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Laboratory environment and bio-medical experience: the impact of administration technique on the quality of immune-behavior data results in stress experience

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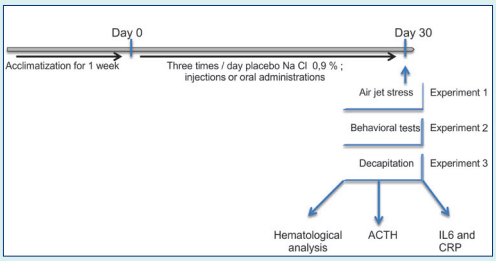
Abstract

Introduction: Often in an experiment, the control group and the intact group are not identified because most scientists neglect the fact that the sets of manipulation as technical administrations may be considered as an undesirable stress on the clarity of the data obtained from a scientific research specifically if it focuses on studying the effects of stress.



Methods: This study was conducted in two parts using 40 male Wistar rats. The first part aimed to treat a group of rats by repeated injections i.p route (1 mL/kg) of placebo or NaCl (0.9%) and the other by direct oral administration of NaCl (0.9%). Both groups spent 1 h of jet air stress with stressed group. Our objective was to consider the effects that these manipulations would have on the validity of behavioral results (the elevated plus maze test, the open field, the light/dark box test) and immune data (immune cell count) during this stress experience. The second part was devoted to the measurement of ACTH, IL6, and CRP in these experimental groups.

Results: Unlike oral administration, repeated intra-peritoneal injections cause a significant increase of plasma obtained levels of the adrenocorticotropin hormone (ACTH), interleukin-6 (IL-6) and the C-reactive protein (CRP) using injections of placebo: NaCl 0.9% (1 mL/kg) and it may have side effect on significant immune and behavioral alterations data quality induced by 1 h of air jet in the animal's cage identified by the leukocyte formula and behavioral tests.

Conclusion: In an experimental protocol conducted on animal models, it is essential to opt for painless techniques such as oral administration instead of painful injections to avoid confusion at the behavioral and immunological results from biomedical experiments specifically one that focuses on the stress study.

Introduction

Homeostasis involves complex interactions between the immune-endocrine system, which is essential for brain functions regulation such as emotion and cognition.[1] External aggressor often appears to change the equilibrium state and is generally associated with pro-inflammatory immune responses due to the activation of thecorticotropic axis (HPA) to modulate the resources of the organism against an agent unknown to the central nervous system (CNS) as it presents itself as a stimulus with sufficient intensity capable of activating pain centers.[2,3]

The responses an animal gives to adverse or stressful stimuli, called stressors, leads to general adaptation syndrome controlled through the hypothalamus-pituitary-adrenal (HPA). The first response which occurs in seconds is the release of catecholamines. This first response is responsible for the increase in blood pressure, heart rate and plasma concentration of free fatty acids and glucose. In parallel, the hypothalamus of activation leads to the secretion of steroid- releasing hormone or CRH (41 amino acid residues) in the hypothalamic-pituitary portal system. The pituitary responds to the release of CRH secretion by adenocorticotropin hormone or ACTH. The second wave of the answer involves the steroid hormones. It develops in a few minutes. The release of glucocorticoids from the adrenal ACTH is stimulated, while the secretion of sex steroids by the gonads decreases. These responses are controlled largely through the HPA axis and secretion of

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corticotropin-releasing hormone (CRH) and corticotropin (ACTH).

Basically, placebo use in animals considered the control group of the experiment for comparison with the active group is required. The placebo administration is used to distinguish the effect caused by the administration itself from the treatment effect and to ensure scientific data analyses' proper execution. However, pseudo–stress imposed by both animal handling and needle-associated repeated narcosis pain can have the opposite effect of an active treatment by triggering an identical physiological and behavioral response to a classic stressor. These advances have been drawn from many critics of placebo classical definitions an inactive substance without any pharmacological effects, thus showing the importance of considering the administration technique itself,[4,5] especially regarding experimentation on rodents where the external social environment is a key factor in the approach with these animals.[6,7] Indeed, the social experience is directly linked to the inflammatory process.[8-10] In mouse and rat, social stressors such as injections are capable of altering the immune system functioning by causing changes of the level of cytokines IL-6 and TNF-α called inflammatory and lead to glucocorticoids resistance.[11,12]

Therefore, handling and infection processes are likely to compromise the quality of blood numbering obtained results and behavioral test in any experimental protocol due to the negligence of choosing an administration technique that minimizes the anxiety state of the animal that is the subject of this study. We studied this effect by using two different administration techniques (intra-peritoneal injection and oral administration) of placebo NaCl to treat leukocyte and behavioral alterations caused by psychogenic stress (the air jet stress) in Wistar rats while trying to explain the results deviation by measuring the associated hormonal (ACTH) and immunity biomarkers (IL6, CRP).

Materials and methods

Species and housing

According to Guidelines for the Care and Use of Laboratory Animals (no. 80–23, 1996), forty Wistar rats weighting between 230-250 g were purchased from the Pasteur Institute (Algiers, Algeria) to carry out this protocol where they were housed in translucent cages and acclimated to the conditions including a constant light/dark cycle turned on at 07:30 am, 25°C. Rats had food and water to drink in ad libitum bottles.

Study protocol

Forty rats were put into four groups (N = 10). The T named group serves as the intact group (no NaCl administrated and no stressed group) while the G group (NaCl oral administrated + stress group) underwent one week training receiving 2 ml of 5% sugar solution directly from the syringe then treated temporarily with the IP group (NaCl injected + stress group) for a period of one month with the vehicle of NaCl 0.9% (1 mL/kg) or placebo three times a day by oral administration and intra-peritoneal injection for the IP group. The placebo treatment duration took one month before air jet stress application, which occurred simultaneously in the S group (stressed group). Recent advances in psychological trauma suggest exploring methods to prevent the onset of anxiety disorders up to 30 days before their apparitions take place.[13]

Air jet stress was chosen due to its recommendation by numerous scientific studies,[14-22] and it is an emotional stressor consisting of creating a 1-h constant air pressure of 1 bar using a compressor equipped with a gauge in the rat cage through a side port. After the air jet stress session, the four groups' behavior was tested in elevated plus-maze, open space and LDB box. Rats decapitation occurred under mild anesthetic diethyl ether and the blood collection was carried out in ethylenediaminetetraacetic acid (EDTA) tubes to determine the lymphocytic composition. After 15 min of centrifugation, the serum is used for adrenocorticotropin hormone measurement (ACTH), interleukin-6 (IL-6), and C-reactive protein (CRP) levels (Fig. 1).

Treatment and experimental groups

The elevated plus maze test

This test is a cross maze with two open arms and closed

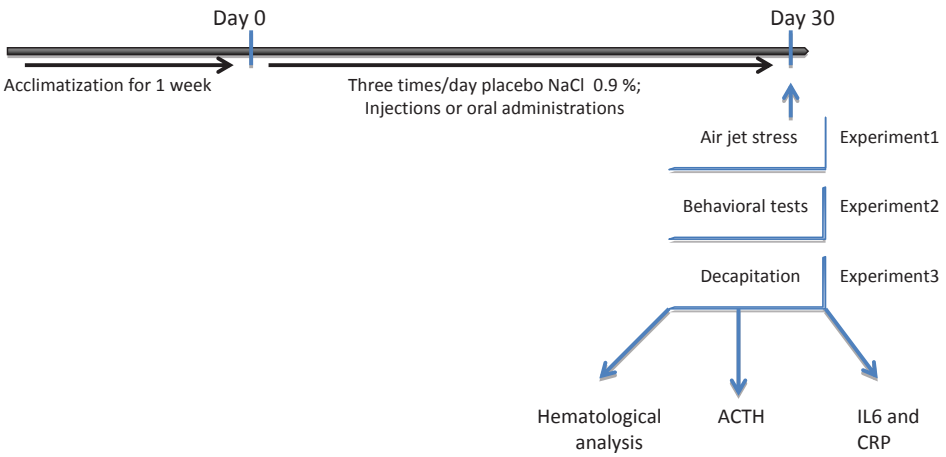

Fig. 1. Scheme of experimental procedure.

arms (50 × 10 cm), (50 × 10 × 45 cm). The apparatus is 50 cm above the ground.[23] The room where the test was performed is illuminated by suspended electric lamp of 65 W (175 cm above the maze center).[24] Each rat was separately placed in the device center, oriented towards an open arm. The exploration was measured for 5 min using the EhtoLog 2.2™ software.[25] The experiment exploits the conflict in rodents between the fear of open spaces and the desire to explore a new environment.[26] The parameters measured in this test were spent time in open and closed arms. At the end of each session, we wiped the device with ethanol.

The open field

This test is considered as a key asset for the measurement of spontaneous exploratory locomotor activity in rodents while reflecting the characteristic fear of these animals from open spaces.[27] The dimensions of this test were selected based on the work of as a cube Plexiglas platform (40 cm 70 cm × 70 cm).[28] During 5 min the experimenter can measure the time spent in each area, the central area (35 cm^2), and the peripheral area. Each session conducted semi-automatically by the EhtoLog 2.2™ software[25] while ensuring the removal of odors by wiping the test with ethanol after each use.

The light/dark box test (LDB)

Many behavioral paradigms based on different conflict situations, social interactions or explorations of new environments have been proposed to model animal anxiety. Costall et al have described (Biochem Behav Pharmacol. 32 (3):777-785, 1989)[29] a new model based on the aversive properties of an open field and on the comparison of exploratory activities in an illuminated and a dark compartment under the influence of anxiolytic substances.[29,30] For the realization of this test, the device after the open field's floor was divided into two compartments: one of them was colored black and the other was left. A bright white light illuminated the transparent compartment. An opening, playing the role of a door, was created between two compartments (10 cm × 10 cm). Each rat was separately installed in the lighted compartment and its behavioral activities were recorded for 5 min and calculated using the EhtoLog 2.2™ software.[25]

Immune cell count

A fully automated blood cell counter (PCE-210 model 2009, Japan) was used to measure the count of lymphocytes, monocytes, and granulocytes.

Biochemical assays

C-reactive protein

According to the protocol provided by the manufacturer (ZK044.L.R, The Binding Site Ltd, Birmingham, U.K.), the CRP serum level was measured by nephelometric methods. 3.51–12 mg/L as approximate measuring range is fixed when the sample dilution is 1/40. Moreover, the sensitivity limit was 0.44 mg/L at 1/5 sample dilution.

IL 6

The protocol followed was provided from the manufacturer standard sandwich enzyme-linked immunosorbent assay kit (EK0412, Boster Biological Technology Ltd, USA). The lowest limit of IL-6 detection levels was under 62.5–4000 pg/mL (The optical densities were read at 450 nm).

ACTH

The ACTH concentration was measured by enzyme-linked assay kit (Phoenix Pharmaceuticals Inc. Burlingame, USA). All steps were followed as they were described in the protocol provided by the manufacturer.

Statistical data

The results of this work are transformed into means ± SEM using MINITAB 15 (Minitab Inc., USA) which aims to calculate the one-way analysis of variance (ANOVA). The value of $p < 0.05$ was regarded as significantly different (post hoc Dunnett test was used when a comparison is required).

Results

Anxious behavior in plus maze

Behavior in S, G, and IP groups increased significantly with the time spent in the maze closed arms ($p < 0.05$ and $p < 0.001$, Fig. 2B) and very significant decrease in the open arms ($p < 0.05$ and $p < 0.001$, Fig. 2A) was compared with the intact group. But no difference was noted between the S and G groups for the first and second parameter compared with the G group ($p < 0.001$).

Anxious behavior in the open field test

Our results show that the stressed S group and both IP and G groups spend more time in the peripheral area (Fig. 3A) and less time in the central area (Fig. 3B) and saves less locomotor activity (Fig. 3C) during the test compared with the intact groups. In contrast to the IP group ($p < 0.01$ Fig. 3A, $p < 0.001$ Fig. 3A and C), oral administration of the placebo in the G group does not seem to change the results obtained in the S group rats concerning the time spent in each of the areas of the test and the distance crossed (Fig. 3).

Anxious behavior in LDB test

In this test, S, G, and IP group rats spent significantly more time in the dark compartment ($p < 0.001$, Fig. 4A) and less time in the light compartment ($p < 0.001$, Fig. 4B) compared with the T group control rats. In contrast, the IP group ($p < 0.01$, $p < 0.001$) was significantly different from the G and S groups. Intra-peritoneal pretreatment does not improve the behavioral performances of IP rats in this test as demonstrated by the non-existence of significant differences between the latter and the S rats (Fig. 4).

Immune cell count

The results of lymphocyte formula show a highly significant increase in the total granulocytes in rats exposed to air jet stress ($p < 0.001$) compared with the intact group T. It seems to be the case that rats treated orally by the placebo were not significantly different from rats in the S group. The opposite of these results is witnessed in the reported rates of lymphocytes and monocytes that we found

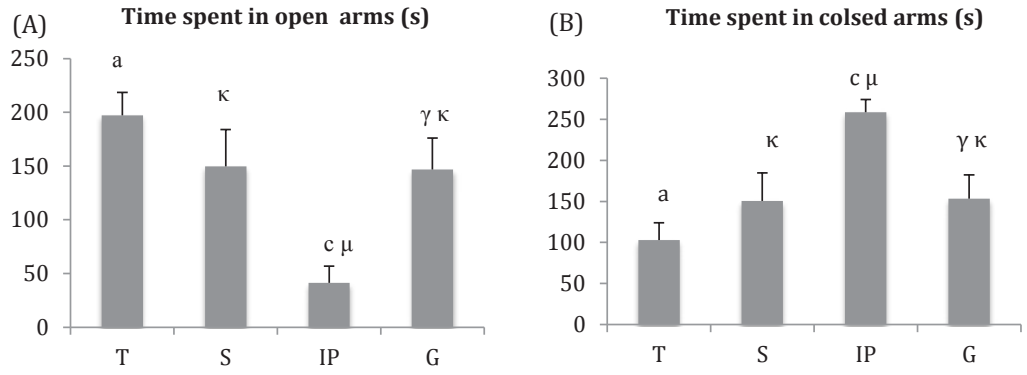

Fig. 2. The parameters in the elevated plus-maze test among rats pretreated with NaCl (placebo) via oral and intra-peritoneal routes and 1 h of exposure to the air jet stress. The results are expressed as the mean ± SEM. $^{a}p < 0.05$ and $^{c}p < 0.001$ vs. S; $^{\alpha}p < 0.05$ and $^{\gamma}p < 0.001$ IP vs. G; $^{\kappa}p < 0.05$ and $^{\mu}p < 0.001$ vs. T.

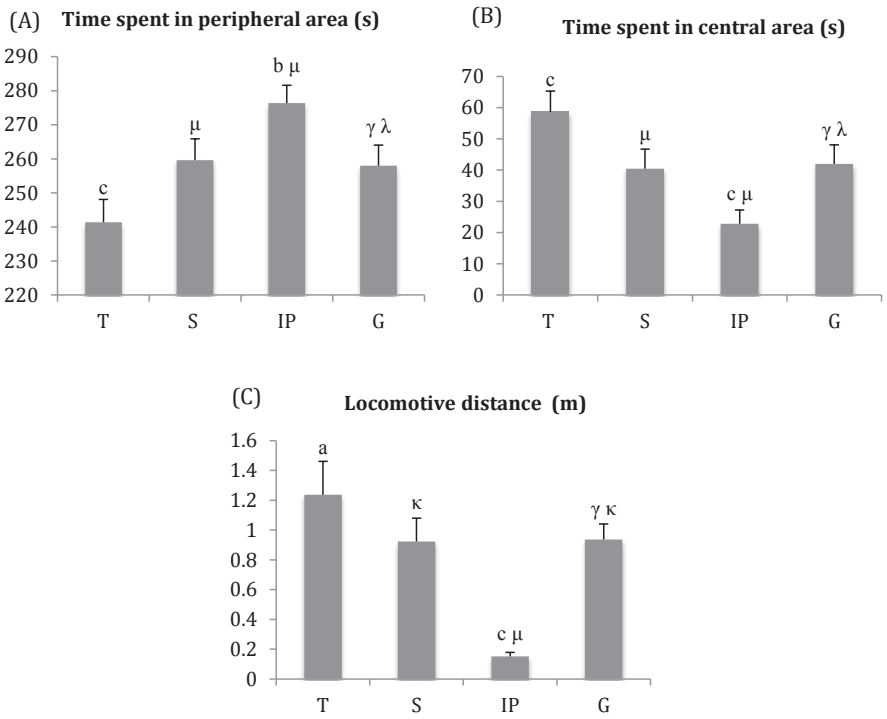

Fig. 3. The parameters in the open field test among rats pretreated with NaCl (placebo) by oral and intra-peritoneal routes and exposed for 1 h to air jet stress. The results are expressed as mean ± SEM. $^{a}p < 0.05$ and $^{b}p < 0.01$ and $^{c}p < 0.001$ vs. S; $^{\gamma}p < 0.001$ IP vs. G; $^{\kappa}P < 0.05$ and $^{\lambda}p < 0.01$ and $^{\mu}p < 0.001$ vs. T.

to be significantly suppressed in the S group ($p < 0.001$) compared with the intact group. Between the G and IP groups, there is a significant difference in the monocyte ($p < 0.01$), granulocyte ($p < 0.01$), and lymphocyte ($p < 0.001$) concentrations (Table 1).

ACTH, CRP and IL 6 plasma levels

The results after one month of intra-peritoneal injection show a significant increase in the plasma levels of ACTH ($p < 0.001$, Fig. 5A), CRP ($p < 0.05$, Fig. 5B) and IL-6 ($p < 0.05$ and $p < 0.01$, Fig. 5C) compared with groups T and G. However, there is no significant difference between the G group and the stressed group (Fig. 5).

Discussion

In this study, we investigated the ability of immune-endocrine mediations triggered after a negative contact with the laboratory animal on the quality of routine scientific stress experience data. The anxiogenic effect of the air jet stress is expressed in the elevated plus maze test by an augmentation past-time in the closed arms compared with the open one. These behavioral changes are probably due to damage in the regions controlling locomotor activity and anxiety. Indeed, sending a compressed air jet into the cage of the animal causes a psychological emotional stress that influences brain function by causing changes in multiple neural systems leading to neurodegenerative disorders.[14-22] The significant difference between IP and G

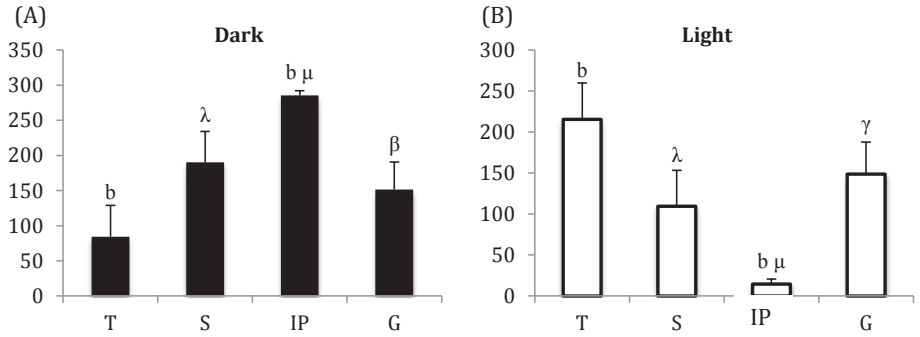

Fig. 4 The parameters of the light/dark box test among rats pretreated with NaCl (placebo) by oral and intra-peritoneal routes and exposed for 1 h to air jet stress. The results are expressed as mean ± SEM. $^{b}p < 0.01$ vs. S; $^{\beta}p < 0.01$ and $^{\gamma}p < 0.001$ IP vs. G; $^{\lambda}p < 0.01$ and $^{\mu}p < 0.001$ vs. T.

Table 1. Immune cell counts in male rats

Parameters	T	S	IP	G
WBC (×10³/μL)	11.56±0.51^{b}	8.35±0.60$^{\lambda}$	10.85±0.82^{a}	7.34±2.09$^{\gamma,\mu}$
LYM (×10³/μL)	8.34±0.23^{c}	3.70±0.41$^{\mu}$	7.52±0.35^{c}	2.85±1.30$^{\gamma,\mu}$
MONO (×10³/μL)	0.52±0.07^{c}	0.28±0.03$^{\mu}$	0.42±0.08^{a}	0.24±0.12β,μ
GRAN (×10³/μL)	2.71±0.32^{c}	4.37±0.32$^{\mu}$	2.91±0.58^{b}	4.22±0.79$^{\beta,\lambda}$

The results are expressed as mean ± SEM. $^{a}p < 0.05$ and $^{b}p < 0.01$ and $^{c}p < 0.001$ vs. S; $^{\beta}p < 0.01$ and $^{\gamma}p < 0.001$ IP vs. G; $^{\lambda}p < 0.01$ and $^{\mu}p < 0.001$ vs. T.

group in the open field test, which can be associated with the i.p treatment, is not due to the distinct route of administration technique but to the handling of animal pain and the repeated narcosis of needles, which inflict a physical and mental stress on the rats, especially when the injection procedure lasts several days throughout the protocol.[31]

Davis and Perusse assumed that the aversive experience of animals weakens their bond with experimenter and affects the quality of behavioral tests used in medical research.[32] Stress caused by negative interactions with the experimenter would damage the learning and cognition abilities of the rat; thereby undermining the usefulness of the animals in scientific research and reducing clarity of the real results issued from the data.[33-35] In opposition, a friendly link such as the administration of drugs directly from the syringe after habituation training of the procedure for one week with a 5% sweet solution, can reduce the stress response associated with experimental practices. This was previously reported in the behavior results (open field, elevated plus maze) in addition to the LDB test, which indicates that the injected group seems to be more anxious than the stressed group through most of the 5 min of the test in the dark compartment. These remarks submit that experimental model interactions with humans during the oral administration imitating the positive social interactions of the species can be used as alternative rewards that replace the aversive effects of the injections.[36]

Secondly exposure to stress air jet caused a particular immune distribution; specifically, the lymphocyte levels were depressed in associated with an important augmentation in the count of granulocytes. In fact, scientific studies have shown that the exposure of rodents to important social challenges, in this case to a 1-h episode of jet air, increases the granulocyte proportion and decreases the lymphocyte proportion.[37] It is possible that the lymphocytes accumulate in the bone marrow.[38] Moreover, psychological stress causes the high level of oxidative damage that can probably disrupt the balance between proliferation and blood cell apoptosis.[39-45] The exact mechanism is not clear; however, researchers suspect the mediation of glucocorticoid-related stress.[46-47]

The main issue our results raise is why, unlike the outcomes of the behavioral tests, did the injected NaCl seem to better restore the values of white blood cells from damage from the oral air jet stress?

Some studies suggest there is a specific window in which the development of specific response may be altered by stress. During a primary response, exposure to stress just before or during the 24 h following vaccination would be a critical period. Stress occurring later would have little or no effect.[48-51] From this perspective, we argue that the handling coupled to the injection procedure repeated for one month is a pseudo-chronic stressor that prevents an immune deviation when exposed to the air jet stress such as the one observed in the group administered placebo orally and wherein the lack of aversive contact with the rats makes them immunologically naive to the stressful session of the air jet following treatment. Neglecting such an immune-resistance process by researchers will likely decrease the quality of collected immunity data by overestimating the immune-pharmacological effectiveness of any product, due to non-consideration of the anxiety impact of the treatment technique on the psychological status of the experimental model.

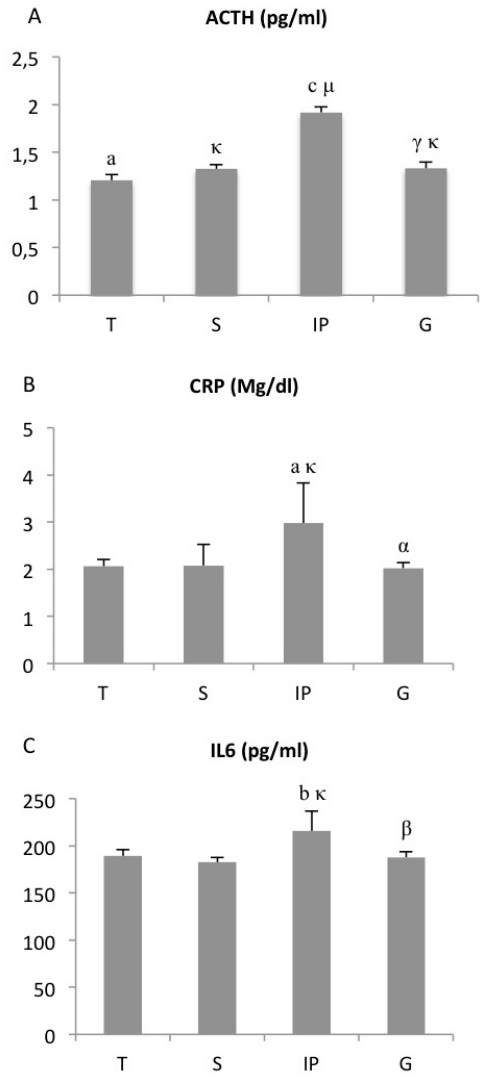

Fig. 5. Plasma levels of ACTH (A), CRP (B), and IL-6 (C) in rats treated with the placebo or vehicle (0.9% NaCL) by oral and intra-peritoneal routes. The results are expressed as the mean ± SEM. [a] p<0.05 and [b] p<0.01 and [c] p<0.001 vs. S; [α] p<0.05 and [β]p<0.01 and [γ] p<0.001 IP vs G; [κ] p<0.05 and [μ] p<0.001 vs T.

The presence of pseudo stress associated with the intra-peritoneal injections preceding the application of the air jet may be the origin of the immune-resistance demonstrated in this protocol in the groups treated only with the vehicle (placebo). We recorded a typical stress response that involves an overproduction in the levels of ACTH and IL-6 at the end of thirty days of vehicle injection with NaCl. The significant associated production of CRP is due to the rate of IL-6 that generally alters the expression CRP, SAA, haptoglobin, fibrinogen, and orosomucoid (acute phase protein). Among these proteins, the CRP is recognized as the selection marker of the inflammatory response that increases the production of inflammatory cytokines and thus amplifies IL-6 rates.[52,53]

The regulation of the immune response is induced by bi-directional inductions that involve the nervous endocrine and the immune system.[54,55] The IL-6 secretion along with other pro-inflammatory cytokines is activated precisely by depression, stressful events and experiences including injections. Additionally, IL-6 is a potential stimulator of the stress axis (HPA).[56] This is translated by the release of ACTH and glucocorticoids, major excitatory hormones. After a session of chronic injection or of physical stress, immune cell sensitivity to the glucocorticoids effect is decreased.[57,58] Processes behind the resistance to glucocorticoids related stress are not fully elucidated.

The stress prevents GR migration from the cytoplasm to the nucleus;[59] it prevents the complex GR hormone from inhibiting gene transcription via the NF-κB routes including proinflammatory cytokines. The physical activity associated with the experimental manipulation of the animals during the injection may be partly involved because physical exercise in humans decreases the sensitivity of blood lymphocytes to dexamethasone, a synthetic steroid.[60] Stress and the physical activity induce a release of IL-6 in to the plasma. Its potential role in the resistance induction was tested in vitro.[61]

Research Highlights

What is current knowledge?

√ Friendly link between animals and experimenter can reduce the stress response related to experimental manipulations.

What is new here?

√ Unlike oral administration, repeated intra-peritoneal injections cause important raise of the obtained plasma levels of the ACTH, IL6 and CRP.

√ The presence of pseudo stress associated with the intra-peritoneal injections may be the origin of immune-resistance

√ Pain of repeated narcosis compromise immune-behavioral data results.

Conclusion

To conclude, this work supports the consideration of non-invasive techniques such as oral administration that provides the animal with a positive contact link with the experimenter and avoids the physical aggressiveness of handling and the pain of repeated narcosis. As we demonstrated with the vehicle injection, a source of undesired pseudo-stress would compromise the data of behavioral tests by triggering a corticotropin response of ACTH and pro-inflammatory cytokines. In addition to air jet stress, it amplifies the animal's anxiety; this is probably due to the increase of IL-6 plasma levels accompanied by the rate of CRP known to affect the immune cell sensitivity to glucocorticoids. Consequently, we support, in an experimental protocol, the distinction between the control group receiving the vehicle (placebo) and the intact group to isolate the effect of the drug on the active group compared with the administration effect itself.

Ethical issues

The study protocol was carried out according to the NIH

revised Guidelines for the Care and Use of Laboratory Animals (no. 80–23, 1996).

Competing interests

Authors declare no competing interests.

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