria invasion, to which these subjects are more susceptible. Our findings can contribute to broaden the understanding of the relationship between phagocytes and two drugs often used in clinical medicine. In the case of the digitalis, it is suggested that this drug may impair the phagocytic function by decreasing the uptake of the particle to be phagocytosed, which represents the first line of defense against several pathogens that complicate the outcome of individuals treated with this therapy. The knowledge of this side effect of digitalis might improve the employment of this drug and, consequently, the outcome of individuals treated with it.

Acknowledgements

The authors gratefully acknowledge Miss Simone Junqueira Medeiros for reviewing the English language of the manuscript, and Mrs. Renê Oliveira Pires and Mr. José Siqueira for technical assistance. Dr. Lícia Maria Mota and Dr. Rodrigo Barbosa Aires were fellowship recipients at the Laboratory of Cellular Immunology supported by PIBIC-UnB/CNPq.

References

[1] Gotschlich EC. Immunity to extracellular bacteria. In: Paul WE, editor. Fundamental immunology. New York: Raven Press; 1993. p. 1287–308.
[2] Lekstrom-Himes JA, Gallin JI. Immunodeficiency diseases caused by defects in phagocytes. N Engl J Med 2000;343:1703–14.
[3] Shearer WT, Anderson DC. The secondary immunodeficiencies. In: Stiehm ER, editor. Immunological disorders in infants and children. Philadelphia: Saunders; 1989. p. 400–38.
[4] Underhill DM, Ozinsky A. Phagocytosis of microbes. Complexity in action. Annu Rev Immunol 2002;20:825–52.
[5] Esposito AL, Poirier WJ, Clark CA. The cardiac glycoside digoxin disrupts host defense in experimental pneumococcal pneumonia by impairing neutrophil mobilization. Am Rev Respir Dis 1989;140:1590–4.
[6] Mason DT. Digitalis pharmacology and therapeutics: recent advances. Ann Intern Med 1974;80:520–30.
[7] Yuengsrigul A, Chin TW, Nussbaum E. Immunosupressive and cytotoxic effects of furosemide on human peripheral blood mononuclear cells. Ann Allergy Asthma Immunol 1999;83:559–66.
[8] Simchowitz L, De Weer P. Chloride movements in human neutrophils. Diffusion, exchange, and active transport. J Gen Physiol 1986;88:167–94.
[9] Fedson DS, Chiarello LA. Previous hospital care and pneumococcal bacteremia. Importance for pneumococcal immunization. Arch Intern Med 1983;143:885–9.
[10] Braunwald E, Grossman W. Clinical aspects of heart failure. In: Braunwald E, editor. Heart disease. Philadelphia: Saunders; 1992. p. 444–63.
[11] Farr BM, Woodhead MA, Macfarlane JT, Bartlett CLR, McCracken JS, Wadsworth J, et al. Risk factors for community-acquired pneumonia diagnosed by general practitioners in the community. Respir Med 2000;94:422–7.
[12] Farr BM, Bartlett CLR, Wadsworth J, Miller DL. Risk factor for community-acquired pneumonia diagnosed upon hospital admission. Respir Med 2000;94:954–63.
[13] Esposito AL. Digoxin disrupts the inflammatory response in experimental pneumococcal pneumonia. J Infect Dis 1985;152:14–23.
[14] Esposito AL. Community-acquired bacteremic pneumococcal

pneumonia. Effects of age on manifestations and outcome. Arch Intern Med 1984;144:945–8.
[15] Brandi MCAC. Padronização de um método de obtenção de fagócitos do sangue periférico para avaliação funcional. Thesis, Universidade de Brasília, Brasília, DF; 1993.
[16] Bakke OM, Aslaksen A, Lehmann V, Lien E. Pharmacokinetics and serum concentration–effect relationship of intravenous deslanoside. J Cardiovasc Pharmacol 1981;3:1015–25.
[17] Ponto LLB, Schoenwald RD. Furosemide. A pharmacokinetic/pharmacodynamic review. Clin Pharmacokinet 1990; 18:381–408.
[18] Muniz-Junqueira MI, Prata A, Tosta CE. Phagocytic and bactericidal function of mouse macrophages to *Salmonella typhimurium* in schistosomiasis mansoni. Am J Trop Med Hyg 1992;46:132–6.
[19] Myrick JE, Hannon WH. A model system for radioimmunoassays: digoxin reference method. In: Rose NR, Macario EC, Folds JD, Lane C, Nakamura RM, editors. Manual of clinical laboratory immunology. Washington: ASM; 1997. p. 30–8.
[20] Moe GK, Farah AE. Digitalis and allied cardiac glycosides. In: Goodman LS, Gilman A, editors. The pharmacological basis of therapeutics. London: Collier-Macmillan; 1970. p. 677–708.
[21] Mollinedo F, Borregaard N, Boxer LA. Novel trends in neutrophil structure, function and development. Trends Immunol 1999;12:535–7.
[22] Mühlberg W, Platt D, Neubig E. Pharmacokinetics and pharmacodynamics of furosemide in geriatric patients. Arch Gerontol Geriatr 1986;5:249–63.
[23] Lachmann PJ, Hobart MJ. Complement technology. In: Weir DM, editor. Handbook of experimental immunology: I. Immunochemistry. Oxford: Blackwell; 1978. p. 1A–5A.
[24] Esposito AL, Clark CA, Poirier WJ. The cardiac glycoside digoxin does not alter the activity of human polymorphonuclear leukocytes in vitro. J Infect Dis 1988;157:1084–7.
[25] Blum A, Miller H. Pathophysiological role of cytokines in congestive heart failure. Annu Rev Med 2001;52:15–27.
[26] Mann DL, Deswal A, Bozkurt B, Torre-Amione G. New therapeutics for chronic heart failure. Annu Rev Med 2002;53: 59–74.
[27] Muniz-Juqueira MI, Santos-Neto LL, Tosta CE. Influence of tumor necrosis factor-α on the ability of monocytes and lymphocytes to destroy intraerythrocytic *Plasmodium falciparum* in vitro. Cell Immunol 2001;208:73–9.
[28] van Vlem B, Vanholder R, De Paepe P, Vogelaers D, Ringoir S. Immunomodulating effects of antibiotics: literature review. Infection 1996;24:275–91.
[29] Epstein FH. Cardiovascular disease epidemiology. A journey from the past into the future. Circulation 1996;93:1755–64.
[30] Sharpe N, Doughty R. Epidemiology of heart failure and ventricular dysfunction. Lancet 1998;352(Suppl.):3–7.
[31] Mendez GF, Cowie MR. The epidemiological features of heart failure in developing countries: a review of the literature. Int J Cardiol 2001;80:213–9.
[32] Kierszenbaum F. Chagas' disease and the autoimmune hypothesis. Clin Microbiol Rev 1999;12:210–23.
[33] Ludewig B, Zinkernagel RM, Hengartner H. Arterial inflammation and atherosclerosis. Trends Cardiovasc Med 2002;12: 154–9.
[34] Guilherme L, Kalil J. Rheumatic fever: the T cell response leading to autoimmune aggression in the heart. Autoimmun Rev 2002;1:261–6.
[35] Cleland JGF, Swedberg K, Poole-Wilson PA. Successes and failures of current treatment of heart failure. Lancet 1998; 352(Suppl.):19–28.
[36] Ginaldi L, De Martinis M, D'Ostilio A, Marini L, Loreto MF, Quaglino D. Immunological changes in the elderly. Aging Clin Exp Res 1999;11:281–6.
[37] Malaguarnera L, Ferlito L, Imbesi RM, Gulizia GS, Di Mauro S, Maugeri D, et al. Immunosenescence: a review. Arch Gerontol Geriatr 2001;32:1–14.
[38] Lord JM, Butcher S, Killampali V, Lascelles D, Salmon M. Neutrophil ageing and immunosenescence. Mech Ageing Dev 2001;122:1521–35.
[39] Lip GYH, Gibbs CR, Beevers DG. ABC of heart failure: aetiology. BMJ 2000;320:104–7.