Glutamine or whey-protein supplementation on alloxan-induced diabetic rats. Effects on CD^{4+} and CD^{8+} lymphocytes[1]

Efeitos da oferta de glutamina ou de proteína do soro de leite sobre os linfócitos CD^{4+} e CD^{8+} em ratos diabéticos aloxano induzidos

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ABSTRACT

Purpose: To evaluate the effects of glutamine (L-Gln) or whey-protein supplementation on CD^{4+} and CD^{8+} lymphocytes in alloxan-induced diabetic rats. **Methods**: Thirty-two healthy male Wistar rats were used in the experiment. Eight rats served as baseline controls (G-1). The remaining 24 animals received alloxan 150mg/Kg intraperitonially dissolved in buffer solution and were equally distributed in 3 subgroups, upon induction of diabetes mellitus, and treated as follows: (G2): saline, 2.0ml; (G3): glutamine solution (0.7g/kg), 2.0 ml; and (G4): whey-protein (WPS) solution (0.7g/kg), 2.0 ml. All solutions were administered by daily 7:00 AM gavages during 30 days. Next, arterial blood samples (3.0 ml) were collected from anesthetized rats for CD^{4+} and CD^{8+} lymphocyte count through flow cytometry technology. **Results**: CD^{4+} and CD^{8+} counts decreased significantly in all groups compared with baseline values (G1). G2 rats CD^{4+}/CD^{8+} ratio decreased significantly compared with G1. CD^{4+}/CD^{8+} ratio increased significantly (>260%) in L-Gln treated group (G3) compared with saline-treated rats (G2). There were no statistical differences in lymphocyte counts (CD^{4+} and CD^{8+}) between L-Gln (G3) and saline-treated (G2) groups. There was a significant reduction in CD^{8+} cell count compared with CD^{4+} cell count in L-Gln treated rats (G3). **Conclusion**: The offer of L-Gln to experimental diabetic rats enhances the immunologic response to infection.

Key words: Glutamine. T-Lymphocytes. CD4-Positive T-Lymphocytes. CD8-Positive T-Lymphocytes. CD4-CD8 Ratio. Rats.

RESUMO

Objetivo: Avaliar os efeitos da suplementação de glutamina ou proteína do soro de leite (PSL) sobre os linfócitos CD^{4+} e CD^{8+} em ratos diabéticos aloxano induzidos. **Métodos**: Trinta e dois ratos Wistar machos, saudáveis, foram utilizados no estudo. Oito ratos foram usados como controles basais (G1). Os 24 animais remanescentes foram equitativamente distribuídos em 3 subgrupos, após indução do diabetes mellitus por injeção intraperitonial de aloxano (150mg/Kg) e tratados como se segue: (G2): salina; (G3): 2,0 ml de solução de glutamina (0,75g/Kg);(G4): PSL, (0,7g/Kg), 2,0ml. Todas as soluções foram administradas por gavagem, diariamente, cada 7:00 h, durante 30 dias. Após esse período, foram coletadas amostradas de sangue arterial para contagem de linfócitos CD^{4+} e CD^{8+} por citometria de fluxo. **Resultados**: A população de linfócitos CD^{4+} e CD^{8+} diminuiu significantemente em todos os grupos em comparação aos valores encontrados no grupo G1. A razão CD^{4+}/CD^{8+} foi significantemente maior (>260%) nos ratos tratados com L-Gln que nos ratos tratados com salina (G2). Não se observaram diferenças significantes na população de linfócitos CD^{4+} e CD^{8+} entre os grupos G3 e G2. Houve redução significante do número de células CD^{8+} quando comparado ao número de células CD^{4+} nos ratos tratados com L-Gln (G3). **Conclusão**: A oferta de L-Gln em ratos diabéticos aloxano-induzidos melhora a resposta imunológica à infecção.

Descritores: Glutamina. Linfócitos T. Linfócitos T CD4-Positivos. Linfócitos T CD8-Positivos. Relação CD4-CD8. Ratos.

Introduction

The major cause of mortality and morbidity in diabetic subjects is due to immune dysfunction [1]. The four important factors that make diabetic subjects more prone to complications are: susceptibility to infections, hyperglycemia, vascular disease and nerve damage [2]. In diabetic patients, infection occurs with greater frequency and severity than in non-diabetics due to the impairment of both humoral and cellular immune responses [3]. The severity of dysfunction increased susceptibility to infections in diabetic patients led the World Health Organization to classify diabetes mellitus as a secondary immunodeficiency disease [4]. In recent years, the molecular biology of lymphocytes ($CD^{4+}$and CD^{8+}), macrophages, and neutrophils and the process of chemical communication between them has attracted considerable interest, and much progress has been made in our understanding of some aspects of fundamental importance not only in preventing or limiting infection, but also in the overall process of repair and clinical conditions of trauma, sepsis, burns, and recovery from surgery [5]. Whey-protein is widely used by athletes due to its high caloric value and its influence on mass gain. Another function of this protein is the immune function through the tissue reparation and stimulation in the production of immunoglobulin [6]. A recent development in the metabolic support of critically ill patients has been the evolution of a concept termed "immunonutrition". Nutrients have been identified that stimulate cells in such a manner as to enhance immunologic responses and potentially improve outcome. The amino acid glutamine is usually included in the list of "immunonutrients"that possess these biological effects [7]. Glutamine is used at a high rate by lymphocytes, even in the resting state [8]; the rate of glutamine use is increased if lymphocytes are activated, for example after stimulation with mitogens [9]. The high rate of glutamine use and its increase upon activation are suggestive that glutamine plays na important role in these cells. Plasma glutamine concentrations are lowered in a variety os "stress" conditions, such as after burns [10], during sepsis [11], after surgery [12] and endurance exercise [13], and in athletic overtraining [14]. These situations are associated with an increased susceptibility to infections, and it has been suggested that this partly may be due to the decreased supply of glutamine to immunocompetent cells such as lymphocytes [15]. The aim of this study is to evaluate the effects of glutamine (L-Gln) or whey-protein supplementation on CD^{4+} and CD^{8+} lymphocytes in alloxan-induced diabetic rats.

Methods

Thirty two healthy male albino Wistar rats obtained from Faculty of Medicine (Federal University of Ceará) Small Animals Laboratory, weighing 150-200g (average 180g) were used in this study. All animals were in compliance with the University of Ceará ethical guidelines for International Organization of Medical Sciences (CIOMS) ethical code for animal experimentation (WHO Chronicle 1985;39(2): 51-6). Approval for experimental use of laboratory animals was obtained from the Commission of Ethics in Animal Research, Federal University of Ceará. The animals were housed in polypropylene cages at ambient temperature of 24°C on a 12 h light-dark cycle. Rats were allowed free access to food (Purina chow) and fasted overnight before the experimental procedure. All procedures were performed under inhalatory diethyl ether anesthesia.

Induction of diabetes

Experimental diabetes was induced by intraperitoneal injection (150mg/kg) of Alloxan monohydrate (Acros Organics Research Laboratories, Inc., New Jersey, USA) dissolved in 0.1M sodium citrate buffer (pH 3,0) . After a period of 2 weeks, rats with marked hyperglycemia (fasting blood glucose > 250mg/dL) were selected and used for study.

Experimental design

Thirty-two healthy male Wistar rats were used in the experiment. Eight rats served as baseline controls (G-1). The remaining 24 animals received alloxan 150mg/Kg ip. dissolved in buffer solution. Upon induction of diabetes mellitus those rats were equally distributed in 3 subgroups and treated as follows: (G2): Saline, 2.0ml; (G3): glutamine solution (0.7g/kg), 2.0 ml; and (G4): whey-protein (WPS) solution (0.7g/kg), 2.0 ml. All solutions were administered by daily 7:00 AM gavages during 30 days. Next, arterial blood samples (3.0 ml) were collected from anesthetized rats for CD^{4+} and CD^{8+} lymphocyte count through flow cytometry technology.

Analysis of blood leukocyte subsets

Samples of 3.0ml of total blood in EDTA were collected through arterial punch until immunophenotyping was made. The rehearsals of Flow Citometrics for immunophenotyping of the peripheral lymphocytes were conducted according to a protocol developed specifically for this study as follows: in 5 ml tubes containing 15µl of anti-CD^{4+} monoclonal antibodies and anti-CD^{8+} marked with fluorescein isothiocyanate 30 µl of blood collected in ethylene diamine tetracetic acid (EDTA) were added. After a 20-minute room temperature incubation, all cell preparations were submitted to lysing of erythrocytes by adding 2ml of a lysing Solution (BD FACS Lysing Solution Catalog number 349202, BD Biosciences, Califórnia, U.S.A.); next, the leucocytes were washed in phosphate buffer solution (PBS) and fixed with 200 µl of a solution containing 1% paraformaldehyde in buffered saline 10g/l,

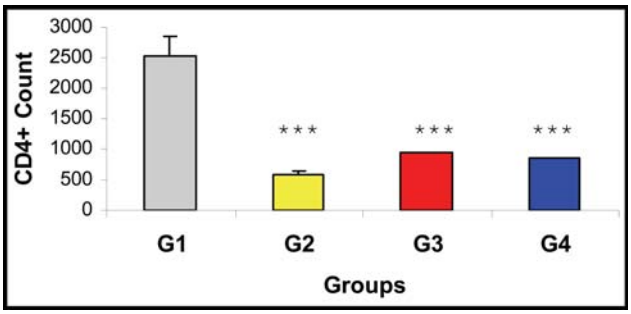
pH 7.2. Analysis was carried out on a FACS Calibur Flow Citometer (Becton Dickinson Instruments, Cambridge, Mass) for a minimum of 10000 gated events.

Statistical analysis

Data were analyzed to assess the significance of differences between groups. ANOVA with *post hoc* Tukey's Multiple Comparison Test and Kruskal-Wallis/Dunn non-parametric tests were used for statistical analysis, as adequate. Statistical significance was accepted as $p<0.05$.

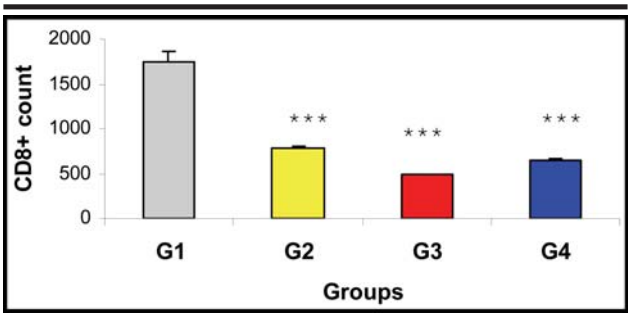
Results

CD4+ and CD8+ counts decreased significantly in all groups compared with baseline values (G1). G2 rats CD4+/CD8+ ratio decreased significantly compared with G1. CD4+/CD8+ ratio increased significantly (>260%) in L-Gln treated group (G3) compared with saline-treated rats (G2). There were no statistical differences between L-Gln (G3) and saline-treated (G2) groups (Figures 1 - 3). CD^{8+} cell count was significantly reduced compared with CD^{4+} cell count in control rats (G1). CD^{4+} and CD^{8+} counts in diabetic rats treated with saline or whey-protein were not different. However CD^{8+} cell count was significantly reduced ($p<0.001$) compared with CD^{4+} cell count in L-Gln-treated rats (Figure 4).



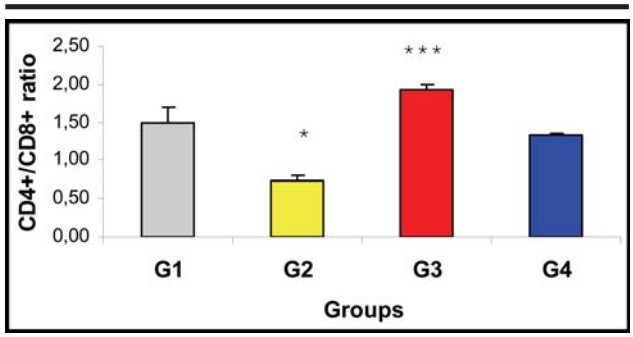
*** p< 0.001 compared with G1

FIGURE 1 - Blood CD^{4+} lymphocyte counts (ANOVA / Tukey's Multiple Comparison Test) Values are presented as means ±S.E.M for 8 animals in each group. Bars represent each group studied



*** p< 0.001 compared with G1

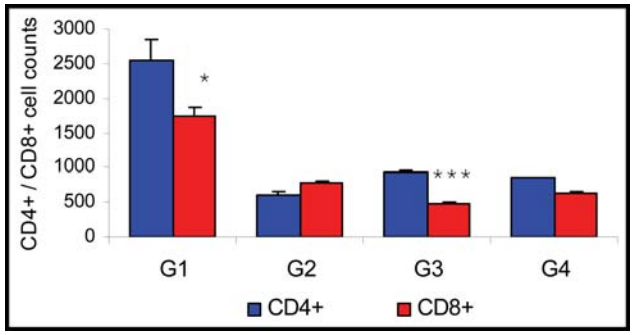
FIGURE 2 - Blood CD^{8+} lymphocyte counts (ANOVA / Tukey's Multiple Comparison Test) Values are presented as means ±S.E.M for 8 animals in each group. Bars represent each group studied



* p< 0.05 compared with G1

*** p< 0.001 compared with G2

FIGURE 3 - Blood CD^{4+}/CD^{8+} ratio. (Kruskal-Wallis/Dunn's Multiple Comparison Test) Values are presented as means ±S.E.M for 8 animals in each group. Bars represent each group studied



* p< 0.05 compared with CD^{4+}

*** p< 0.001 compared with CD^{4+}

FIGURE 4 - Blood CD^{4+} and CD^{8+} lymphocyte counts (Student's *t* Test). Values are presented as means ±S.E.M for 8 animals in each group. Bars represent each group studied

Discussion

High CD^{4+} and CD^{8+} lymphocyte counts are seen in healthy individuals (G1). It has been demonstrated that diabetes mellitus leads to a reduction in the number of CD^{4+} and CD^{8+} lymphocytes [16-17] Insulin administration to diabetic individuals may change this picture by stimulating the glucose transporters and enabling the passage of the carbohydrate to the interior of the cell, and by inhibiting proteolysis and bloking the liberation and oxidation of aminoacids [16]. Low levels of $CD^{4+}$and CD^{8+} lymphocytes found in all diabetic rats was expected, as no insulin treatments were carried out in this study. It has been widely demonstrated that the decrease in lymphocyte cells play an important role in the immunologic response to infections. Diabetic patients show functional loss in the defense mechanism attributed to these cells such as quimiotaxy, adhesion and fagocytosis [17]. *In vitro* studies using rat macrophage cells have demonstrated that fagocytosis is directly related to the concentration of glutamine available[10]. Juretic et al. [18] have demonstrated that glutamine acts as an activator of Killer cell promoting the lysing of the aggressing

agent. Malfunctions found in macrophages are associated with the depletion of NADPH and with the increase in the concentrations and the advance of proteic glicolisation which lead to the induction and liberation of cytocines like the interleukines-1 (IL-1) and tumor necrosis factor [10]. The first (IL-1) promotes the liberation of the free radicals resulting in cell injury [19]. Diabetic patients have reduced capacity to produce interferon gama (IFN-á), resulting in decreased T lymphocytes production with direct effects on in the CD^{4+} and CD^{8+} cells [20]. *In vitro* studies have showed an increase in CD^{4+} lymphocytes and a decrease in CD^{8+} lymphocytes counts when glutamine is added to the culture media; this effect is not noticed when another amino acid is added [7]. The offer of glutamine to G3 rats promoted a significant reduction in CD^{8+} lymphocytes count. According to Ardavi *in vitro* lymphocytic proliferation of rat cells is dependent on glutamine [21]. Glutamine stimulates the synthesis of nucleic acids and provides energy to the proliferation of mononuclear cells like the CD^{4+} lymphocytes. Besides, it is an important source of nitrogen for the synthesis of some intermediate constituents [22] CD^{4+}/CD^{8+} ratio has been used for assessment of individuals immunodeficiency state[10]. The significant increase in G3 CD^{4+}/CD^{8+} ratio (Fig. 3) compared with G2 rats and the significant reduction in CD^{8+} lymphocytes count in L-Gln treated rats (G3) found in this study suggests that the offer of L-Gln to diabetic individuals may alter the immunologic response to infection.

Conclusion

The offer of L-Gln to experimental diabetic rats enhances the immunologic response to infection.

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