B-4491
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RESEARCH ARTICLE Indian Journal of Animal Research

# Vestibular Stimulation as An Interventional Approach for Cold Water Stress Induced Immunological and Histopathological Changes in Rats

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10.18805/IJAR.B-4491

## ABSTRACT

**Background:** Chronic exposure to stress results in immuno-suppression. The present study was carried out to explore the therapeutic benefits of vestibular stimulation on the immune system of stress-induced Wistar rats.

**Methods:** Stress was intervened in the Wistar rats by cold water swimming method for a period of 14 days. Following stress, caloric vestibular stimulation was induced in the rats by irrigating external auditory meatus bilaterally with hot water (temperature 41°C) for 2 minutes for a period of 15 days and 30 days in respective groups. At the end of the experiment, blood samples were obtained and serum corticosterone, Interleukin 2 (IL2), Immunoglobulin M (IgM) levels were analyzed using ELISA method and histopathology of brain was assessed. Brain CD4 and CD8 cells were analyzed by Immunohistochemistry.

**Result:** Stress significantly increased serum corticosterone, IL2 levels and reduced IgM levels. Stress group showed increased dendritic arborization in prefrontal cortex, neuronal atrophy, nuclear pyknosis with congested blood vessels in hippocampus and mononuclear cell inflammatory infiltrate in hypothalamus. Brain extravascular CD4 and CD8 showed positive in stress group. Caloric vestibular stimulation effectively reduced serum corticosterone levels, histopathological changes, brain extravascular CD4 and CD8 cells and improved IgM in the present study.

**Key words:** Caloric vestibular stimulation, Cold water swimming stress, CD4, CD8, Histopathology, IgM, IL2, Stress.

## INTRODUCTION

Stress is essential for survival. The most important mechanisms by which body responds to stress is through activation of hypothalamic-pituitary adrenal (HPA) axis and release of glucocorticoids (GCs) which trigger physiological, biochemical, immunological and behavioral responses to cope up with the demanding situation (Schneiderman *et al*., 2005). Sustained exposure to GCs induce deleterious responses and affects an individual. Interleukin 2 (IL2) synthesized by activated T cells is a major immuno-regulatory cytokine and is essential for the proliferation and activation of CD4 and CD8 cells (Liao *et al*., 2013). It is a key factor responsible for both growth and death of antigen activated T lymphocytes and controls autoimmunity (Malek, 2003). The literature lacks clarity in substantiating the effect of stress on IL2 levels.

Blood brain barrier (BBB) and Blood cerebrospinal fluid (BCSF) barrier are largely impermeable to T lymphocytes under physiological conditions. Many studies have reported altered CD4 and CD8 count in peripheral circulation and mobilization in response to stress (Atanackovic *et al*., 2006). However, studies correlating stress and CD4, CD8 infiltration in brain samples are scarce.

Controlled vestibular stimulation has been traditionally used for the diagnosis of neurological disorders involving brain stem (Wilkinson *et al*., 2013), which could also be used to investigate and treat clinical conditions (Miller and Ngo, 2007). Controlled vestibular stimulation is reported to be beneficial in the modulation of neurotransmitters related to brain ageing, in dementia and also in the improvement of depression and anxiety (Kumar *et al.,* 2014; Jinu *et al*., 2018). But, limited knowledge exists about the effects of vestibular stimulation on stress-induced immune cell dysfunctions in brain. The objective of the current study is to evaluate the effect of cold water stress on (1) CD4, CD8 cell infiltration into the brain parenchyma, (2) on serum corticosterone, IL2 and IgM levels (3) histopathology of brain and (4) to evaluate the effects of caloric vestibular stimulation on the observed stress-induced changes.

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**How to cite this article:** Kranthi, T.R., Archana, R. and Sivanesan, S.K. (2021). Vestibular Stimulation as An Interventional Approach for Cold Water Stress Induced Immunological and Histopathological Changes in Rats. Indian Journal of Animal Research.
DOI: 10.18805/IJAR.B-4491.

**Submitted:** 19-04-2021 **Accepted:** 07-07-2021 **Online:** 16-08-2021

## MATERIALS AND METHODS

### Animals

Male, Wistar rats of 3 to 6 months age (200 to 300g) were included in the study. Animal experiments were performed as per the guidelines of Committee for Control and Supervision of Experiments on Animals (CPCSEA). Four animals were housed per cage and provided with a commercial pellet diet and water *ad libitum*. The present study was carried out at Prathima Institute of Medical Sciences during the years 2018 and 2019 after obtaining Institutional animal ethics committee clearance (1/PIMS/ 2017 Dated 24/08/2017). All the experiments and sample collection were done between 9.00 AM and 12.00 Noon.

### Experimental design

The animals were randomly selected and grouped as follows.

Group I: (n = 4) Control.

Group II: (n = 4) CWS group, administered with cold water swimming stress for 14 days.

Group III: (n = 4) CWS +15 R group rats were subjected to stress and left untreated for 15 days to check for natural recovery.

Group IV: (n = 6) CWS+15 days CVS group rats received cold water swimming stress for 14 days followed by caloric vestibular stimulation for 15 days as intervention.

Group V: (n = 4) CWS +30 R group rats were subjected to stress and left untreated for 30 days to check for natural recovery

Group VI (n = 6) CWS+30 days CVS group rats received cold water swimming stress for 14 days followed by caloric vestibular stimulation for 30 days as intervention.

### Cold water swimming stress

The animals were subjected to stress for a period of 14 days. Stress was induced by allowing the rats to swim in cold water maintained at 10°C for 30 minutes once a day. Circular plastic containers of 60 cm height and 40 cm diameter were used for the study and water level was maintained at 30 cm height (Nagaraja and Jeganathan, 1999).

### Caloric vestibular stimulation (CVS)

CVS was induced in rats by irrigating external auditory meatus for a duration of 2 minutes using warm water of (41°C). In a polyethylene tube 2 mL water was taken and pushed at constant speed into the external auditory meatus of rats to induce caloric vestibular stimulation.

After 14 days of stress, caloric vestibular stimulation (CVS) was bilaterally given as intervention for a duration of 15 and 30 days in the respective groups (Varghese *et al.*, 2015). Blood samples were collected by retro orbital puncture (Ravikumar *et al.,* 2021)and animals were sacrificed as per the guidelines provided by Control and Supervision of Experiments on Animals (CPCSEA).

### ELISA (Solid phase enzyme-linked immunosorbent assay)

Separation of serum was done after clotting of the blood and subjected it to centrifugation using Remi-8M centrifuge for 10 min at a speed of 503 x g.

### Determination of serum corticosterone

Corticosterone was determined using Demeditec corticosterone rat ELISA kit as per the instructions provided by the manufacturer.

### Measurement of serum IL2

IL2 was determined using Boster rat IL-2 Picokine™ ELISA kit as per the instructions provided.

### Analysis of serum IgM

IgM was determined by using LSbio rat IgM ELISA kit as per the instructions provided by the manual.

### Histopathology of brain

After removal, immediately brains were fixed in formaldehyde buffered with 10% phosphate. The brains were placed in 10% neutral buffered formalin (NBF) for 24-48 hours and then dehydrated with ethanol (70% for 24 h, 90% for 1 h and 100% for 1 h), later cleaned with xylene. 20:1 ratio of formalin to tissue was maintained. For trimming, tissue sections of 3-5 mm were made using forceps, cutting board, single edge razor blade. Tissue was submitted in one cassette. Dehydration was done using increasing grades of ethyl alcohol (70%, 80%, 95%) in tissue processor (YORCO; semi-automated) for 10 minutes each. Clearing was done for removing alcohol and allowed the wax to enter the tissue section. This was done by using xylene in tissue processor. Impregnation was done using paraffin wax in tissue processor. Tissue embedding involves removal of processed tissue from the cassettes, placing them in a mould (Leukhart's mould) made up of brass maintaining their original orientation. Then the mould was filled with paraffin wax which produces a paraffin block. After embedding in paraffin wax, coronal sections were cut using a microtome (Leica RM 2025, Germany) at 5 µm thicknesses. Sections were mounted on glass slides and followed by staining. Sections were stained using hematoxylin and eosin technique (Makwana *et al,* 2019). Slides were mounted on DPX Moutant and kept dry without air bubbles.

### Determination of CD4 and CD8 expression by immuno--histochemistry

Immunohistochemistry was performed on formalin fixed paraffin embedded brain tissue. Flex monoclonal mouse anti-human CD4 clone 4B12 antibody was used to determine CD4. For estimating CD8, Flex monoclonal mouse anti-human CD8 clone C8/144B antibody was used. It was followed by Mayer's haematoxylin counterstaining for 30 seconds. The slides were dried, mounted using DPX and covered with coverslips. The immunoreactivity was visualized using the LABOMED-300 microscope (Zettl *et al*., 2004).

### Statistical analysis

Data was analyzed using SPSS statistical software package version 20.0 (SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple comparisons. All the variables are expressed as Mean ±SEM p-value < 0.05 was considered to be statistically significant.

## RESULTS AND DISCUSSION

### Serum corticosterone

The results of corticosterone measurements are shown in Table 1. Significantly ($p<0.05$) higher serum corticosterone level was found in the CWS group, CWS R15 and CWS R30 recovery groups in comparison to the control group. Whereas, CVS intervention for 15 days and 30 days showed a significant ($p<0.05$) decrease of corticosterone levels when compared to the recovery groups (CWS R15 and 30). There was statistically significant reduction ($p<0.05$) of corticosterone level in rats which received CVS intervention for 15 days and 30 days in comparison to its respective control groups and other stress groups which did not receive CVS.

### Serum Interleukin 2

The results of IL2 values are shown in Table 1. As compared to the control group, the CWS group showed a significant ($p<0.05$) increase in IL2 levels. In contrast, recovery groups (CWS R15 and CWS R30) showed significantly lower ($p<0.05$) IL2 levels compared to CWS group. However, in comparison to stress group (CWS), groups which received CVS intervention for 15 days and 30 days did not show a significant difference ($p>0.05$).

### IgM

The results of the IgM assessment are shown in Table 1. As compared to the control group, the CWS group, CWS R15, CWS R30, and CWS+CVS15 which received CVS intervention for 15 days showed a significant ($p<0.05$) decrease in IgM levels. However, there was no significant difference between control and CWS+CVS 30 group which received a 30 day CVS intervention.

### Histopathology of prefrontal cortex, hippocampus and hypothalamus

Following stress, prefrontal cortex showed increased dendritic arborization. Hippocampus showed neuronal atrophy, nuclear pyknosis with congested blood vessels. Mononuclear cell inflammatory infiltrate was seen in hypothalamus. Following 15 days of caloric vestibular stimulation, prefrontal cortex, hippocampus and hypothalamus showed normal morphology, whereas, the control 15 days recovery showed mononuclear inflammatory cell infiltrate in hippocampus. The 30 days recovery group and interventional (CWS+CVS 15 and 30) groups showed normal morphology (Fig 1 and 2).

### CD4, CD8 cells

Immunohistochemistry of the brain has shown extravascular CD4 and CD8 cells in CWS group rats. Rats that received CVS for 15 days (Fig 3) showed no CD4 and CD8 cells in brain parenchyma while the CWS+R15 group, which did not receive CVS showed CD4 cells. Animals in the CWS+CVS 30 group which received CVS intervention for 30 days and CWS+R30 group showed normal parenchyma.

Immune system was once believed to be autonomic, but currently there is substantial evidence which proves the role of CNS in regulation of immune system. Environmental stimuli like sensory, psychosocial factors and stress can be translated into signals by nervous system which modulates the functions of immune system (Felten and Felten, 1994). CNS can modulate immunity by both neuronal and hormonal pathways.

Stress triggers the hypothalamic - pituitary adrenal (HPA) axis (Joëls *et al*., 2004) and consequently raises corticosterone levels. It binds to the GC receptors on the immune cells and interferes with the role of NF-kB, which limits the number of cytokine instigated immune cells. Catecholamines bind to adrenergic receptors, which restrict cytokine-induced gene transcription (Padgett and Glaser, 2003). In the current study, we witnessed a significant increase in serum corticosterone in stress and non-interventional (recovery) groups. We observed a significant decrease in serum corticosterone levels in the interventional group which received CVS intervention which is an indicative that caloric vestibular stimulation is effective in the reduction of serum corticosterone levels.

The T cells invasion into brain parenchyma is monitored by the Blood Brain Barrier (BBB). However, in the absence of BBB damage and neuro-inflammation, T-cells show less motility and exit quickly, even if it enters the cerebral circulation (Morawski *et al*., 2017). In the current study, in the stress group, the resident CD4 and CD8 cells were

**Table 1:** Serum corticosterone, serum IL2 and IgM levels in cold water stress induced rats.

| Parameters | Control | CWS | CWS + R 15 | CWS + CVS 15 | CWS + R 30 | CWS + CVS 30 |
|---|---|---|---|---|---|---|
| serum corticosterone (ng/ml) | 11.74 ± 0.52 | 181.88 ± 36.32[a] | 171.46 ± 35.78[a] | 73.65 ± 8.84[e] | 111.93 ± 15.72[c] | 54.76 ± 9.54[d] |
| serum IL2 (pg/ml) | 1506.4 ± 69.47 | 2449 ± 295.89[c] | 1471.2 ± 76.83[f] | 1822.4 ± 252.77 | 1398.5 ± 59.09[f] | 1639.1 ± 88.34 |
| IgM (mg/ml) | 0.90 ± 0.05 | 0.43 ± 0.03[b] | 0.40 ± 0.03[b] | 0.46 ± 0.07[b] | 0.49 ± 0.01[c] | 0.64 ± 0.11 |

Results are expressed as Mean± SEM; [a]p < 0.001 as compared to control, [b]p <0.01 as compared to Control Group; [c]p < 0.05 compared to Control Group; [d]p < 0.001 as compared to Stress Group; [e]p < 0.01 as compared to Stress Group; [f]p < 0.05 as compared to Stress Group.

positive in brain extravascular tissue. It demonstrates the differential ability of lymphocytes movement in CNS during stress. However, 15 days of caloric vestibular stimulation has prohibited such accumulation of CD4 and CD8 cells into CNS. After 30 days both interventional and non-interventional groups showed no CD4 or CD8 cells in extravascular tissue which implies caloric vestibular stimulation is helpful in early recovery. Stress modifies the expression of tight junction and adherens junctions proteins (occludin, ZO-1, VEGFα, claudin-5 and VE-cadherin) of BBB and changes microvascular endothelial cells ultrastructure. These changes enlarge gaps in tight junctions of BBB. The flow of lymphocytes into CNS augmented during stress might be due to enhanced permeability of BBB (Xu *et al*., 2019; Lee *et al*., 2018). The exact mechanisms and factors involved in the regulation of these BBB proteins are not yet concrete.

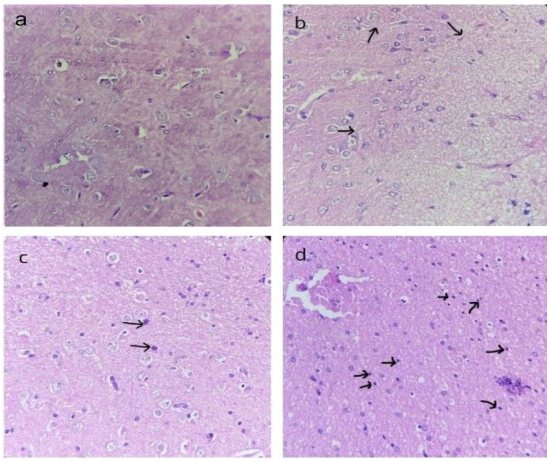


**Fig 1:** Histopathology of brain of Wistar rats at 40x magnification under microscope. **a** control hippocampus **b.** dendritic arborisation in prefrontal cortex in stress group **c.** nuclear pyknosis in hippocampus in stress group **d.** mononuclear inflammatory cells in hypothalamus in stress group

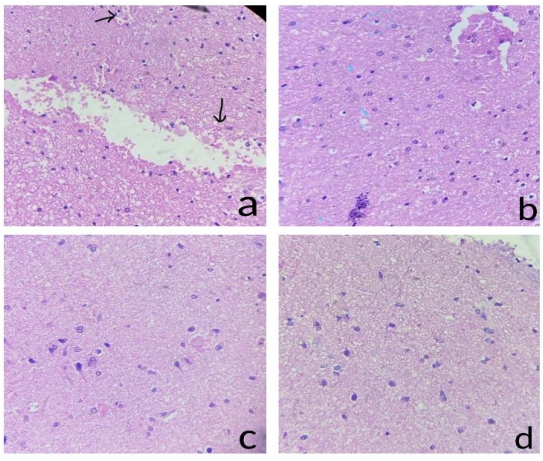


**Fig 2:** Histopathology of brain of Wistar rats viewed at magnification 40x under microscope. **a.** congested blood vessels in hippocampus in stress group **b.** mononuclear inflammatory cells in 15 days recovery group **c** and **d.** normal cortex and hypothalamus following 15 days of CVS.

Interleukin 2 is a major immuno-regulatory cytokine released from activated T cells. It is essential for the proliferation and activation of CD4 and CD8 cells (Liao *et al*., 2013). It is an essential element for both growth and death of antigen-activated T lymphocyte, which controls autoimmunity (Malek, 2003). Repeated stress and restraint stress has decreased IL2 in previous studies (Batuman *et al*., 1990; Sheridan *et al*., 1991), whereas in the current study, serum IL2 levels increased in stress group. The findings of the present study are in agreement with the report of Himmerich *et al*. (2013). The release of IL2 is controlled by CD4+, CD25+ regulatory T cells. Stress inhibits these regulatory T cells and subsequently increases IL2 production (Himmerich *et al*., 2013). In the present study, serum IL2 levels were found to be elevated in animals that received CVS intervention (CVS 15 and CVS 30) in comparison to rats which did not receive CVS intervention. Role of this elevated IL2 in interventional groups has to be further explored.

Immunoglobulin M (IgM) serves as the first line of host's body defense, secreted from plasma cells. It activates the classical pathway of the complement system after binding with an antigen, triggers phagocytosis and antigen presentation (Ehrenstein and Notley, 2010). In the present study, serum IgM reduced significantly in the stress group, and this finding is in accordance with the previous studies (Moazzam *et al.,* 2013). After 30 days of CVS intervention, IgM levels improved when compared to the non-interventional groups. The fall in IgM could be due to the indirect influence of corticosterone on B cells which prevent B cell differentiation and suppress immunoglobulin production (Sapolsky *et al*., 2000).

HPA axis is the primary pathway involved in the regulation of stress induced CNS alteration of immune system. Vestibular stimulation can influence HPA axis by vestibulo-paraventricular polysynaptic pathways. Hypothalamus is involved in diverse endocrinal functions. Vestibular system has connections with lateral and posterior hypothalamus (Rajagopalan *et al*., 2017). Vestibular stimulation can decrease cortisol and stress reactivity in infants (White-Traut *et al*., 2009). Caloric vestibular stimulation can inhibit sympathetic-adrenal-medullary axis and hypothalamic-pituitary-adrenal (HPA) axis by direct pathways and indirectly by increasing the release of GABA in substantia nigra which inhibits HPA axis at the level of paraventricular nucleus (PVN) and reduces ACTH secretion. Vestibular stimulation also activates hippocampal formation and stress axis can be inhibited by activated hippocampal formation (Sailesh and Mukkadan, 2013). Thus vestibular stimulation can inhibit stress axis and reduces glucocorticoid levels and stress induced changes mediated by glucocorticoids.

Chronic stress causes both structural and functional alterations in prefrontal cortex. Stress affects different areas

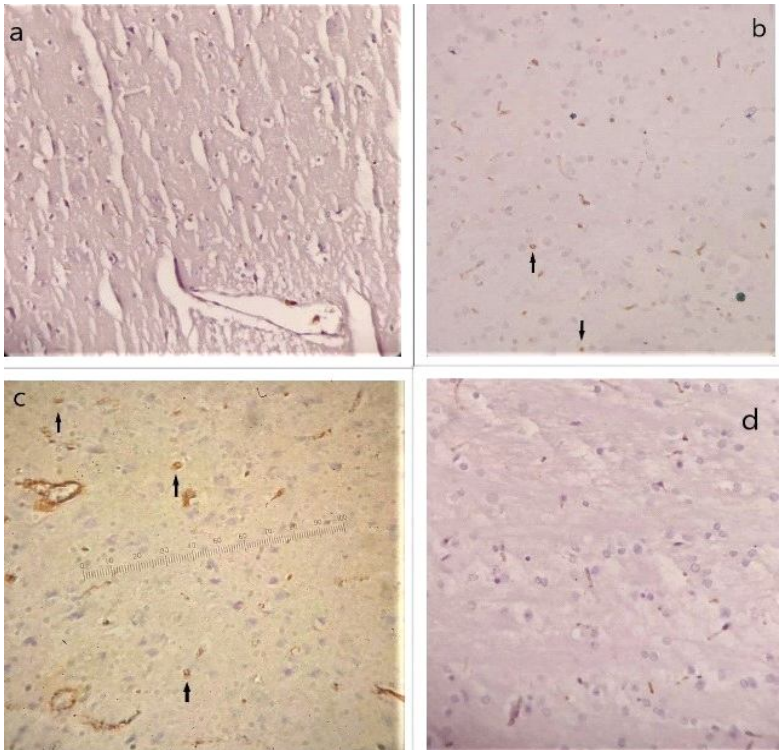


**Fig 3:** Immunohistochemistry section of rat brain under 40 x; **a.** Control b and **c.** stress group showing CD4 and CD8 cells **d.** Interventional group showing no extravascular CD4 or CD8 cells in Parenchyma.

of prefrontal cortex differentially. It causes shortening of dendrites in medial prefrontal cortex while producing growth of dendrites in orbitofrontal cortex. In the present study, we have observed increased dendritic arborization following 14 days of stress which is in accordance with previous studies (McEwen and Gianaros, 2010).

Hippocampus is a vulnerable target of number of hormones which include thyroid, gonadal, adrenal hormones and modulates dendritic structure and synapse formation. Repeated stress causes CA3 dendritic atrophy and there is suppression of neurogenesis (Khan *et al*., 2020). In the current study, we observed neuronal atrophy, nuclear pyknosis with congested blood vessels in hippocampus which clearly indicate reduced blood supply, neuronal damage and inflammation in stress group. In the present study, mononuclear cell inflammatory infiltrate was seen in hypothalamus. Following 15 days of caloric vestibular stimulation, prefrontal cortex, hippocampus and hypothalamus has shown normal morphology. After 30 days, both interventional and non-interventional (recovery) groups showed normal histology. Hence, the present study proved that caloric vestibular stimulation (CVS 15 days to stress group) helps in early recovery.

A clear link exists between vestibular system and cortex. Vestibular system is extensively connected with cerebral cortex and lesions of vestibular system leads to atrophy of cortex and hippocampus (Brandt *et al*., 2005). Previous studies have also proven that bilateral loss of vestibular function is associated with decreased hippocampal volume, cell number, proliferation, reduced dendritic length and altered morphology leading to memory deficit, anxiety and autonomic disorders (Stackman and Herbert, 2002; Zheng *et al*., 2012; Sangeetha *et al*., 2016).

Controlled vestibular stimulation enhances dendritic arborization in pyramidal cells of hippocampus (Devi and Mukkadan, 2017) and promotes enhances cell proliferation in dentate gyrus and possibly neurogenesis (Smith *et al*., 2010). Because of extensive connections with brain structures, vestibular stimulation influences the physiology of cortex. The present study provides preliminary data on hippocampal, prefrontal cortical and hypothalamic morphological changes induced by stress and effects of caloric vestibular stimulation on stress induced changes.

## CONCLUSION

Though many studies are available depicting the relationship between stress and CD4, CD8 cells in the blood, to the best of our knowledge, the present study unraveled the effect of stress-induced changes in CD4 and CD8 cells of brain extravascular tissue. As seen in the present work, the levels of serum IL2 from several other studies were contradictory implicating further validation and exploration of mechanisms involved in it. The present study highlights CVS as a non-invasive physiological intervention, which can be incorporated in daily life to reverse the stress-induced immunological and histopathological changes.

### Conflict of interest disclosure

The authors declare no conflict of interest.

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