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A higher anxiety state in old rats after social isolation is associated to an impairment of the immune response

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ABSTRACT

Social isolation is common in the elderly exerting negative effects on neuroimmunoendocrine communication. Nevertheless physiological responses to a stressful situation may vary according to diverse factors. This work studies the differences in the immune response of aged male rats socially isolated depending on the anxiety levels produced. Social isolation impaired certain immunological parameters, but a more anxious response to isolation was associated to global severe immunosuppression and greater oxidative state. Thus, responding anxiously to isolation may suppose a more potent risk of morbidity and mortality further than isolation and anxiety by themselves, particularly in elderly subjects.

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1. Introduction

The existence of a bidirectional relationship between the neuroendocrine and the immune systems, *i.e.* the regulatory systems, ensures the maintenance of homeostasis and hence, mental and physical health (Blalock, 1989; Besedovsky and Del Rey, 1996, 2007). Given the complexity of this neuro-immuno-endocrine communication any *stimuli* that affect the neuroendocrine system have consequent effects on the immune system and *vice versa*. Thus, the neuroendocrine modifications induced by chronic psychological stress also alter immune response (Segerstrom and Miller, 2004; Costa-Pinto and Palermo-Neto, 2010; Cruces et al., 2014). Living in groups is essential for the survival and reproductive success of social species since the maintenance of these structures provides protection from environmental threats. Indeed, strong social bonds between partners are positively correlated with health and increased life expectancy in social species (House et al., 1988; Kiecolt-Glaser and Newton, 2001). Given this concept, social isolation and loneliness are considered potent chronic emotional stressors, impairing the normal functioning of the neuroendocrine and the immune systems and disrupting the neuro-immuno-endocrine communication and the consequent loss of homeostasis leads to the appearance of pathologies (Adam et al., 2006; Cole et al., 2007; Cole, 2008; Arranz et al., 2009a; Cacioppo and Hawkley, 2009; Hawkley et al., 2012). Accordingly, social isolation and loneliness may be considered risk factors of morbidity and mortality (House et al., 1988; Holt-Lunstad et al., 2010; Pantell et al., 2013), so it is necessary to study its underlying mechanisms in order to prevent its deleterious effects on health. Given the long life expectancy of human species and the difficulty of determining when an individual is suffering loneliness (a subjective feeling), it is important to develop animal models of social isolation in social species, such as rodents. This allows us to study the effects of social isolation on the neuroendocrine and the immune systems as well as on the communication between them.

Feeling lonely is by no means uncommon in the elderly. In today's world, aged individuals frequently suffer a lack of social support due to the loss of close relatives, *i.e.* widowhood, or the difficulty in establishing and/or maintaining quality social relationships. All these situations may increase the probability to suffer loneliness. In addition, aging leads to a deterioration of the neuro-immuno-endocrine system and hence, to the loss of homeostasis (De la Fuente, 2008a). This age-related physiological deterioration is due to the establishment of an oxidative and inflammatory chronic stress (*oxi-inflamm-aging*) where the accumulation of oxidants and inflammatory compounds together with a decrease of the anti-oxidant and anti-inflammatory defenses leads to an impairment of the physiological systems, especially the regulatory systems (De la Fuente and Miquel, 2009). In this context, the immune system may contribute to the rate of *oxi-inflamm-aging*, slowing down or speeding up this process (De la Fuente, 2008b; De la Fuente and Miquel, 2009). Moreover, the immune system has been proposed as a good

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marker of health and predictor of life expectancy, since a good maintenance of several immune functions is related to a longer life span (Wayne et al., 1990; Guayerbas et al., 2002a; Guayerbas and De la Fuente, 2003; De la Fuente and Miquel, 2009).

According to the above mentioned facts, the first aim of the present work was to study, in an animal model of social isolation in elderly, the effects of this chronic psychological stress on the function and the redox state of immune cells.

Since the aging process is very heterogeneous, each individual of a population with the same chronological age could show a different rate of aging, *i.e.* a different biological age (Shock, 1980; De la Fuente and Miquel, 2009). Additionally, it is known that the mental and physiological responses to a particular stress may vary according to a wide range of factors acting throughout the life of each subject (Cruces et al., 2014). Anxiety may be one of these factors modifying the stress response. Anxiety by itself may compromise the normal functioning of the neuroendocrine and immune systems impairing the oxidative/inflammatory balance (Hovatta et al., 2010; Vida et al., 2014). Furthermore, it is known that social isolation could increase anxiety levels (Rodgers and Cole, 1993; Moragrega et al., 2003). Thus, individual differences in anxiety in response to a certain stressor must be taken into consideration. In this regard, our research group developed an animal model in which mice of the same strain, sex and age confronted by the same stressful situation showed variable anxiety responses. These individual differences are reflected in the functionality of the regulatory systems and even in the life span of the individuals (Viveros et al., 2001; Guayerbas et al., 2002a; Guayerbas et al., 2002b; De la Fuente et al., 2003; Guayerbas and De la Fuente, 2003; Pérez-Alvarez et al., 2005).

Taking everything into account, the second aim of the present work was to assess the inter-individual differences in response to social isolation based on the anxiety levels induced by this sort of psychological stress, in old male rats.

2. Materials and methods

2.1. Animals

Eighteen male Wistar rats (Harlan Iberica, Barcelona, Spain) were received between six to seven weeks of age (230–250 g). They were housed two per cage and maintained with *ad libitum* access to food and tap water under light (12 hour light/dark cycle; lights on at 8:00 AM) and temperature (22 ± 2 °C) controlled conditions. Animals were weighed once a week, and were left to age till they were 20 months old, when the social isolation procedure started. The animal care procedures were approved by the Ethics Committee of the UNED and efforts were made to minimize the number of animals used. All handling was in accordance with the guidelines set out in the European Community Council Directives (86/609/EEC).

2.2. Chronic social isolation stress protocol

At the age of 20 months (582 ± 12 g body weight), animals were randomly divided into two groups: control and isolated animals. Half of the rats were kept housed in pairs and used as controls (n = 8) and the other ones were isolated in individual cages covered with black plastic bags on the sides in order to prevent visual contact with the rest of the colony (isolated group n = 9) for eight weeks.

2.3. Body weight

During the isolation protocol, both control and isolated animals were weighed weekly in order to analyze the possible body weight changes of the individuals.

2.4. Behavioral tests

Behavioral experiments were conducted between 09:00 h and 13:00 h, in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Community Council Directives (1201/2005) on this subject.

Three days before evaluating anxiety levels using an elevated plus maze, the rats were handled daily to habituate them to experimental manipulations.

2.4.1. Elevated plus maze

Four weeks after the beginning of the social isolation procedure, anxiety-related behavior was evaluated using the elevated plus-maze (EPM) test. The EPM consists of two opposing open arms (45 × 10 cm) and two enclosed arms (45 × 10 × 50 cm) that extend from a central platform (10 × 10 cm), elevated 65 cm above the floor. The rats were placed individually on the central platform facing an enclosed arm and were allowed to freely explore the maze for 5 min. The behavior of each rat was monitored using a video camera, and the movements of the rats were automatically registered and analyzed with a computerized tracking system (Ethovision 1.90, Noldus IT, The Netherlands). Entry into an arm was defined as entry of all four paws into one arm. The total distance traveled as well as the time spent in the open and closed arms was recorded. In addition, EPM results were used to classify isolated rats according to the time they spent in the open arms of the maze as LA – low anxiety – (n = 5), with values above the mean of all rats, and HA – high anxiety – (n = 4), with values below the mean.

2.5. Collection of tissue samples and obtaining of leukocyte suspensions

Eight weeks after the beginning of the social isolation protocol, all animals were deeply anesthetized by injection of tribromoethanol (200 mg/kg) and sacrificed according to the guidelines of the European Community Council Directives 1201/2005 EEC. Spleens were removed aseptically and freed from fat. A fragment of the organ was stored at −80 °C in order to further study oxidative stress parameters. Another fragment of spleen was minced with scissors and gently pressed through a mesh screen (Sigma, St Louis, USA). The cell suspensions were centrifuged in a gradient of Ficoll-Hypaque (Sigma) with a density of 1.070 g/mL. Cells from the interface were re-suspended in RPMI 1640 medium enriched with L-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated fetal calf serum (Gibco, Canada) and 1% gentamicin (10 mg/mL, Gibco) and washed, and the number of leukocytes was determined and adjusted to 1×10^6 cells/mL. Cellular viability, routinely measured before and after each experiment by trypan-blue exclusion test, was higher than 95% in all experiments. All incubations were performed at 37 °C in a humidified atmosphere of 5% CO_2.

2.6. Immune function parameters

2.6.1. Chemotaxis assay

The assay was carried out following a modification of the method of Boyden (1962) according to the technique previously described by Guayerbas et al. (2002c). Chambers with two compartments separated by a filter (Millipore, Ireland) of 3 μm pore diameter were used. Aliquots of 300 μL of leukocyte suspensions were placed in the upper compartment, and aliquots of 400 μL of the chemoattractant fMet-Leu-Phe (Sigma) at a concentration of 10^{-8} M were placed in the lower compartment. The chambers were incubated for 3 h, the filters were fixed and stained and the Chemotaxis Index (C.I., the number of leukocytes in the lower face of the filter) was calculated by counting using an optical microscope the total number of leukocytes in one-third of the lower face of the filters. All C.I. was assayed in duplicate.

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