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A New Approach to Manual Therapy for the Immune System: an Experimental Study

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ABSTRACT

Objective: The aim of the present study was to analyze the effects of micro-physiotherapy on the acute stress induced in rats by analyzing the cytokines Th1 and Th2. **Methods**: Forty-five wistar rats (weighing approximately 200 grams) were divided into three groups (3, 14 and 21 days) and then sub-divided into groups of five (control group, the placebo group and treated group). The animals were deprived of sleep for a period of four days. The treatment applied involved soft touches on determined points of the referred organs and tissues. Analysis of the Th1 and Th2 systems was performed using flow cytometry. **Results**: Upon analysis of the pro-inflammatory interleukin 2 cytokines and the Tumor Necrosis Factor, the lowest concentration levels were observed in the group that was treated for 21 days. The anti-inflammatory cytokine, interleukin 4, recorded similar concentration levels as the pro-inflammatory cytokines, with low values in the group treated for 21 days. In the statistical analysis, a significant difference was found between the 21-day control group and the 21-day treated group for the concentration of interleukin 4. **Conclusion**: Based on the analysis of serum from rats submitted to micro-physiotherapy, the levels of pro-inflammatory and anti-inflammatory cytokines remained below the levels of the other groups. Thus, this technique influenced the immune system in terms of treating the mechanism of acute stress.

1. INTRODUCTION

All of the functions of a living organism depend on adequate equilibrium with the environment. This balance is maintained by an adaptive response that consists of a series of physical and emotional reactions that limit the deleterious effects of stressors in an attempt to maintain homeostasis in the body. Homeostasis is constantly challenged or threatened by intrinsic and extrinsic factors[1]. In order to maintain this equilibrium, an adaptive response is necessary. According to Selye[2], this depends on the quality (physical or emotional), intensity and duration of the stimulus, in which any disturbance that alters homeostasis is considered a stressor. This response to stress is necessary for survival. When the intensity of any stressor goes above the body's threshold, a "stress syndrome" is the result[1].

Therefore, stress can be defined as any situation capable of disturbing our physiological or psychological homeostasis and can lead to repercussions that affect behavioral, endocrine and immunological properties depending on the intensity and duration of the stressor[3]. Nowadays, we are exposed to a wide range of adverse situations that have a significant impact on many aspects of our day-to-day lives, depending on how the body reacts to a stressor. Homeostasis in the immune system is completely dependent on the adequate interaction between regulator cells and costimulatory molecules[4]. The main mediators involved in adaptive responses to stressors are glucocorticoids and catecholamines, while the equilibrium between the cytokines Th1 (pro-inflammatory) and Th2 (anti-inflammatory) is also significant[5].

The Th1/Th2 equilibrium is fundamental to the protection of the body against external agents. For example, Th1 cells become active in response to intracellular bacterial and viral attacks, while also playing a role in the activation of macrophages and in the appearance of antigens through the liberation of interferon-γ (IFN- γ), interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α). Conversely, the immune cytokines that are characteristic of Th2, such as IL-4, IL-5, IL-10 and IL-13, promote humoral defense by stimulating mastoids, eosinophils and B cells against extracellular pathogens[6].

Several previous studies have shown that adults submitted to acute stress exhibit increased production of Th1 cytokines[4], as well as greater Th2 activity, with more production of pro-inflammatory agents, such as TNF-α, which are notable in Th1/Th2 imbalances. According to Xiang et al.[4] this imbalance may persist for up to a week after the end of a stressful event. These mechanisms are partly responsible for abnormalities in the immune system, with an increase in pro-inflammatory activity leading to a significant risk of developing a disease or disorder if it is not duly regulated.

A number of treatment options exist, including the use of drugs to reestablish the Th1/Th2 equilibrium[7, 8]. However, conservative and non-pharmacological treatment protocols represent a good alternative for patients, given that they have little or no side effects. Manual therapy is one of these options and involves a wide range of techniques in which the therapist uses their hands to make contact with the patient´s skin in order to assess, identify and treat a variety of clinical conditions and reestablish the body´s normal function[9]. The literature contains little information about the effects of manual therapy on the immune system, particularly in relation to Th1 and Th2 cytokines[10, 11].

Micro-physiotherapy involves manual therapy that acts directly on the surface of the body to identify and treat clinical conditions, with global results[12, 13]. The technique involves identifying the primary cause of the disease and/or symptoms and allowing the body to "heal itself". Thus, the body will recognize the aggressor (antigen) and begin the elimination through cellular and tissue reprogramming[14]. Assuming that micro-physiotherapy is a holistic approach, and since stress affects the body as a whole, including several different systems, we hypothesize that micro-physiotherapy would act on the protective systems, in this case, the immune system, to promote a rebalance of the components such as the cytokines Th1 and Th2. These cytokines respond to an acute or chronic stressful event by deregulating the immune system. Therefore, the aim of the present study was to analyze the effects of micro-physiotherapy on the acute stress induced in rats by analyzing the cytokines Th1 and Th2.

2. METHODS

2.1. Samples

In total, 45 wistar rats (weighing approximately 200 grams) were used in the present study. Before the induction of acute stress, the rats had free access to food and water and were kept in a light/dark cycle of 12 hours. They were randomly divided into three groups as follows: Control Group (CG), which involved stress without treatment (n=15); the Placebo Group (PG), which involved stress and caressing the animals back (n=15); and the Treated Group (TG), in which the animals were treated using micro-physiotherapy (n=15). These groups were sub-divided into CG3, CG7 and CG21; PG3, PG7 and PG21; TG3, TG7 and TG21;

where 3, 7 and 21 means the analysis made after the treatment. The present study received approval from the Animal Use Ethics Committee under protocol number 034/2014.

2.2. Induction of acute stress

A sleep deprivation model was used to induce acute stress[15]. The animals were caged individually and alternatively submitted to the following conditions: 16 hours without water; two nights of continuous illumination; two periods (7 and 17 h) of 45°C inclination of the cage; one period of 17 hours in a dirty cage (100 ml of water in a sawdust bedding); one period (8 h) without food; and a period of 17 h paired with another animal in a cage (the animals were always paired with the same partner).

2.3. Manual therapy procedure

2.3.1. Treated group

The application of the micro-physiotherapy begun with palpation assessment and the micro-physiotherapy treatment were performed at the same time by one of the researchers (DG), who had prior experience of this technique. The assessment involved a palpation examination in which was carried out on the animals' skin in an attempt to find a decrease in cutaneous tissue mobility. Such method could be demonstrated by viscerosomatic reflex where this reflex is initiated by afferent impulse from a visceral disorder to somatic tissues resulting in a sensory and motor changes in skeletal muscle, and skin, for example[16]. This was shown in a study where the authors[17] found consistent findings of viscerosomatic reflex where osteopaths throughout palpation detected changes in somatic tissues such as decreased mobility of the skin.

The researcher used their two hands or their middle and index fingers, depending on the size of the body area under investigation. Locations of the decreased mobility of the skin found in the animals are shown in figure 1. After these restrictions were encountered, the aim of the therapy was to decrease the tension or restriction in the mobility of skin tissue. This was conducted by bringing both hands together on the hypomobile points found and maintaining this position until the tension was relieved.

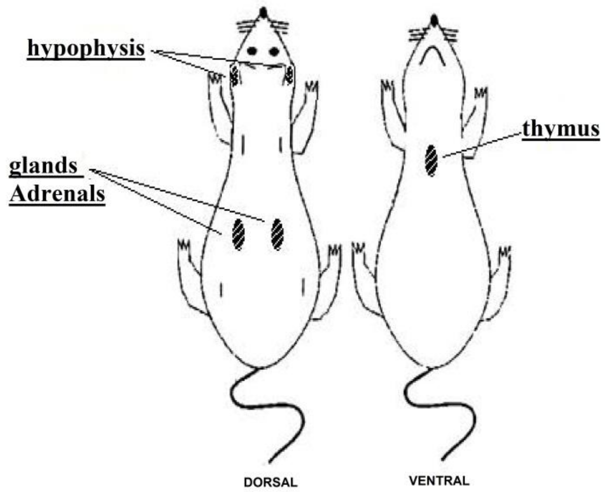

Figure 1. Localization of decrease mobility of the skin in the animals.

2.3.2. Placebo and Control Group

Each animal from the placebo group was removed from its cage, placed on the researcher´s hand (AH), and with other hand had its neck and backstroked gently during 10 minutes in a 1 Hz cycle approximately. This researcher (AH) was not aware about the micro-physiotherapy technique, to exclude any possible effects of this technique. In this manner, we were trying to show two distinct effects. The control group was left undisturbed throughout the entire protocol, except for regular cage cleaning, which was done for all animals by the same researcher.

2.4. Flow Cytometry

In total, 1 ml of blood was collected from each rat for the flow cytometry analysis (BD Accuri C6 - Becton Dickinson, USA). This content was deposited in microtubes of 1.5 ml and kept in a water bath for 15 minutes, before being centrifuged at 1500 RPM for 5 minutes at 18 degrees Celsius. After being centrifuged, the supernatant (serum) was separated for later use. The BD™ cytometric bead array (CBA) mouse th1/th2 cytokine kit was used to detect IL-2, IL-4, IL-6, TNFα and IFN, following the manufacturer’s instructions. Initially, we added 50ul of the serum sample from each rat to 50ul of the mix containing an equal proportion of the specific beads for each cytokine, with 50ul of detection reagent and 50ul of standard diluent. after incubation for two hours in the dark, 1 ml of water was added and centrifuged for 2000 RPM for 5 minutes at 4 degrees Celsius. Subsequently, the supernatant was discarded and resuspended in 300ul of water.

The theoretical limit of detection for each cytokine using the *BD™ cytometric bead array* (CBA) mouse th1/th2 cytokine Kit was defined as the concentration corresponding to two standard deviations above the mean fluorescence of 30 repetitions of the negative control (0pg/ml). The following limits were applied: IL-2 = 0.1pg/ml; IL-4 = 0.03pg/ml and TNF = 0.9pg/ml. The cytometry reading was conducted manually, with the acquisition of 10,000 events from each sample.

2.5. Analysis of the cytometry data

FCap 3.0 Array software was used to analyze the samples after the performance of the flow cytometry. The results were displayed in graphs, including the mean and standard deviations values.

2.6. Euthanasia

All rats were euthanized 1 day after the procedures. The animals were anesthetized with 80 mg/kg ketamine and 15 mg/kg xylazine and euthanized with an intraperitoneal injection of a lethal dose of thiopental. The procedure was performed in an experimental room without the presence of other animals.

2.7. Statistical Analyses

The Shapiro-Wilk normality test was used and the sample was analyzed using the Kruskal-Wallis test, with Dunn´s post-test.

3. RESULTS

Figure 2 displays the mean and standard deviation values for interleukin 2 in the control, placebo and treated (micro-physiotherapy) groups. Note that these values were 648.8±144,2 pg/ml in CG3, 863.9±33.19 pg/ml in CG7 and 978.8±12.01 pg/ml in CG21. There was an increase of interleukin 2 (pg/ml) in the CG. The mean values were 1191.0±591.8 pg/ml in PG3, 635.6±284.3 pg/ml in PG7 and 1484.0±71,92 pg/ml in PG21, with a notable variance between the interleukin values in PG3 and PG21. The mean values were 907.8±1156 pg/ml for TG3, 156.4±140.3 pg/ml for TG7 and 50.35±49.07 pg/ml for TG21. No statistically significant values were found in the Kruskal Wallis test (*P*=0.1483).

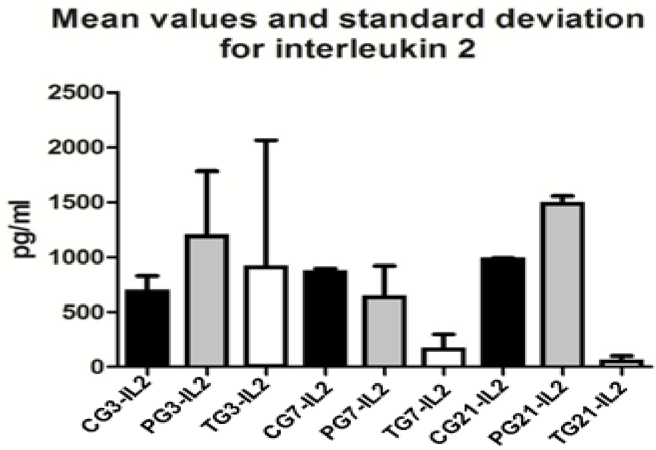

Figure 2.

Figure 3 displays the mean and standard deviation values for IL-4: CG3 = 845.5±219.4; CG7 = 1204.0±381.0; and CG21 = 1718.0±197.8. Note that the presence of this interleukin increased in the CG during the study period. The mean and standard deviation values in PG3 were 929.2±266.7, while those in PG7 were 755.4±70.9 and those in PG21 were 953.9±474.9. In the PG, there was a clear maintenance of the pg/ml of interleukin 4. In TG3, the mean values were 49.15±36.23, while in TG7 they were 74.79±70.09 and in TG21, they were 67.37±27.92. Note that the levels of interleukins increased slightly in TG7 and remained low in the other two periods. A statistically significant difference was recorded in the statistical analysis (p=0.0008). Dunn´s post-test confirmed significant differences between the groups, the largest of which occurred between CG21 and TG21 (*P*=0.0078).

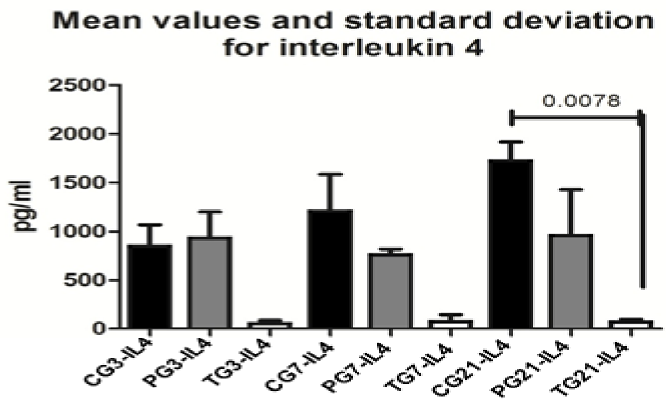

Figure 3.

The pg/ml levels for TNF-α were 1788.0±85.50 pg/ml in CG3, 2464.0±295.3 pg/ml in CG7 and 2657.0±7.6 pg/ml in CG21. The TNF-α levels were found to increase in the CG and the increase was continuous. The mean and standard deviation values were 3052.0±1496.0 pg/ml in PG3, 2536.0±88.71pg/ml in PG7 and 2636.0±593.8pg/ml in PG21. In this case, for the PG, the TNF-α levels exhibited a slight decrease in the concentration. The mean and standard deviation values were 62.28±72.87pg/ml in TG3, 1039.0±670.7pg/ml in TG7 and 74.85±68.41 pg/ml in TG21. Notably, the concentration of TNF-α was higher in TG7 than in TG3 and TG21. TG3 and TG21 exhibited similar levels. In the statistical analysis, the values exhibited significant data, with a p-value of p=0.0033. A statistically significant difference was recorded in Dunn´s test in CG21 ≠ TG21 and PG21 ≠ TG21, with p-values of 0.0078 and 0.0144, respectively (Fig 4).

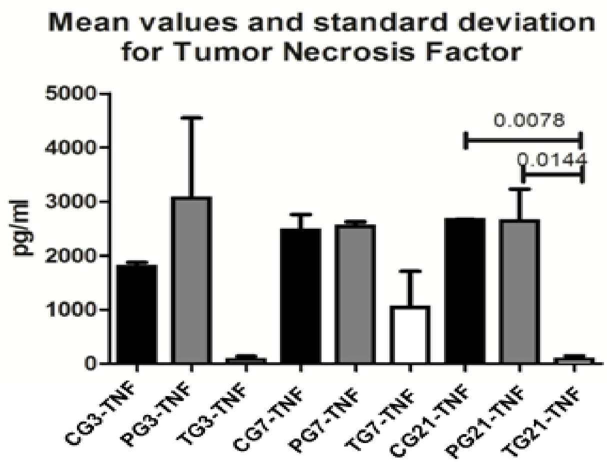

Figure 4.

4. DISCUSSION

Chennoui et al.[18] analyzed the presence of TNF-α during sleep deprivation and reported that this cytokine plays a role in this situation. The same authors confirmed that the concentrations of TNF-α and IL-6 were significantly higher among sedentary sleep-deprived rats than among sedentary control rats. In addition, the IL-6 concentration was statistically lower in sleep-deprived rats that performed exercise than among sedentary sleep-deprived rats. In the present study, the TNF values were high in the CG and PG groups (3, 7 and 21 days), when compared with the TG groups. This suggests that the technique used to treat the animals was effective in terms of controlling the liberation of TNF by the cells. No significant correlation was found between the CG and the TG. The present study confirmed that TNF-α (pro-inflammatory cytokine) can activate immune-mediated inflammation in the brain, thereby increasing the laboratory evidence that TNF-α activates the molecular inflammatory mechanisms that induce neurotoxicity, for example.

The technique of micro-physiotherapy promoted immediate and delayed activity (for two months). It is possible to confirm the influence of pro- and anti-inflammatories by comparing TNF-α and IL-2 with IL-4, which increased in the 21-day period in the treated group. Subtle tactile stimulation is capable of maintaining the homeostasis of the metabolism of the skin, thereby demonstrating the interaction between the neuroendocrine system and cutaneous stimulation[19, 20]. This subtle contact (promoted in micro-physiotherapy) alters the HPA axis through variations in the immune system, given that the skin and its appendages can generate the same mediators used during the responses to systemic stress[21]. Through the HPA axis of the skin, the active stress of the mastoid cells leads to a selective suppression of the TH1 response, altering humoral immunity[22]. A number of authors have demonstrated that the skin and its appendages are richly innervated and their efferent signals are well represented by the sensory cortex[20,23], which may explain the improvement in the regulation of cytokines in the present study. Treatment protocols that involve subtle tactile stimulation have provided favorable results among humans and animals in relation to stress[24], the autonomous nervous system[25] and neuro immunomodulatory systems[26].

Meltzer et al.[27] and MacNeil et al.[28] reported that the high SNS tone is a response to the inflammatory alterations to the system. High circulating concentrations of TNF-α, IL-1 and IL-6 act on the hypothalamus to stimulate the pathways of the central nervous system that drive the sympathetic system to expend energy in the relevant target tissues, including secondary lymphoid tissues and the inflammation sites. In the present study, the subtle contact involved in the micro-physiotherapy identified restrictions in tissue mobility in areas corresponding to the adrenal glands, pituitary gland, and thymus. After the intervention involving light touch (approximately 5 to 10 grams), there was a significant reduction in the quantity of the cytokines IL-4 and TNF-α. These responses to stress may occur as result of the effective functions that these subtle touches promote when compared with discriminative tactile functions. Type-C afferent fibers (Cf) respond with minimal contact in a pleasant manner[29]. Furthermore, Milne et al.[30] have previously demonstrated that spinothalamic neurons converge these nociceptive inputs towards the skin when faced with a visceral stimulus. According to Craig[31], the Cf can be considered as a large extension of the afferent system that is involved in the monitoring of tissues (in skin, muscles, and viscera). This system integrates several internal signals (of the body) and the cutaneous tissue, which is vital for the maintenance of homeostatic balance.

In addition, psychological stress can impair several aspects of the immune system. Reiche et al.[32] reported that communication between the CNS, the endocrine system, and the immune system involves chemical messengers, soluble mediators which are secreted by nerve cells, cells from the endocrine organs or immune cells, and psychological stressors may disturb this communication network. Palumbo et al.[15] found that the concentration of IL-8 was significantly lower among patients with post-traumatic stress disorder. In a second assessment, they confirmed higher concentrations of IL-2 and IL-6. In the present study, it was found that the level of IL-2 remained high in CG3, PG3 and TG3, when compared with the level found in CG7, PG7 and most notably, TG7. It was notable that the level of IL-2 was low in TG21. Based on these results, it is possible to hypothesize that the micro-physiotherapy affected the liberation of IL-2 during the study period. This inhibition related to the technique decreased over the 21-day period.

Some limitations may be raised in this study. Lack of another researcher to see the validity and reliability of palpation in areas of poor skin mobility. Regarding the placebo group, the same researcher who performed the technique in the experimental group could have performed on the placebo, however, he would not be "blind" to the applied technique and this could interfere with the results. In relation to analyzes, histochemical data from the suprarenal and pituitary gland would be interesting to see the effects of the micro-physiotherapy technique on cortisol and acetylcholine respectively. Future studies should address these issues to better understand the mechanisms behind the micro-physiotherapy technique, as well as research in humans where the effect of psychological stress is closest to reality.

5. CONCLUSION

Based on the analysis of serum from rats submitted to micro-physiotherapy, the levels of pro-inflammatory and anti-inflammatory cytokines remained below the levels of the other groups. Thus, this technique influenced the immune system in terms of treating the mechanism of acute stress.

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