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# Different Duration of Cold Stress Enhances Pro-Inflammatory Cytokines Profile and Alterations of Th1 and Th2 Type Cytokines Secretion in Serum of Wistar Rats

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**Abstract:** Cold stress is generally considered to suppress the immune system and may lead to an increase in the occurrence of disease in the presence of a pathogen. The immune system is ordinarily brought back to a baseline response level after immune challenge through homeostatic processes in part regulated by the cytokines. Often, findings reported from various studies investigating the effects of stress on the cytokines are conflicting. This is due to some conditions has limited the accumulation of information on effects of cold stress on cytokine profile. The present study investigated the effects of multiple cytokines in response to a given stressor to understand the role of cytokines in the immunological responses to different duration of cold stress exposure in rats. Thus, researchers simulated animals' hypothermia life environment, using Luminex xMAP and evaluated the effect of cold stress for pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), chemokine interferon-γ-inducible protein-10 (IP-10), Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) type of cytokine in serum of SPF Wistar rats. Irrespective of the duration, cold stress enhanced levels of pro-inflammatory cytokine IL-6. Acute cold stress up-regulated TNF-α, IP-10, IL-2 and IL-4 levels. TNF-α, IL-2 and IL-4 levels showed down-regulate trends compared with the acute cold stress treatment groups in the duration of chronic cold stress. Both IFN-γ and IP-10 levels increased significantly in cold stress 9 and 12 days. These data demonstrate that cold stress enhances pro-inflammatory cytokines profile and alterations of Th1 and Th2 type cytokines secretion in serum of rats. With the extension of the cold stress time, the cellular adaptive immune response and to some extent, the nonspecific humoral immune response was also affected.

**Key words:** Cold stress, cytokines, Luminex xMAP, immune function, rat, China

## INTRODUCTION

Animals will face various kinds of environmental stressors every day and cold stress as one of the stressors commonly exists in the cold region. Researchers have demonstrated that cold stress dramatically affected the health and welfare of animals in the cold region (Li *et al*., 2006; Wang *et al*., 2009; Yildirim and Yurekli, 2010a, b). Therefore, there is a need to understand how cold stressors affect health status of animals. Moreover, studies also proved that acute or chronic cold stress induces immunomodulatory effects in animal models as well as in human, mice and chicken (Banerjee *et al*., 1999; Brenner *et al*., 1999; Padgett and Glaser, 2003; Hangalapura *et al*., 2004).

Cold stress as a natural stressor may have its own unique pattern of neuroendocrine changes because of the accompanying body temperature variations that may influence immune functions (Shu *et al*., 1993). Cold stress affects various aspects of immune function depending on the nature and duration of the stress (Dhabhar, 2003). For example, stressors can directly affect the cells of the immune system and modulate the secretion of pro-inflammatory cytokines, Th1 or Th2 cytokines (Ruzek *et al*., 1997; O'Connor *et al*., 2003; Hangalapura *et al*., 2006). Cytokines play a key role in bidirectional communication between the neuro-endocrine and immune systems (Mahbub-E-Sobhani *et al*., 2008, 2011). It has been suggested that the interplay between

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hormones and cytokines during thermal stress may influence immune homeostasis in response to environmental challenges (Dugue, 2000; Russwurm *et al*., 2002). Most cytokines have pleiotropic, redundant and multifunctional functions and the level of one cytokine is tightly regulated by other cytokines (Adibhatla *et al*., 2008). Thus, some factors elevated by stress exposure could be responsible, in part for stress-induced suppression of other factors. It is important to examine multiple cytokines in response to a given stressor to understand the role of cytokines in the physiological response to cold stress exposure.

To date, there are a number of ways to administer cold stress and this may account for the different effects on the cytokines reported by various studies. However, there are some conditions had limited the accumulation of information on effects of cold stress on cytokine profile included the cold stimulus conditions, regulatory environment temperature, methods of cytokines detection and the choice of experimental animals in the studies. For example, some studies used cold water stress (Cheng *et al*., 1990; Shu *et al*., 1993; Dugue, 2000) which could not simulate animals' hypothermia life environment and the ambient temperature, humidity, carbon dioxide content could not be controlled precisely. Cold stress did not have a significant effect on IL-1β levels in man (Jansky *et al*., 1996) while in mice, cold exposure enhanced IL-1 cytokine levels (Cheng *et al*., 1990). Moreover, in order to correlate a specific physiological, pathology or disease process with changes environment temperature using traditional methodologies (real-time PCR, ELISPOT, ELISA and intracellular cytokine staining), limited sample sizes or budget restrictions can often prohibit this kind of testing (Hangalapura *et al*., 2006; Rhind *et al*., 2001).

In the present study, a climate room was described for precise regulation of temperature, humidity and carbon dioxide content. The precision of regulation for the temperature is ±0.05°C and the relative humidity is ±1%. The homogeneous cold temperature could be obtained (±0.1°C) as cold stressor. To determine cytokine profiles in serum, one possible multiplex platform is the Luminex xMAP, a bead array coupled with discrete fluorescent molecules to detect multiple soluble analytes (Wong *et al*., 2008). xMAP technology offers ideal speed and sensitivity for performing multiplexed cytokine measurements. Luminex's breakthrough approach offers comparable sensitivity to traditional ELISA-based systems (Elshal and McCoy, 2006) but with additional advantages including extended dynamic range and smaller sample size. With their small volume, low cost per test and multiplex capacity, Luminex-based cytokine assays have potential utility for present studies. The Specific Pathogen Free (SPF) rats were also a better choice in the studies which could reduce individual differences, to improve the accuracy of the experiment results.

The main aims of the present study was to determine the effect of different duration of cold stress (acute and chronic cold stress) on level of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), chemokine interferon-γ-inducible protein-10 (IP-10), Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) type of cytokines in serum of SPF Wistar rats.

## MATERIALS AND METHODS

**Treatment of animals:** The study protocol was approved by the Animal Care Committee of Jilin University and was performed according to Chinese animal care guidelines. Rats were obtained from the Laboratory Animal Center, Academy of Military Medical Science. Ninety male SPF Wistar rats (12 weeks old, 190-240 g) were housed individually under diurnal lighting conditions at the climatic room (12-12 h light/dark) with free access to drinking water and a standard pellet diet. The environment in the climatic room was maintained at a temperature of 24±0.05°C, relative humidity 45±0.1%. The rats were divided into the following ten experimental groups: 1 group for the control group experiment, 4 groups for the acute cold stress experiment and 4 groups for the chronic cold stress experiment. The 8 stress treatment groups were maintained at 4±0.05°C (3, 6, 12 and 24 h treatment groups for the acute cold stress and 3, 6, 9 and 12 days treatment groups for the chronic cold stress). After the treatments, rats are anesthetised by 75 mg $kg^{-1}$ sodium pentobarbital. In order to determine cytokines levels in blood, 4 mL blood taken from the anesthesized rats by entering right ventricule from their hearts. The bloods were allowed to clot for at least 30 min before centrifugation for 10 min at 1000×g and then remove serum and assay immediately or aliquot and store samples at ≤-20°C.

**Cytokine measurements:** Eight cytokines interleukin-1β (IL-1β), IL-2, IL-4, IL-6, IL-10, IFN-γ interferon-γ-inducible protein-10 (IP-10) and Tumor Necrosis Factor-α (TNF-α) were measured using the MILLIPLEX MAP 8-plex Cytokine kit (Millipore, Billerica, MA) at the Institute of Pharmacology and Toxicology, Academy of Military Medical Science, Beijing, China. Median fluorescence intensity calculated from duplicates for each sample was collected using the Luminex-100 System Version 1.7 (Luminex). The StatLIA Software package (Ver. 3.2, Brendan Scientific Inc.) incorporating a weighted five-parameter logistic curve-fitting method was used to calculate sample cytokine concentrations.

**Statistical analysis:** Statistical analysis was carried out using the SAS Version 9.1 statistical program. All data were expressed as arithmetic mean±standard deviation. One-way Analysis of Variance (ANOVA) tests were used to evaluate cytokine production across different cold stress time with overall significance for factors of cold stress time/concentration reported as such. Statistical significance was considered at the 5% level ($p<0.05$).

## RESULTS AND DISCUSSION

**Effect of cold stress on pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) levels:** The effects of cold stress on pro-inflammatory cytokine IL-1β levels were investigated in different cold stress time from 3 h to 12 days. As shown in Fig. 1, IL-1β levels had not significant difference among different duration of cold stress. As shown in Fig. 2, IL-6 levels increased significantly in cold stress 12 and 24 h, 3, 6, 9 and 12 days treatment groups when compared to control group ($p<0.05$), IL-6 levels showed up-regulate volatility among different duration of cold stress. The concentration of TNF-α were upward trend in duration of acute cold stress. As shown in Fig. 3, acute cold stress significantly enhanced the concentration of TNF-α in stress 3, 6, 12 and 24 h treatment groups when compared to control group ($p<0.05$). The concentration of TNF-α was significantly higher in acute cold stress 3, 6, 12 and 24 h compared with chronic stress 6, 9 and 12 days treatment groups ($p<0.05$).

**Effect of cold stress on chemokine (IP-10) levels:** The effects of cold stress on chemokine IP-10 levels were investigated in different cold stress time from 3 h to 12 days. As shown in Fig. 4, IP-10 levels increased significantly in 24 h cold stress group when compared to cold stress 3, 6, 12 and 24 h, 3 and 6 days and control groups ($p<0.05$). The levels of 9 and 12 days cold stress groups increased significantly compare with 3, 6 and 12 h, 3 and 6 days and control groups ($p<0.05$) but IP-10 levels had not significant difference between 9 and 12 days cold stress groups.

**Effect of cold stress on Th1 cytokines levels:** In present study, we measure Th1 cytokines (IL-2 and IFN-γ) levels in different duration of cold stress. The concentration of IL-2 was upward trend in duration of acute cold stress. Effects of acute and chronic cold stress on levels of the IL-2 are shown in Fig. 5. Compared with the corresponding control group, acute cold stress in 3, 6, 12 and 24 h treatment groups significantly increased

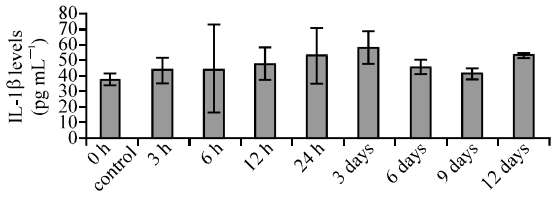


Fig. 1: The effect of different duration of cold stress treatment on interleukin-1β levels in rat serum. Analysis of variance followed by one-way Analysis of Variance tests

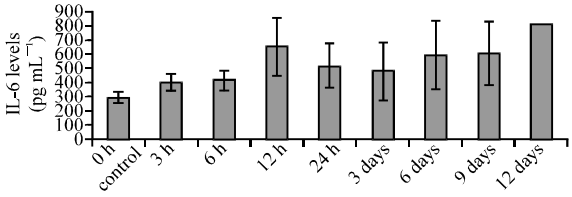


Fig. 2: The effect of different duration of cold stress treatment on interleukin-6 levels in rat serum. Analysis of variance followed by one-way analysis of variance tests

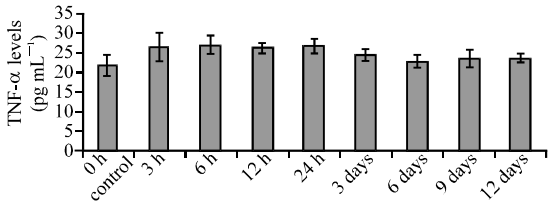


Fig. 3: The effect of different duration of cold stress treatment on TNF-α levels in rat serum. Analysis of variance followed by one-way analysis of variance tests

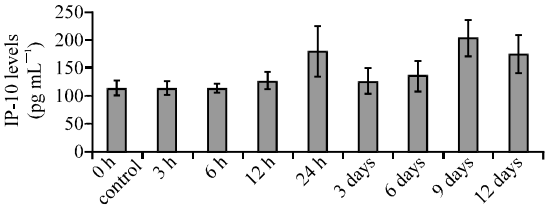


Fig. 4: The effect of different duration of cold stress treatment on Interferon-γ-inducible protein-10 levels in rat serum. Analysis of variance followed by one-way Analysis of Variance tests

($p<0.05$). IL-2 level of 3 days chronic cold stress group was higher significantly than control group ($p<0.05$) but there were not difference significantly in the other chronic stress groups. IL-2 levels returned to normal

concentration in chronic stress groups from 6-12 days compared with control group. As shown in Fig. 6, IFN-γ level increased significantly in chronic cold stress 9 and 12 days treatment groups when compared to any other acute or chronic cold stress treatment groups ($p<0.05$). IFN-γ levels were higher significantly in the 9 days cold stress compared with 12 days cold stress treatment group ($p<0.05$). Compared with the corresponding control group, acute cold stress in 12 and 24 h treatment groups significantly increased ($p<0.05$).

**Effect of cold stress on Th2 cytokines levels:** Th2 cytokines (IL-4 and IL-10) levels were investigated in different cold stress time from 3 h to 12 days. As shown in Fig. 7, cold stress significantly enhanced the concentration of IL-4 in stress 6 and 24 h, 3 and 6 days treatment groups when compared to control group ($p<0.05$). As shown in Fig. 8, IL-10 levels have not significant difference among different duration of cold stress.

The reactive mechanism of cold stress is very complicated and the results from different studies are not consistent. These may be attributed to the duration of cold exposure, the temperature of cold exposure, the genetic background of experimental animal and so on. But lots of studies have proved that cold stress could influence the energy metabolism and immune responses (Rybakina *et al*., 1997; Cichon *et al*., 2002; Haman *et al*., 2002). Pro-inflammatory cytokines are important in the recruitment of immune cells to the site of infection. Production pro-inflammatory cytokines can be stimulated directly by depression and other negative emotions and stressful experiences. Indeed, both physical and psychological stressors can provoke transient increases in pro-inflammatory cytokines (Glaser and Kiecolt-Glaser, 2005). In the present study, researchers examined pro-inflammatory cytokines IL-1β, IL-6 and TNF-α which were the principle cytokines that mediate acute inflammation. Irrespective of the duration, cold stress did not induct the change significantly of IL-1β levels, IL-1β levels showed slightly up-regulate trend in acute cold stress from 3-24 h and still showed increase to chronic cold stress 3 days. The results of IL-1β were consistent with man underwent cold and wet exposures (Jansky *et al*., 1996). While in mice and chicken, cold exposure enhanced significantly IL-1β levels (Cheng *et al*., 1990; Hangalapura *et al*., 2006).

Cold stress up-regulated expression of both IL-6 and TNF-α and this effect was determined by the duration of cold stress. With the strengthening of the cold stress (from acute cold stress to chronic cold stress), IL-6 levels still showed increase trend. Many studies have also demonstrated that restraint stress could induce elevation of the plasma IL-6 level. Yildirim and Yurekli (2010a) found that cold stress induced the increase of IL-6 levels in rat liver, lung and brain and heart tissues. Takaki *et al*. (1994) found that immobilization stress may increase plasma IL-6 via central and peripheral catecholamines (Nukina *et al*.,

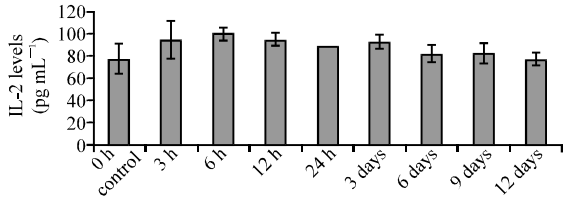


Fig. 5: The effect of different duration of cold stress treatment on interleukin-2 levels in rat serum. Analysis of variance followed by one-way Analysis of Variance tests

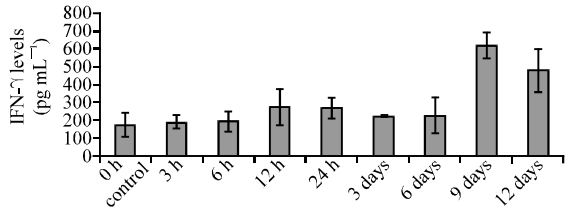


Fig. 6: The effect of different duration of cold stress treatment on IFN-γ levels in rat serum. Analysis of variance followed by one-way Analysis of Variance tests

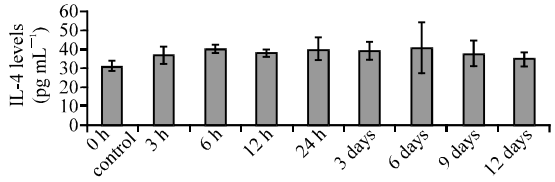


Fig. 7: The effect of different duration of cold stress treatment on interleukin-4 levels in rat serum. Analysis of variance followed by one-way Analysis of Variance tests

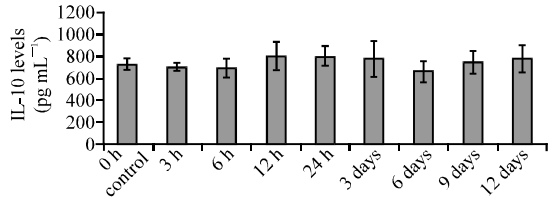


Fig. 8: The effect of different duration of cold stress treatment on interleukin-10 levels in rat serum. Analysis of variance followed by one-way Analysis of Variance tests

2001). Brenner examined the human immunological responses to cold exposure and they found a rise in circulating levels of IL-6 (Brenner *et al*., 1999).

Acute cold stress induced the increase significantly of TNF-α but TNF-β showed down-regulate trend in chronic cold stress from 3-12 days until the concentration of TNF-α down to normal range compared with control group. So, researchers predicted that TNF-α could do a stress maker in the duration of acute cold stress. To date, IL-1, IL-6 and TNF-α cytokines are the major pro-inflammatory cytokines that are responsible for early response of inflammation and either act as endogenous pyrogens (Netea *et al*., 2000). They help protect against infection and prepare injured tissue for repair by enhancing phagocytic cell recruitment and activation. Furthermore, pro-inflammatory cytokines released by recruited cells regulate the ability of fibroblasts and epithelial cells to remodel the damaged tissue (Kiecolt-Glaser *et al*., 1995). In the present study, the oversecretion of pro-inflammatory cytokines might to reduce the damage caused by cold stress.

To date, the patients with primary Sjogren's syndrome showed increased IL-6 levels of serum were correlated with poor quality of life in these individuals and the study suggested that the patients with concentrations of IL-6 higher than those of the healthy controls showed a significantly lower score in the dimensions of bodily pain and physical functioning (Baturone *et al*., 2009). So, researchers predicted that IL-6 levels increased significantly from acute to chronic cold stress compared with control group which may be related to against the pain and damage caused by cold stress. Furthermore, Stevenson suggested that IL-6 was an important early indicator in evaluating piglet health status (Stevenson *et al*., 2006). Andrew analyzed the levels of cytokines after psychological stress and they found that IL-6 was the most representative indicator (Steptoe *et al*., 2007). Thus, we suggested that IL-6 could do as a hallmark cytokine when rats were in the environmental challenges.

Cytokines produced by the innate immune system lead to differentiation of the T-helper 1 (Th1) and 2 (Th2) immune pathways. Activation of Th1 often involves stimulation of cellular immunity while Th2 is associated with humoral immunity. These results of present study, together with changes in IL-6 and TNF-α level, imply that cold stress may result in dysregulation of the Th1/Th2 cytokine profiles break the Th1/Th2 balances and then affect immune response.

The Th1 response is associated with the release of the cytokines IFN-γ and IL-2. IFN-γ is a key cytokine which is central to stimulatory and inhibitory roles of a Th subset. IFN-γ does not directly inhibit differentiated Th2 cells. Instead, they were inhibited by blocking the differentiation of these subsets from naive precursors. Essentially, IFN-γ has been shown to inhibit Th2 response (Coffman, 2006). In this present study, we also found that IFN-γ and IP-10 had similar trends in duration of cold stress. Yue *et al*. (2011) found that elevated IFN-γ concentration could induce macrophages to secrete the IP-10. IP-10 is the production of the Th1-attracting chemokines which preferentially promotes Th1 immune responses (Yue *et al*., 2011). The results suggest that IFN-γ and IP-10 also had synergic action to promote cell-mediated immunity in the duration of cold stress.

IL-2 level is an important indicator of cellular immunity (Chen *et al*., 2011). In the present study, IL-2 levels increased significantly in acute cold stress treatment groups compared with control group. The result would increase an evidence indicates that acute cold stress could have a stimulating effect on cell-mediated immunity which is consistent with study results of Dhabhar and McEwen (1996) (Dhabhar *et al*., 1996; Jiang *et al*., 2007). To date, mild stress or hormone related with stress treatment to animals, the immune level of animals would be enhanced. Numerous studies showed that small amounts of harmful or cold stressful agents could be beneficial for the health of laboratory animals and improve immunity (Dhabhar *et al*., 1996; Dhabhar and McEwen, 1999). In this present study, IL-2 levels returned to normal concentration compared with control group in the duration of chronic cold stress which indicated that the body has adapted gradually to the cold stress. But with the strengthening of the cold stress and immunosuppression would be induced. Rhind found that cold stress enhanced concentration of IL-6, IL-2 and TNF-α in human serum (Rhind *et al*., 2001). Liu Ya-Li suggested that oral (gavage) administration of compound nutrients was found to attenuate the acute and chronic immobilization and cold water immersion stress-induced increase in serum IL-6 levels and decrease in IL-2 levels (Liu *et al*., 2007). The result of IL-2 was converse to the present study. It was showed that cold stressors can directly affect the secretion of cytokines, under different stress conditions, cytokines showed different trends.

The Th2 response is associated with the release of IL-4 and IL-10. These cytokines tend to enhance the production of antibodies. To the data showed that cold stress up-regulated the IL-4 levels from duration of cold stress 24 h to 6 days and did not affect IL-10 levels. IL-4 is a key regulator in humoral and adaptive immunity and decreases the production of Th1 cells and macrophages. IL-4 antagoizes the effects of IFN-γ and thus inhibits cell-mediated immunity (Coccia *et al*., 2000). IL-4 as the

hallmark cytokine of Th2 immunity if it is overexpressed, it negatively interferes with the immune defense mechanisms thus decreasing the recruitment, expansion or activity of major effector cells such as the Th1 cells (Salak-Johnson and McGlone, 2007). In the duration of chronic cold stress from 3-6 days, IL-4 and IL-6 (Th2 related) levels sharply higher compared with the other treatment groups, IL-2 and IFN-γ (Th1 related) levels returned to normal range which indicated that humoral immunity was becoming dominant.

Acute and chronic stressors tend to affect the immune responses differently whereby chronic stress most often leads to suppression of the immune system. Often, acute stress has limited suppressive effects on immune function (Salak-Johnson and McGlone, 2007). To the knowledge, the data provided herein are the first to examine whether different duration of cold stress affects the role of multiple cytokines in the response (s) to cold stressor exposure in rats.

## CONCLUSION

In this study, acute cold stress enhanced the immune level of rats. When cold stress was prolonged, the cellular adaptive immune response and to some extent, the nonspecific humoral immune response was also affected. These results suggest that eight cytokines reflects archetypal Th1- and Th2-type responses and provide insight regarding the polarization of cytokine activity at different cold stress levels. Moreover, the underlying factors seem to be estimable and can be used to represent divergent profiles in cold stress analyses. However, it should be emphasized that none of the cytokines specific to one particular subset are exclusive products of Th cells because other leukocytes can contribute to Th1 or Th2-type responses. It is important to point out that like many other researchers, we studied only one specific part of the immune function. We lack information on whether the function of the whole immune system was affected by cold stress. Clearly, the pattern of multiple cytokines induction and the role of multiple cytokines in the response (s) to cold stressor exposure require further attention.

## ACKNOWLEDGEMENTS

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