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Effects of dietary restriction or swimming on lymphocytes and macrophages functionality from old rats

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Although aging compromises the functionality of macrophages (MΦ) and lymphocytes (LY), and dietary restriction (DR) and exercise partially counterbalance immunosenescence, it is unknown what effects of both strategies have on the functionality of these immune cells. Rats were randomly distributed into adult control (AD), older group (OLD), older submitted to 50% of DR (DR) and older submitted to swimming (EX) ($n = 10$ in each group). The function of immune cells (proliferative index, phagocytic capacity and H_2O_2 production), the weight and protein content of lymphoid organs (thymus and spleen), plasma glutamine concentration, interleukins (IL-1, IL-2, IL-6) and, immunoglobulins (IgA and IgG) were analysed. There was an increase of 74% in body weight in aged animals as compared with the AD group, while body weight reduced 19% in the DR as compared with the OLD group. Swimming training stimulated MΦ phagocytosis, while the EX group presented a decrease of the proliferative capacity of LY from the mesenteric lymph nodes (44% and 62%, respectively), when stimulated with ConA and LPS as compared with the old rats. These data demonstrated that DR and exercise affects differentially MΦ and LY function.

Keywords Aging, caloric restriction, cell function, exercise, immune system, weight loss

INTRODUCTION

The increase in average lifespan observed in the last 50 years has not been accompanied by a reduction in the period of illness experienced toward the end of life (Wick et al., 2000). This phenomenon is partially accounted for by the

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aged-associated decline in innate and adaptive immunity (Pawelec et al., 1999; Plowden et al., 2004; Venkatraman & Fernandes, 1997). Dietary restriction (DR) and chronic exercise training have been utilized to counterbalance the effect of aging on the immune system and seem to improve a diverse array of functional parameters with regard to macrophages (MΦ) and lymphocytes (LY) (Strasser et al., 2006).

Although the most notable effect of both DR and exercise is a body weight reduction, these effects upon different immune parameters are diverse (Rogers et al., 2008; Strasser et al., 2006). Moreover, it is possible that the effects of DR and exercise on innate and adaptative immunity would be tissue-specific. It is interesting to note that peritoneal MΦ are highly activated with aging which suggests modulation at distance by adipocytes (Wu et al., 2007). Although theoretically DR and exercise modulate the immune system, at least in part, through influencing the systemic levels of cytokines, the consequence of both strategies is far more complex and is not restricted to the factors produced by a specific tissue (Bacurau et al., 2007; Fontana & Klein, 2007).

In this sense, it is interesting to investigate whether the differential effects of DR and exercise on immunity are related to a specific set of changes induced on plasmatic levels of cytokines. Among cytokines and interleukins (IL) that trigger important inflammatory response are: IL-1, IL-6, IL-8, tumor necrosis factor-α and IL-2 (Pedersen, 2000). Moreover, reductions in immunoglobulins (Ig), such as IgA-producing lymphoid cells and plasma IgG can increase susceptibility to infection (Pedersen & Hoffman-Goetz, 2000).

As age advances, the capacity of cells to synthesize macromolecules (eg. DNA and RNA) is altered and this could be related to a reduced ATP supply (Buttgereit et al., 2000). MΦ and LY consume glucose and glutamine in a manner strictly related to their function (Newsholme, 2001), and we previously demonstrated that the metabolism of both substrates accompanied the alterations induced in cell functionality by stimuli as diverse as cancer, experimental arthritis and undernutrition (Bacurau et al., 2007; Cunha et al., 2003; Navarro et al., 2010). Thus, the clarification of the effects of DR and exercise on the functionality of MΦ and LY from aged animals would be of value.

In this sense, the aim of the present study was to investigate the effects of DR and exercise on the function of MΦ and LY of aged rats. The initial hypothesis was that DR and exercise would result in differential effects on the function of immune cells from old rats.

MATERIALS AND METHODS

Animals

Adult and old male Wistar rats (2 and 18 months old, respectively) from the animal Breeding Unit, Institute of Biomedical Sciences, at the University of Sao Paulo, Sao Paulo, Brazil, were housed in a temperature controlled room at 23 °C under an inverted photoperiod regimen of 12:12 h light:dark cycle (lights on at 6:00 h p.m.) with water and commercial food available *ad libitum*. These animals were maintained in accordance with the guidelines of the Brazilian Association for Laboratory Animal Science, and all experimental procedures

were approved by the Ethical Committee on Animal Experimentation of the Institute of Biomedical Sciences, University of Sao Paulo.

All reagents, unless otherwise specified, were purchased from Sigma (St Louis, MO, USA). Glucose and glutamine were purchased from Merck (Damstadt, Germany).

Experimental Groups

Adults (AD) rats were maintained with chow *ad libitum* and without exercise ($n = 10$). The old rats were divided into three groups, the first group received chow *ad libitum* without exercise (OLD, $n = 10$). The second group was submitted to dietary restriction under 50% of diary food intake for 6 weeks without exercise (DR, $n = 10$). The third group performed aerobic swimming training (EX) for 6 weeks with chow provided *ad libitum* ($n = 10$).

The dietary restriction animals received chow once per day, and different hours in each day. The quantities were equal to 50% of chow *ad libitum* consumption measurement one week before experimental assay beginning. The animals were weighed weekly, and the final measurement was used to determine the Lee Index calculation (cube root of weight per length) (Bernardis & Patterson, 1968).

Training Protocol

The animals were initially submitted to a week of familiarization. During this week, the animals were familiarized to the aquatic environment in daily sessions of 5–10 min without loading. It is important to note that because of the reduced time of exposure to water and the absence of a load, no physiological adaptation (ie, immune) was induced during the familiarization week. After this familiarization week, the swimming training protocol was initiated. The animals were subjected to training for 6 weeks, in which animals performed a swimming session for 1 h, 5 days/week, between the hours of 7:00–9:00 h p.m., in individual tanks with flowing water maintained at 30 °C, as described by Lancha et al. (1995). Following the first week, the overload resistance was gradually increased to 2% of weight of the animal.

Taking into account that the effects of an acute exercise session on circulating leukocytes (on number and functions) remain for 3–24 h, all evaluations were initiated 48 h after the last training session. Animals were sacrificed by decapitation.

Peritoneal Macrophages

PBS (0.14 M NaCl, 4.7 mM KCl, 10 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4 mM Na_2HPO_4, 1.3 mM NaH_2PO, 1.3 mM $CaCl_2$, pH 7.2) was injected (6 mL) intraperitoneally, and after 30 s, peritoneal MΦ were collected. Cell viability was confirmed by trypan blue exclusion (> 95%). At least 92% of the peritoneal exudate cells were MΦ, as determined by differential counting. Peritoneal MΦ were utilized in the experiments of consumption (glucose, glutamine), production (lactate, gluta- mate and aspartate), phagocytosis, and H_2O_2 production.

Lymphocytes from Mesenteric Lymph Nodes

Cells from the mesenteric lymph nodes were pressed against a steel mesh, as described by Ardawi and Newsholme 25. The cell suspension was filtered

(Whatman PLC, Middlesex, UK) and centrifuged at 150 g for 15 min at 4 °C. The total contamination with MΦ was lower than 1%. Mesenteric LY were used for the measurement of proliferative index, in the experiments of consumption (glucose, glutamine), and production (lactate, glutamate, and aspartate).

Lymphocyte Proliferation

Mesenteric LY were cultivated in 96-well plates (1×10^5 cells per well; Corning, One Riverfront Plaza, NY, USA) under sterile conditions in Gibco RPMI 1640 medium for 48 h at 37 °C in an artificially humidified atmosphere of 5% CO_2 in a microprocessor incubator (Lab Line, Boston, MA, USA). Cells were also cultivated in the presence of concanavalin A (ConA; 5 mg mL^{-1}) or lipopolysaccharide (LPS; 10 mg mL^{-1}). After 48 h in culture, more than 98% of the LY were still viable, as measured by trypan blue exclusion. The cells were labelled with 7400 Bq 14C- thymidine (Amersham-GE Healthcare, Uppsala, Sweden) diluted in sterile PBS, yielding a final concentration of 1 mg·ml^{-1}. The cells were maintained under these conditions for an additional 15 h and automatically harvested using a multiple-cell harvester and filter paper (Skatron Combi, Sulfolk, UK). The paper discs containing the labelled cells were counted in 5 ml Bray's scintillation cocktail (Sigma, St Louis, MO, USA) in a Beckman-LS 500 liquid scintillator (Beckman Instruments, Fullerton, CA, USA).

Hydrogen Peroxide Release and Phagocytosis

The release of hydrogen peroxide (H_2O_2) was measured using a modification of the method described by Pick & Mizel (1981). The cells were incubated in siliconized flasks (25 ml) in 1 ml PBS in the presence of glucose (5 mM) under an atmosphere of 5% CO_2/95% air at 37 °C. After 1 h of incubation, 100 mL of a mixture of phenol red (200 mL^{-1}) and horseradish peroxidase (19 U ml^{-1}, final concentration) were added. After 10 min, the reaction was stopped with 100 mL 1 M NaOH and the absorbance measured at 620 nm. The rate of H_2O_2 release was linear over a period of 1 h, and there was no significant difference in the H_2O_2 release if cells were incubated in the presence or in the absence of bicarbonate-containing medium. The rate of phagocytosis was measured in MΦ incubated in siliconized flasks (25 mL) in 1 mL PBS in the presence of glucose (5 mM), glutamine (2 mM) and zymosan particles under an atmosphere of 5% CO_2/95% air at 37 °C. After 40 min, an aliquot was obtained, and after dilution (10:1) in PBS (pH 7.2), the aliquot was evaluated by light microscopy and the rate of phagocytosis was determined.

Cytokines

Plasma levels of interleukins (IL-1, IL-2, IL-6) were measured using commercially available ELISA kits (Amersham-GE Health- care, Uppasala, Sweden). The plasmatic levels of the immunoglobulins A and G were measured using commercially available immunoprecipitation kits (Master – Bioclin, Quibasa).

Thymus and Spleen

The spleen and thymus were removed and immediately weighted prior to freezing. For analysis, organs were submitted to protein extraction as describe

by Folch et al. (1957), and the protein quantity was determined according to the procedures of Lowry et al. (1951).

Protein Measurement

The protein content of all samples was measured utilizing the method of Bradford. BSA was used as standard.

Statistical Analysis

Analysis was performed using GraphPad-Prism. When differences among groups were detected by two-way factorial ANOVA, the Tukey *post hoc* test was used to determine which groups were significantly different. The level of significance of $p \leq 0.05$ was chosen for all statistical comparisons. Data are presented as means $\pm$ SEM.

RESULTS

Body Composition

Aging resulted in 74% of increase in body weight as compared with the AD group, as EX and DR groups decreased body weight as compared with the OLD group. There was no difference in body weight between DR and EX groups (Table 1).

The lymphoid organs were significantly affected by aging. There was a reduction in thymus weight (11%, $p \leq 0.05$) as compared with AD group, with no effect on spleen weight. In addition, thymus protein content was also reduced (28%) in the OLD group as compared with AD group ($p \leq 0.05$). Thymus protein content was reduced by 35.14% in the exercised animals as compared with the OLD group ($p \leq 0.05$). DR also promoted a reduction in thymus weight (16%, $p \leq 0.05$) as compared with the OLD group. Chronic swimming training resulted in higher spleen protein content as compared with DR (Table 1).

Plasmatic Parameters

There was no statistically significant effect of aging, DR or exercise on cytokines, glutamine and immunoglobulins (Table 2).

Table 1. Body weight, food intake, weight and protein content of thymus and spleen.

	AD	OLD	DR	EX
Body Weight (g)	271.7 ± 15.50	473.0 ± 15.10*	383.9 ± 11.80*#	405 ± 8.60*#
Food Intake (g)	21.8 ± 1.1	22.9 ± 0.8	11.7 ± 0.3 *#	22.0 ± 0.7&
Thymus				
Weight (g)	0.31 ± 0.02	0.28 ± 0.02*	0.09 ± 0.01*#	0.20 ± 0.02*
Protein (mg/g of tissue)	89.30 ± 4.10	64.80 ± 5.10*	56.50 ± 2.30*	42.00 ± 4.90*#
Spleen				
Weight (g)	0.62 ± 0.03	0.87 ± 0.05	0.80 ± 0.08	0.79 ± 0.06
Protein (mg/g of tissue)	45.80 ± 2.80	50.10 ± 2.10	43.70 ± 2.10	58.30 ± 5.50&

AD= Adults; OLD = Older group; DR= Dietary restriction; EX= Exercise; *$p < 0.05$ for comparison with the AD group; #$p < 0.05$ for comparison with the old group; &$p < 0.05$ for comparison with DR group.

Lymphocytes

The proliferative capacity of blood LY presented a tendency to be elevated when stimulated with ConA (42%) and LPS (58%) in older rats compared to AD animals (Table 3). The proliferative capacity of LY from the mesenteric lymph node presented a tendency to increase in cells of DR animals with ConA (36%) and LPS (49%) when compared to cells of AD rats. In trained animals, the proliferative capacity of LY from the mesenteric lymph nodes was reduced by 44% and 62%, respectively, when stimulated with ConA and LPS in comparison to values obtained in OLD rats.

Macrophages

Regarding their functional parameters, MΦ from OLD animals presented a tendency to increase phagocytosis by 76% compared to values from AD cells (Figure 1). DR promoted a tendency of increased phagocytosis (2.42-fold) in cells of DR animals in comparison to MΦ from OLD rats. Hydrogen peroxide production presented a tendency of increase equivalent to 1.28-fold (Figure 2). Exercise training promoted a 4-fold increase in phagocytosis when compared to values observed in macrophages of OLD animals (Figure 1). Chronic exercise training increased MΦ hydrogen peroxide production by 1.35-fold (Figure 2).

Table 2. Serum concentration of the interleukins IL-1, IL-2 and IL-6, glutamine (nmol/ml) and immunoglobulins IgA and IgG.

	AD	OLD	DR	EX
IL-1 (pg/mL)	17.54 ± 1.85	14.00 ± 0.84	13.19 ± 0.86	16.61 ± 2.26
IL-2 (pg/mL)	112.67 ± 37.69	87.70 ± 24.22	114.40 ± 36.03	72.38 ± 17.81
IL-6 (pg/mL)	115.14 ± 36.39	122.94 ± 37.10	84.57 ± 20.66	51.94 ± 12.53
Glutamine (nmol/mL)	0.62 ± 0.03	0.87 ± 0.05	0.80 ± 0.08	0.80 ± 0.06
IgA (mg/mL)	ND	22.20 ± 5.30	29.80 ± 5.20	ND
IgG (mg/mL)	387.10 ± 7.40	522.90 ± 121.90	424.40 ± 12.50	406.40 ± 11.00

AD= Adults; OLD = Older group; DR= Dietary restriction; EX= Exercise; ND= No detectable.

Table 3. Proliferation index of lymphocytes from peripheral blood and mesenteric lymph nodes.

	AD	OLD	DR	EX
Peripheral blood				
CONA	1.10 ± 0.03	1.56 ± 0.25	1.50 ± 0.19	0.84 ± 0.04*&
LPS	1.31 ± 0.08	2.07 ± 0.51	1.95 ± 0.50	1.28 ± 0.07
Mesenteric lymph nodes				
CONA	1.40 ± 0.12	1.47 ± 0.18	1.27 ± 0.14	1.08 ± 0.03
LPS	1.40 ± 0.08	1.23 ± 0.10	1.32 ± 0.10	1.22 ± 0.07

AD= Adults; OLD = Older group; DR= Dietary restriction; EX= Exercise; CONA = concanavalin; LPS = lipopolysaccharide. *$p < 0.05$ for comparison with the AD group; &$p < 0.05$ for comparison with DR group.

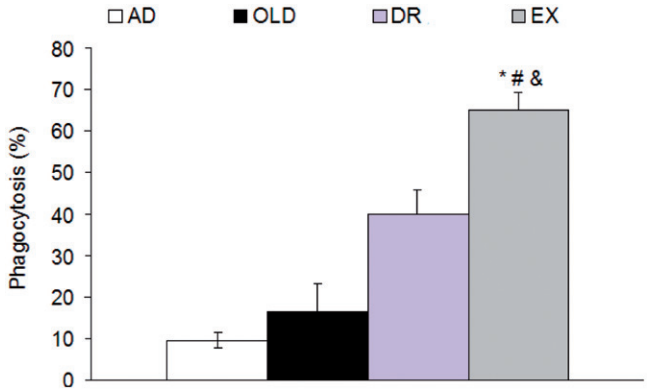

Figure 1. Phagocytosis (%) from macrophages present in the peritoneal cavity. AD= Adults; OLD = Older group; DR= Dietary restriction; EX= Exercise. *$p < 0.05$ for comparison with the AD group (AD); #$p < 0.05$ for comparison with the OLD group; &$p < 0.05$ for comparison with DR group.

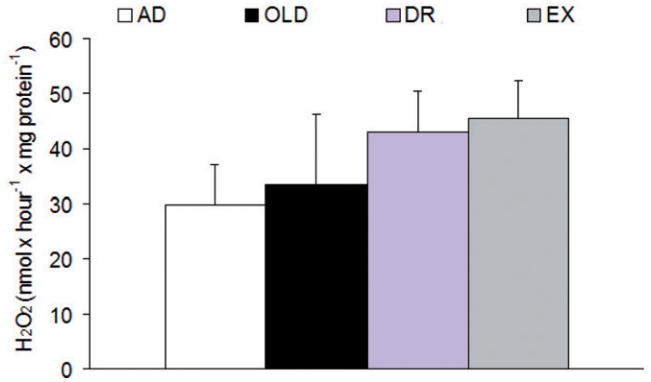

Figure 2. Hydrogen peroxide (nmol $\times$ h^{-1} $\times$ mg $protein^{-1}$) production from macrophages present in the peritoneal cavity. AD = Adults; OLD = Older group; DR = Dietary restriction; EX = Exercise.

DISCUSSION

We investigated the metabolism of MΦ and LY in aged animals submitted to DR or exercise. As expected by the initial hypothesis, results revealed that DR and exercise had differential effects on the function of MΦ and LY of aged rats. In opposition to aging, exercise training increased the phagocytosis of MΦ and decreased the proliferative capacity of LY from the mesenteric lymph nodes (44% and 62%, respectively), when stimulated with ConA. Aging resulted in increased body weight as compared with the adult rats, while body weight reduced with DR as compared with the OLD group.

MΦ and LY exert an important role on inflammatory and immune responses (Curi et al., 1999). Immunosenescence is related to the deterioration of certain MΦ and LY functions (Plowden et al., 2004), while chronic exercise and DR are partially able to reverse the effects of aging on the immune system. In the present study, independently of exercise, DR affected only/mainly the immune parameters related to LY. The increased production of immunoglobulins could account in part this "synthetic" need. LY proliferation is another process that

imposes an increased demand for macromolecules, although this it was not observed in LY from mesenteric lymph nodes in the present investigation. It is possible that the absence of an increased proliferative index could be explained by the effect of DR on antigen-specific T lymphocytes in specific LY subfractions (Jolly, 2007).

Evidence of a possible benefit of nutrition on immune function and risk of infection during periods of exercise stress and DR is limited (Murphy et al., 2008). To note, it has been shown that a solution of oat β-glucan (solution of oat bran concentrate enriched to 68% soluble β-glucan) for the 10 days before inoculation with Herpes simplex virus Type 1 (HSV-1) blunted the increase in morbidity and mortality in exercise-stressed mice. This was associated with an increase in macrophage antiviral resistance to HSV-1 but not NK cytotoxicity following 3 consecutive days of exhaustive treadmill running at a speed of $36\,m\,min^{-1}$ and a grade of 8% until volitional fatigue (Davis et al., 2004). Moreover, it has been suggested that lung macrophages play a direct role on the benefits of oat β-glucan in offsetting the increased risk for infection following exercise stress (Murphy et al., 2008).

Both exercise and DR modulate the immune system through a complex interplay among hormones and cytokines (Bacurau et al., 2007; Fontana & Klein, 2007), while immune system and metabolic systems are tightly coupled. In obesity, the secretion of several humoral factors (ie, hormones, cytokines and chemokines) is an important initiator of the immune and inflammatory response, and in this scenario, resident MΦ in adipose tissue are considered to be the main contributor (Olefsky & Glass, 2010). However, the higher inflammatory state of aged adipose tissue seems to be mainly determined by adypocytes while resident MΦ are not strongly affected.

Of note, the peritoneal MΦ of aged rats presented an increased capacity to produce TNF-α and IL-6 (Wu et al., 2007); thus, the investigation of a possible relation between the changes imposed by exercise/DR in the different adipose tissue depots and metabolism/function of immune cells is further justified. In the present study, systemic levels of cytokines displayed no change with aging, DR or exercise. Possibly, this difference in results may be associated with the systemic nature of our measures, as local cytokines production could be altered.

Regarding the thymus, this organ is responsible for providing naïve T lymphocytes compartment throughout life, thus ensuring that a diverse repertoire is maintained to help combat invading novel antigens. However, with aging the thymus suffers an atrophy process along with a marked decline in naïve T lymphocytes (Simpson et al., 2008). As a consequence elderly severely impair their immune system to recognize and respond to novel pathogens (i.e., influenza), increasing morbidity and mortality as a result of infectious disease (Simpson, 2011). Interestingly, results from the present study confirmed the aging induced-atrophy in the thymus. Moreover, DR induced the most pronounced thymus atrophy as compared with aging alone, which was not observed for the exercised group. Additionally, exercise increased phagocytosis index, while DR did not, thus confirming some benefits of exercise. To note, the decrease of T-cell proliferative index with exercise was not accompanied by alterations in cytokines, immunoglobulins nor glutamine.

Nevertheless, we demonstrated that 8 weeks of moderate treadmill training in rats increased the utilization and metabolism of glutamine in T and B lymphocytes. Possibly this metabolic shift favoring aerobic metabolism in these lymphocytes helps to explain the beneficial immunomodulations observed with chronic exercise (Navarro et al., 2013). Another study from our research group revealed that phagocytosis of macrophages and neutrophils were increased in animals submitted to four swimming adaptation sessions (Ferreira et al., 2010).

Some important limitations of the present study include the lack of specific subset lymphocytes analysis, combination of a DR and exercise old group and lack of cytokines measures in tissues.

In summary, although DR and exercise were able to counterbalance several effects of aging, the effects of these strategies were diverse. Our data demonstrated a differential action of DR and exercise on MΦ and LY metabolism and function. Chronic swimming training improved the phagocytosis of MΦ and decreased the proliferative capacity of LY from the mesenteric lymph nodes. Dietary restriction and exercise reduced body weight in aged rats, although the most pronounced decrease in thymus mass induced by DR should be considered as a negative effect.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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