# MACRORHAGE PHAGOCYTIC ACTIVITY AT ACUTE COLD-RESTRAINT STRESS EXPOSED RATS: POSSIBLE ROLE OF NITRIC OXIDE

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## ABSTRACT

*In this study, the effect of acute cold-restraint stress (ACRS) on macrophage phagocytic activity and the possible role of inducible nitric oxide synthase (iNOS) was investigated. The phagocytic activity and plasma total nitrate levels were increased by administration of ACRS. These effects were blocked by injection of a specific iNOS inhibitor aminoguanidine (AG) (100 mg/kg, i.p.). Taken together, these findings indicate that the increase of phagocytic activity at ACRS exposed rats is related to the iNOS originated nitric oxide (NO).*

## Introduction

Influences of the stress-response on the immune system are diverse and usually depend on the animal species considered, the nature of the stressor and pathogen, the immune parameter studied and the physiological status of the animal. However, it is generally admitted that although certain acute stressors can be immunostimulating, stress usually exerts immunosupressive effects and increases the susceptility to diseases in vertebrates (1-4), Stress involves activation of both of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system (5). Repeated studies have demonstrated that both glucocorticoids and norepinephrine (NE) can modulate immune responses at different levels. In vitro and ex vivo, glucocorticoids suppress macrophage phagocytosis, T cell proliferation in response to mitogens or antigens, natural killer cell activity, and cytokine secretion from T cells and macrophages (6-9). In vitro, NE alters cytokine, chemokine and nitric oxide (NO) production by macrophages (10-12). NO is known to participate in free radical-mediated damages (13,14). It has been found that NO produced from inflammatory cells by iNOS has antimicrobial, antitumor and cytotoxic effects (15). The release of NO and other reactive nitrogen intermediates is an important effector function of the macrophages directed against a variety of pathogenic microorganisms(16,17). This nitrogen intermediate has antimicrobial effects against several fungi (18) and activated macrophages that produce reactive nitrogen intermediates are efficient at killing both the yeast and the hyphal forms of C.Albicans (19). Restraint stress increases the release of various iNOS inducers such as cytokines and excitatory amino acids in both central and peripheral compartments (20-23).

Restraint stres is widely used as a stress model in physiology, pharmacology, immunology, and behavioral neurobiology (24). Acut cold-restraint stres (ACRS) is considered a physical and psychological stres. Although ACRS has been the traditional model for studying ulcer origin and progression, and for testing of possible

therapeutic drug for ulcers (25), the effect of ACRS on host resistance have not been well documented. In fact, unlike more extensive investigations of chronic stress/infection models (26,27), the effects and mechanisms associated with acute stress modification of host resistance have been less often reported. The mechanisms by which stress modulates host resistance to infectious diseases have not been well delineated.

In the light of these observation we aimed to investigate the effect of ACRS on rat peritoneal macrophage pahagocytic activity. In addition, to investigate the possible involvement of iNOS in ACRS applied rats, aminoguanidine was used as a specific inhibitor of iNOS (28,29 ).

## Materials and Methods

### Animals and procedure

Male Wistar rats weighing 200–250 g were divided into 3 groups containing 8 animals each. All the rats housed at under a 12-hr dark/light cycle and allowed food and water were available ad libitium. The committee for animal experiments of the Dicle University Medical Research Center (Diyarbakır, Turkey) gave its approval for the project (project number: 30.2.D C.0.01.00. 00/2906).

The Groups were as follows:

Group 1. (C). Control

Group 2. (Stress group). saline i.p. 30 min before ACRS

Group 3. (AG +stress group). AG 100 mg/kg i.p. 30 min before ACRS

### Chemicals

Specific iNOS inhibitor aminoguanidine, all the chemicals and reagents that were used for the measurement of phagocytic activity and plasma total nitrite levels were purchased from Sigma (Steinheim, Germany). Aminoguanidine dissolved in 0.9% NaCI and stored at 4°C.

### ACRS Protocol

Stress and AG+stress groups were starved for 24 hours, and the drinking water was removed 1 hour before starting experiments. For ACRS, the rats were restrained in individual close-fitting tubular wire-mesh cages at 4°C (30) and the non- stress control group was kept in starvation cages at 22 ± 1°C. At the end of 4 hours, all the rats were sacrificed.

### Phagocytic activity assays

Peritoneal macrophages were obtained by lavage with a phosphate-buffered saline solution (PBS) (pH=7.2-7.4) were suspended in cold (4°C) Hanks' Balanced Salt Solution (HBSS) with 5% fetal calf serum (FCS) at an approximate concentration of $2xl0^6$ cells/ml. The viability of the macrophage was always >95% as determined by trypan blue exclusion. The suspension of macrophages in HBSS were incubated at 37°C for 30 min in a water bath, with stirring. A suspension of heat killed saccharomyces cereviscea yeast cells ($40x10^6$ cells/ml) in HBSS added at same volume with the macrophage solution was incubated in a separate tube for 60 min. After the the incubation, phagocytic activity was evaluated in terms of phagocytosis number (% phagocytic cell in population, macrophages that ingested at least 1 yeast particle) and phagocytosis index (mean number of yeast particles absorbed by one cell) (31) were microscopically enumerated by counting 200 cells (the nonphagocytosing cells too).

### Measurement of plasma levels of total nitrite

Since NO is a very labile molecule, its direct measurement in the biological samples is very difficult (32). In an aqueous solution, NO reacts with molecular oxygen and accumulates in the plasma as nitrite and nitrate ions. Therefore, the stable oxidation and products of NO, nitrite ($NO_2^-$) and nitrate ($NO_3^-$), can be readily measured in biological fluids and have been used in vitro and in vivo as indicators of NO production (33). Plasma nitrite levels were measured with the Griess reaction (34). Briefly, samples were initially deproteini-

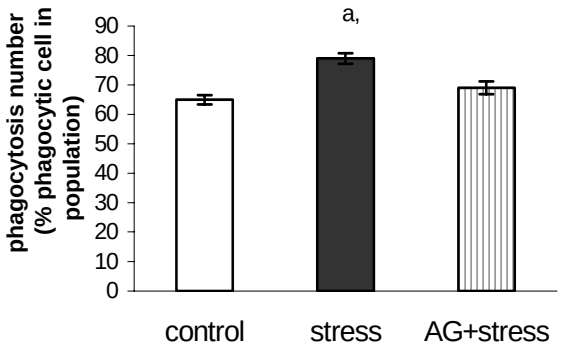


**Fig. 1.** The yeast phagocytosis number at ACRS exposured rats and the effect of AG on this activity. Data represent mean ± SE, [a] $p<0.01$ vs. control group, [b] $p<0.05$ vs. AG+stress group.

zed with Somogyi reagent (35). Total nitrite (nitrite +nitrate) was measured after the reduction of nitrate to nitrite by copporized cadmium granules by a spectrophotometer at 545 nm. A standard curve was established with a set of serial dilutions ($10^{-8}$–$10^{-3}$ mol/l) of sodium nitrite. Linear regression was made by using the peak area from the nitrite standard.The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as µmoles per liter plasma. Therefore, plasma nitrite and nitrate (total nitrite)concentrations were accepted as an index of NO.

**Statistical Analysis**

Results are expressed as the mean±SE. The Kruskal–Wallis test was used to compare the three groups. In two-group comparisons, the Mann–Whitney U-test was performed. $p< 0.05$ was considered statistically significant.

## Results and Discussion

**Effect of ACRS on macrophage phagocytic activity**

The phagocytic activity levels of studied groups are shown in **Figs. 1 and 2**. The phagocytic activity was higher in the stress group than the control group. Yeast phagocytosis number increased from %65±1.6 to %79±1.8 ($p<0.01$, **Fig. 1**) and phagocytosis index was increased from 1.75±0.1 to 2.67±0.19 ($p<0.01$, **Fig. 2**). AG, an inhibitor with a high specifity for iNOS, reduced the phagocytic activity of macrophages at AG+stress group. njection of AG before ACRS procedure reduced the yeast phagocytosis number from %79±1.8 to %69±2.2 ($p<0.05$) and phagocytic index from 2.67±0.19 to 1.95±0.15 ($p<0.05$).

**Effect of ACRS on plasma total nitrite levels**

ACRS administration increased plasma total nitrite levels (**Fig. 3**) when it is compared with control group (20.4±0.8 to 25±1.5 µM/L) ($p<0.05$). AG administration before ACRS procedure reduced the increased total plasma nitrite levels from 25±1.5 to 19.4±1.0 ($p<0.05$).

Many studies indicated that stresses eventually result in either supression or enhancement of immune system (36-38). Eventhough, most of the studies have focused on the alteration of lymphocyte functions and a few of them have investigated the alteration of macrophage functions by stress. In addition, the mechanisms of the immune system participated in the increase of an organism's resistance under stress is still poorly understood.

In the present study, it was shown that

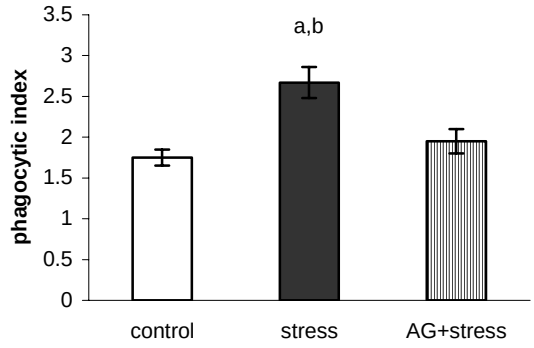


**Fig. 2.** The yeast phagocytic index at ACRS exposured rats and the effect of AG. Data represent mean ± SE, [a] $p<0.01$ vs. control group, [b] $p<0.05$ vs. AG+stress group.

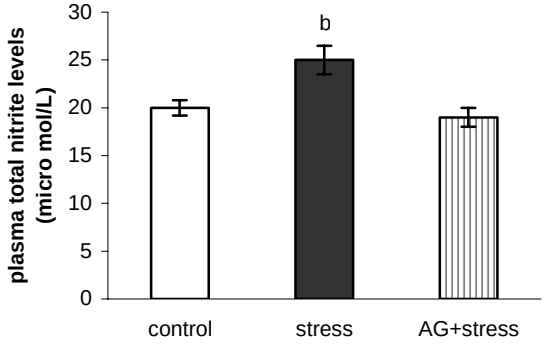


**Fig. 3.** Plasma total nitrite levels at ACRS exposured rats and the effect of AG on these levels. Data represent mean ± SE, [b] $p<0.05$ vs. control and AG+stress group.

ACRS activated the phagocytic activity (yeast phagocytosis number and phagocytic index) in the peritoneal macrophages. Ortega et al. reported that relatively intense acute stressor (swimming to exhaustion) increased phagocytic activity in peritoneal macrophages (39). Likewise, cold swim stress results with the activation of peritoneal macrophages (40). Quindos et al demonstrated that the phagocytic activity of rats which had exposured restraint and fasting stress for 15 h was increased (41). Shilov et al. obtained similar results with Quindos et al, indicated that restraint stress increased phagocytic activity of blood cells after 6 h (42). In contrast to our results, zgüt-Uysal et al. (43) and Davydova et al. (44) findings indicate that the phagocytic activity in peritoneal macrophages of restraint stressed (4h and 14 h) rats is decreased.

Restraint stress increases the release of various iNOS inducers such as cytokines and excitatory amino acids in both central and peripheral compartments (20-23). Kizaki et al. demonstrated that the increased phagocytic activity was abolished by the inhibition of iNOS at cold swim stressed

rats (40). Likewise, Jeon et al demonstrated that cold stress increases NO production in mouse peritoneal macrophages (45). Following the ACRS, NO that was generated from the gastric mucosa (46,47) and central nervous system (48) is rapidly metabolized to nitrite, nitrate or to the other metabolites and transferred to the circulation. Nevertheless, practically it is difficult to know the actual amounts of NO generated in the peritoneal macrophages.

In our study, ACRS caused over production of NO as seen by the increase of total nitrite concentration in plasma. In order to asses the possible role of iNOS in increased phagocytic activity, aminoguanidine was used as an specific inhibitor of iNOS. Administration of aminoguanidine decreased the ACRS-induced peritoneal macrophage phagocytic activity and plasma total nitrite concentration.

Different and sometimes conflicting results have been reported as to the influence of physiological stress in phagocytosis by macrophages, depending on the kind of stress, the animal species, the source of the macrophages (peritoneal vs splenic), the chronicity of the stressor procedures employed and the particles used in the assays which means the involvement of different types of macrophage receptors ( 49).

In conclusion, the result of this study indicated that an increased at macrophage phagocytic activity due to ACRS administration for 4 h increased NO levels via iNOS in macrophages.

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