A STUDY ON THE ROLE OF PROINFLAMMATORY CYTOKINES AND THEIR MODULATION BY CHOLINERGIC ACTIVITY IN CHRONIC STRESS INDUCED CARDIAC DYSFUNCTION

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1. Introduction

Heart diseases are the leading cause of mortality and morbidity worldwide. Among various risk factors for heart diseases, chronic stress is considered to be an important one. Majority of cardiologists believe that chronic stress and improper diet are key factors in the progression and onset of cardiac abnormalities. The studies had reported strong link between chronic mental stress and coronary heart diseases (Rosengren *et al.*, 2004; Yusuf *et al.*, 2004). Prolonged exposure to stressful life events has been associated with development of hypertension (Rosengren *et al.*, 2004; Mucci *et al.*, 2016; Liu *et al.*, 2017). Moreover, high intensity mental stress has shown to precipitate acute cardiac complications such as myocardial ischemia, arrhythmias and cause sudden cardiac death (Levine, 2022). Despite overwhelming evidences of association between stress and heart dysfunction, the precise neurobiological mechanism underlying this association is not clearly understood.

The stress response is the result of a complex interaction of physiological, genetic, and environmental variables. Standard stress response quantification approaches, such as hormonal and immunological assessments, often evaluate a particular component and fail to link downstream biological consequences to underlying physiological processes (Perera, Soos and Machin, 2022). Understanding this complex system requires better insight into how the cellular and metabolic systems are altered during chronic stress. Blood serves as a primary homeostatic component of the body and assists in maintaining a stable microenvironment. The functional characteristics of each and every single component of blood change dynamically depending on the strength and duration of external stimuli (Saravanan and Sarumathi, 2013). The neurological, circulatory and metabolic systems are often the first to be affected by stress. Therefore, investigating the underlying processes of haematological and metabolic alterations that occur as a result of chronic stress would provide a molecular level understanding of the various physiological mechanisms that connect different stressors to stress responses and downstream pathological alterations. Thus, one of the aims of the current study was to examine the effects of prolonged stress on haematological, and metabolic changes that ultimately causes cardiac abnormalities. Moreover, the early detection of chronic stress in clinical practice is challenging due to its complicated aetiology. This study also aimed to develop a biomarker for the early identification of chronic stress based on metabolite alterations in serum.

The chronic stress causes long-term dysregulation of stress hormones (such as catecholamines and cortisol), which changes the pattern of cytokine release from immune cells. This alteration promotes the production of proinflammatory cytokines over anti-

inflammatory cytokines resulting in chronic inflammation (Liu, Wang and Jiang, 2017). The proinflammatory cytokines reportedly confirmed elevated following chronic stress including Interleukin-6 (IL-6), Interleukin-1β (IL-1β) and Tumor necrosis factor- α (TNF-α) (Miller, Maletic and Raison, 2009; Maydych, 2019). Literature suggests that various cardiac disorders are also associated with increased levels of proinflammatory cytokines (Amin *et al.*, 2020). Kapadia and colleagues reported clinical evidence of TNF-α contributing to left ventricular dysfunction, cardiomyopathy and pulmonary edema (Kapadia *et al.*, 1998). Infusion of IL-6 in rats caused concentric hypertrophy, myocardial fibrosis, and diastolic dysfunction (Meléndez *et al.*, 2010). IL-6 and CRP are also linked to increased morbidity in unstable angina pectoris and depressed myocardial function in heart failure (Gabriel *et al.*, 2004). The resulting inflammation mediated by various proinflammatory cytokines is one of the common hallmarks of chronic stress and various cardiac abnormalities. Thus, inhibition of the pro-inflammatory cytokines produced during chronic stress might be a helpful strategy for combating chronic stress induced cardiac dysfunction.

Diacerein, an anthraquinone derivative, belonging to the class of non-steroidal anti-inflammatory drug possess unique pharmacological actions on several cytokines such as IL-1β and IL-6 and is clinically used to treat osteoarthritis (Martel Pelletier and Pelletier, 2010; Pavelka *et al.*, 2016). While the effect of diacerein is known on IL-1β, docking studies revealed that diacerein also disrupts IL-6 signalling by interfering with the binding of IL-6 to its receptor (Bharti *et al.*, 2016). Tofisopam is a broad spectrum, benzodiazepines (BZDs) derivative having 2, 3-vicinal structure, differs from other benzodiazepines. It does not exert its action via binding to the Gamma Amino Butyric Acid (GABA) receptors. Instead, the drug increases the level of secondary messengers i.e. cGMP and cAMP in the brain via selectively inhibiting the PDE-4A1 enzyme followed by phosphodiesterase (PDE)-10A1, PDE-3, and PDE-2A3 respectively (S. Srivastava *et al.*, 2014). Various literature has reported that PDE-4 inhibits the production of TNF-α by altering CAMP/PKA pathway (Palladino *et al.*, 2003; Li, Zuo and Tang, 2018). Moreover, a study by Kalashnikov SV et al., 2002 showed tofisopam enhanced dexamethasone-induced suppression of lymphocyte proliferation and depressed TNF-α production (Kalashnikov, Kalashnikova and Kokarovtseva, 2002). Thus, another aim of the study was to assess the cardio-protective effects of diacerein (IL-6 inhibitor) and tofisopam (TNF-α inhibitor) in a model of chronic unpredictable stress (CUS) induced cardiac dysfunction.

Recently, vagus nerve-mediated cholinergic signalling has been reported to control immune function and proinflammatory responses via the inflammatory reflex, termed as cholinergic

anti-inflammatory pathway (CAP) (Pavlov and Tracey, 2012; Koopman *et al.*, 2016). CAP is a neurological mechanism that controls the production of proinflammatory cytokines through signals that involve the vagus nerve and α-7 nicotinic acetylcholine receptors (α7nAChR) (Czura, Rosas-Ballina and Tracey, 2006). Experimental activation of CAP, either by direct electrical stimulation of the efferent vagus nerve (VNS) or by an α7-nAChR agonist, is beneficial in attenuating severity of various diseases by suppressing the production of several proinflammatory cytokines (Liu *et al.*, 2020; Kania *et al.*, 2021; Shi *et al.*, 2022). VNS treatment is minimally invasive; however, there are preoperative risks involved with device implantation. Transcutaneous vagus nerve stimulation (taVNS) is a non-invasive approach that has recently been developed to address these shortcomings (Yap *et al.*, 2020). Moreover, PNU-282987(N-[(3R)-1-azabicyclo [2.2.2]oct-3-yl]-4-chlorobenzamid-hydrochloride) is a selective α7nAChR agonist (Pinheiro et al., 2017). Based on these findings, our last aim was to assess the effect of PNU-282987 (α7nAChR agonist that modulates CAP) and taVNS (CAP modulator) on CUS induced inflammation and heart damage.

2. Objectives

- To test the contribution of CUS to spontaneous cardiac dysfunction in albino Wistar rats.
- To assess the contribution of cholinergic flow to spatio-temporal localization of central and peripheral proinflammatory cytokines (IL-6, TNF-α) in CUS rats.
- To assess restoration potential of TNF-α /IL-6 inhibitor and VNS in CUS induced cardiac dysfunction.

3. Experimental Design

3.1. Study Design

A total of 102 animals were used in this study. The animal studies were performed in six stages, and with exception to the first and last stages in which only two groups viz control and CUS were used, all the rest of the stages involved usage of three groups i.e., control, CUS and treatment groups. In first stage the animals were validated for induction of stress. In second and third stage, studies involved evaluating the effect of IL6-inhibitor (Diacerein) and TNF-α inhibitor (Tofisopam) respectively, in chronic stress induced cardiac dysfunction. In fourth and fifth stage studies involved studying the effect of activation of CAP using taVNS and an α-7nAChR agonist (PNU-282987) respectively, in chronic stress induced cardiac dysfunction. The last stage study examined the effect of chronic stress on haematological and metabolic changes that could lead to cardiac abnormalities.

3.2.CUS Protocol

The animals of CUS group were subjected to a random stressor daily for 8 weeks. The stressors for CUS paradigm were: **restraint** – rats were held in restrainers for 1 h; **tilted cage** – rat cages were tilted at 45° for 1 h; **shaking** – rats were shaken on a mechanical shaker for 1 h; **hot air stream** – rats were subjected to hot air from a hair dryer for 10 min; **social defeat** – rats were exposed to a hostile rat, and after being overwhelmed, they were returned to their original home cages with a cut-off time of 5 min; **overnight illumination** – rats were subjected to regular room light during the night period; **inverted light cycle** – regular room light in rats holding area was inverted (off during day time and on during night) (Monteiro *et al.*, 2015)**.**

3.3. Validation of CUS

Post 8 weeks of stress exposure, control and CUS rats were subjected to body weight assessment, behavioural tests and corticosterone measurement for assessment of induction of stress.

3.3.1. Body Weight Analysis

The animals were weighed at the beginning of the investigation and every week thereafter until the end of the animal study. Moreover, % weight variation, obtained using Eq. (1), was used to indicate the change in body weight (Mishra *et al.*, 2016).

$$\text{Weight Variation (\%)} = [(\text{Final weight} - \text{Initial weight})/\text{Final Weight}] \text{ X } 100 \quad (1)$$

3.3.2. Behavioural Analysis

Anxiety/depressive-like behavioural response in rats is a strong predictor of stress-related conditions, and these behavioural changes were assessed using Novelty Suppressed Feeding Test (NSFT), Elevated Plus Maze Test (EPMT) and Forced Swimming Test (FST) (Hattiangady *et al.*, 2014; Arndt, Peterson and Cain, 2015; Rau *et al.*, 2015).

3.3.3. Corticosterone Measurement

Corticosterone levels in serum were measured using a commercial ELISA test kit procured from Wuhan Fine Biological Technology Co., Ltd., China, as per instructions provided by the manufacturer (El-Fattah *et al.*, 2018).

3.4. Molecular Docking Studies

Docking studies were performed to assess the interaction between IL-6 receptor and diacerein using AutoDock 4.0 and TNF-α receptor and tofisopam using AutoDock Vina (Morris *et al.*, 1998).

3.5. Treatments

The CUS rats were treated with diacerein (50 mg/kg/day x 20 days P.O) days (Abd Allah, 2017; Almezgagi *et al.*, 2020), tofisopam (50 mg/kg/day x 14 days P.O) (Üçel *et al.*, 2020), PNU-282987 (1mg/kg x 7 days, i.p) (Vicens *et al.*, 2011), and taVNS (1.0 ms, 6 v, 6 Hz, for 40 min x 2 weeks, alternatively) (Rawat *et al.*, 2019) in separate studies.

3.6. Serum Proinflammatory Cytokines Assessment

Proinflammatory cytokines levels in serum were measured using a commercial ELISA test kit procured from BD Biosciences, USA, as per instructions provided by the manufacturer (Singh *et al.*, 2012; P. Srivastava *et al.*, 2014).

3.7. Central Proinflammatory Cytokines Assessment

IL-6 and TNF-α levels in the homogenate of brain tissue were determined using ELISA kit procured from Abcam, USA, as per manufacturer's instruction. Moreover the expression of IL-6 and TNF-α in hippocampus and hypothalamus was determined using immunohistochemistry (IHC) (Santana-Krímskaya *et al.*, 2020).

3.8. Electrocardiography-Heart Rate Variability (ECG-HRV) Assessment

ECG of the groups under study was recorded for 10 min and then analyzed using the ECG and HRV modules included with Lab Chart Pro-8 (AD Instruments, Australia). The obtained result was further processed by MetaboAnalyst 5.0 internet server for multivariate analysis (Rawat *et al.*, 2019).

3.9. Assessment of cardiac IL-6, TNF-α, caspase 3, inducible nitric oxide synthase (iNOS), and transforming growth factor-β (TGF-β) expression

The expression of IL-6, TNF-α, caspase 3, iNOS, and TGF-β in cardiac tissue of respective groups was determined using IHC(Santana-Krímskaya *et al.*, 2020).

3.10. Blood Pressure Assessment

The systolic blood pressure measurement of rats was determined by non-invasive blood pressure (NIBP) module of the Power Lab system (AD instrument, Australia) (Alamgeer *et al.*, 2013).

3.11. Histopathological Assessment

The histopathological assessment of rats was performed using hematoxylin and eosin (H&E) staining for morphological assessment as well as with Masson’s trichrome staining for fibrosis detection (Helms *et al.*, 2010; Slaoui and Fiette, 2011; Ma *et al.*, 2016; Al-Rasheed *et al.*, 2018; Mayyas, Alsaheb and Alzoubi, 2019).

3.12. Cardiac Troponin I (cTn1) Assessment

cTnI levels in serum were measured using a commercial ELISA test kit procured from Wuhan Fine Biological Technology Co., Ltd., China, as per instructions provided by the manufacturer (Ismail, 2022).

3.13. Cardiac Caspase 3 Assessment

Caspase 3 level in heart homogenate of the respective groups was determined using an ELISA test kit from G Biosciences USA, as per instructions provided by the manufacturer (Gautam *et al.*, 2022).

3.14. Measurement of Haematological, Serum Lipid and Electrolyte Indices

Haematological parameters were determined using an automated cell counter analyser (Horbia, France). The level of TG was determined using standard biochemical kit from Agappe Diagnostic Ltd., India. VLDL levels were calculated using TG level by Friedwald formula (Kanthe *et al.*, 2012). Calcium, potassium and sodium analysis were performed using commercial kit, as per the manufacturer's protocol.

3.15. ^{1}H NMR-based Serum Metabolic Profiling

For NMR studies, 10 samples from each group were used for the validation of metabolic biomarker analysis. High resolution one-dimensional (1D) 1H Carr−Purcell−Meiboom−Gill (CPMG) NMR spectrums were obtained for all the serum samples for characterization of various metabolites, using NMR spectrometer (800 MHz Bruker, Avance III, equipped with Cryoprobe). All peaks were identified in the 1D and ^{1}H CPMG NMR spectra and annotated, followed by the concentration profiling of different serum metabolites by CHENOMX NMR suite (Chenomx Inc., Edmonton, AB, Canada) (Dubey *et al.*, 2021; Ramappa *et al.*, 2023; Singh *et al.*, 2023).

4. Results & Discussion

4.1. Validation of CUS

Before exploring the effects of CUS on cardiac dysfunction in rats, we ensured the proper induction of chronic stress in our animal model. Weight loss is considered an early sign of stress in animal models. The chronically stressed rats showed stunted growth in body weight post 3rd week and as the CUS paradigm continues, a decrease in body weight was observed till the end of the paradigm. The control rats gained weight over time. The restricted growth is due to the increased release of corticotrophin releasing hormone (CRH), which has appetite suppressor properties (Herman and Tasker, 2016; Ans *et al.*, 2018). Several studies have shown that chronic stress stimulates hypothalamic-pituitary-adrenal (HPA) axis, leading to

elevated serum corticosterone levels. Following chronic stress, the CUS animals showed a significant increase in serum corticosterone levels compared to control. A clear relationship exists between chronic stress and depression. The 8 week stress paradigm resulted in a significantly higher immobility time in CUS rats compared to control rats in FST. The EPMT revealed that CUS rats spent significantly more time in closed arms compared to control rats, suggesting induced unconditioned anxiety-like behaviour in rats. The NSFT, on the other hand, showed increased latency to first bite in CUS exposed rats, indicating decreased motivation levels and depression-like behaviour. The overactive HPA and SAM axis caused elevated corticosterone levels and anxiety/depressive-like behaviour, respectively (Sharpley, 2009).

4.2. Effect of CUS on Peripheral and Central Proinflammatory cytokines

Inflammation has emerged as a central link as it gets activated by chronic stress and contributes to the early progression of cardiac complications. Our study demonstrated significantly elevated IL-6 and TNF-α levels in serum of CUS rats, which persisted even after 3 weeks of last exposure to stress. Similarly, the expression of IL-6 and TNF-α was also significantly increased in cardiac tissue of CUS animals. The brain tissues of CUS animals also showed increased expression of IL-6 and TNF-α. The release of proinflammatory cytokines from macrophages in the periphery and microglia in the brain is caused via the hyperactivated adrenergic system and corticosterone resistance (Leonard, 2010).

4.3. Effect of CUS on Cardiac Function

Chronic stress leads to detrimental effects on heart contractility and conduction properties. The cardiac functions assessment of CUS rats by ECG analysis revealed persistent elevation of heart rate, which is consistent with several reports of resting tachycardia in animal models of chronic stress (Crestani, 2016). An increase in average R amplitude, T Amplitude, P Amplitude and ST Height of the sinus rhythm was observed in animals exposed to chronic stress, suggesting remodeling of the heart's electrical conduction system. These changes in the sinus rhythm are associated with altered morphology and function of the heart.

Vagal (cholinergic) flow has also been shown to regulate the release of pro-inflammatory cytokines in the brain and periphery (Pavlov and Tracey, 2012). Assessment of HRV is a non-invasive approach for evaluating the functionality of the autonomic nervous system. Among time domain variables, the RMSSD reflects the beat-to-beat variance in heart rate and is used to assess the vagally mediated changes reflected in HRV. Moreover, the high frequency (HF) component in frequency domain variables of HRV reflects parasympathetic activity. The SD1 is the non-linear measurement of HRV reflecting parasympathetic flow

(Shaffer and Ginsberg, 2017). All these HRV indices viz. RMSSD, HF, and SD1 were reduced in chronically stressed animals, suggesting a decline in parasympathetic activity.

Furthermore, literature suggests that hypertension facilitates remodeling of the structure and functions of the heart, which ultimately causes alterations in the ECG-HRV paradigm. We observed persistent high systolic blood pressure in CUS rats, even post 3 weeks of last exposure to stress, which might have lead to cardiac abnormalities observed in CUS rats.

4.4. *In-Silico* Studies

The binding affinity of diacerein with IL-6 receptor, as determined by AutoDock 4.0, was found to be -8.4 kcal/mol, which reveals diacerein high binding affinity towards IL-6 receptors. These binding affinities were attributed to 2 hydrogen bonds and 2 pi-alkyl bonds. The amino acids involved in the interaction include LYS 45, GLY 73, PRO 46, ALA 47, SER 72, LEU 90, HIS 70, VAL 91, GLN 68, LEU 69, ASP 92, THR 124, LEU 123, VAL 93, SER 122.

The binding affinity of tofisopam to TNF-α receptor was found to be -6.8 kcal/mol, suggesting tofisopam high affinity for TNF- α receptors. These binding affinities were attributed to 1 hydrogen bond. The amino acids involved in the interaction include LEU57, TYR59, TYR 151, GLY 122, GLN 61 and SER 60.

4.5. Restoration potential of IL-6 Inhibitor (Diacerein) in CUS Induced Cardiac Dysfunction

Treatment of CUS rats with diacerein prevented the CUS induced rise in serum IL-6 levels along with the expression of cardiac IL-6. Moreover, the CUS induced alterations in ECG parameters were also prevented in diacerein treated CUS rats. The increase in R, T and P amplitude of sinus rhythm was prevented; however, it failed to prevent the elevated ST height and T amplitude. Moreover, the decline in vagal tone in CUS rats was also prevented with diacerein treatment, evidenced by normalized HRV indices viz RMSSD, SD1 and HF values.

The diacerein treated CUS rats also showed reduced cardiac histopathological features observed in CUS rats such as disarrangement of cardiac myofibrils, few pyknotic nuclei and nuclei aggregation. Another marker of cellular inflammation TGF-β was also found to be elevated in cardiac tissues of CUS-VEH animals. This elevation of TGF-β was not observed in cardiac tissues of diacerein treated CUS rats.

Exposure to cytokines causes expression of iNOS in majority of cardiovascular tissues, such as endothelial cells, vascular smooth muscle, and cardiac myocytes. iNOS plays an important role in cellular development, migration, and fibrosis. Literature also suggests that inflammation induced cardiac dysfunction is driven by increased iNOS activity and the

resulting NO (Zhang *et al.*, 2022). Immunohistochemical analysis demonstrated a significant increase in the expression of iNOS in cardiac sections of CUS-VEH rats. Moreover, the diacerein treatment restored the iNOS expression.

cTnI is a component of the troponin regulatory complex only found in cardiac muscle and released into the circulation following cardiac damage. cTnI is a well-established marker routinely utilized as the gold standard for detecting myocardial damage (Park *et al.*, 2021). Elevated cTnI has been linked to a 2.4-fold greater risk of cardiovascular mortality (Park *et al.*, 2021). Our study also showed a significantly elevated circulating cTnI level in CUS-VEH rats indicating myocardial damage. This elevation of cTnI levels was significantly reduced in diacerein treated CUS rats.

Apoptosis of cardiomyocytes due to chronic stress has been recognised as an important mechanism in the development of cardiac dysfunction (Van Empel *et al.*, 2005). Caspase 3 has been reported to be involved in the apoptosis of cardiomyocytes (Liu, 2018). Our study demonstrated increased caspase 3 expression in cardiac histological sections of CUS-VEH rats. The diacerein treatment significantly prevented the increase in expression of caspase 3 observed in cardiac tissues of CUS rats.

Thus, diacerein demonstrated protective effects against chronic stress induced functional and structural cardiac abnormalities. This effect could be attributed to the inhibition and reduced IL-6 expression in diacerein treated CUS rats.

4.6. Restoration potential of TNF-α Inhibitor (Tofispam) in CUS Induced Cardiac Dysfunction

Tofisopam treatment for 14 days in CUS rats reduced the CUS induced increase in serum TNF-α levels along with the expression of cardiac TNF-α**.** Moreover, tofisopam administration for 2 weeks post CUS paradigm substantially prevented the CUS induced increase in heart rate and R amplitude along with CUS induced reduction in RR interval and S amplitude. Tofisopam administration, however, was unable to restore the changes in T amplitude and ST height caused by CUS. Moreover, the decline in vagal tone in CUS rats was also prevented with tofisopam treatment, evidenced by normalized HRV indices viz RMSSD, SD1 and HF values.

The tofisopam treated CUS rats also showed improved cardiac histopathological features observed in CUS rats, including disarrangement of cardiac myofibrils along with few pyknotic nuclei. Moreover, Masson trichrome staining revealed that CUS rats treated with tofisopam exhibited less collagen deposition compared to CUS-VEH rats indicating protective effects of tofisopam against CUS induced cardiac dysfunction. However, the value

of %area of fibrosis in tofisopam treated animals fails to reach to the significant value, when compared to CUS-VEH animals.

The increased fibrosis is generally mediated by TGF-β/Smads signaling in CUS rats. TGF-β1 activates the development of cardiac fibrosis by phosphorylating Smad2/Smad3, a downstream mediator (Saadat *et al.*, 2021). The expression of TGF-β in cardiac tissue was increased in CUS-VEH animals, which was restored by tofisopam treatment.

Similar to other studies, the expression of iNOS in cardiac tissue was increased in CUS-VEH animals, which was restored by tofisopam treatment. Moreover, this elevation of CUS induced cTnI levels was significantly reduced in tofisopam treated CUS rats. Expression of caspase 3, an apoptotic marker, was also increased in cardiac tissue of CUS-VEH animals. The tofisopam treatment significantly prevented the increase in expression of caspase 3 observed in CUS rats.

Thus, tofisopam demonstrated protective effects against chronic stress induced functional and structural cardiac abnormalities. This effect could be attributed to the inhibition and reduced TNF-α expression in tofisopam treated CUS rats

4.7. Restoration potential of taVNS in CUS Induced Cardiac Dysfunction

TaVNS treatment in CUS rats reduced the CUS induced increase in serum proinflammatory cytokines levels along with the expression of cardiac IL-6 and TNF-α. It is in line with previous studies demonstrating vagus nerve stimulation resulting in decreased monocyte and macrophage activity as well as the production of proinflammatory cytokines (Johnston and Webster, 2009).Moreover, treatment with taVNS post CUS substantially prevented the CUS induced increase in heart rate, R amplitude, and T amplitude along with CUS induced reduction in RR interval. However, taVNS was unable to restore the changes in S amplitude caused by CUS exposure. Moreover, the decline in vagal tone in CUS rats was also prevented with taVNS treatment, evidenced by normalized HRV indices viz RMSSD, SD1 and HF values

The taVNS treated CUS rats also showed reduced cardiac histopathological features observed in CUS rats such as disarrangement of cardiac myofibrils and interstitial edema between myocytes, along with few pyknotic nuclei. Moreover, CUS group showed increased collagen deposition, along with some degeneration of myocardial fibres, which was significantly prevented by taVNS treatment, indicating cardio-protective effects of vagus nerve stimulation.

TGF-β was also found to be elevated in cardiac tissues of CUS animals. This elevation of TGF-β was not observed in cardiac tissues of taVNS treated CUS rats. This regulating effect

of taVNS is thought to be mediated via modulation of CAP (Go *et al.*, 2022). Moreover, Immunohistochemical analysis demonstrated a significant increase in the expression of iNOS in cardiac sections of CUS rats. Moreover, taVNS treatment restored the iNOS expression to basal levels in CUS rats, possibly by suppressing NF-κB, thus suggesting protective role of taVNS against NO mediated effects on cardiac tissue (Hamann *et al.*, 2013; Wang *et al.*, 2020).

Our study showed a significantly elevated circulating cTnI level in CUS rats indicating myocardial damage. This elevation of cTnI levels was significantly reduced in taVNS treated CUS rats. suggesting a protective role of taVNS against CUS induced cardiac dysfunction. Moreover, caspase 3 expression was increased in cardiac tissue of CUS animals which was prevented by taVNS treatment.

Thus, taVNS demonstrated protective effects against chronic stress induced functional and structural cardiac abnormalities. This effect could be attributed to the activation of CAP. This non-invasive technique can be used as a adjuvant therapy for the management of CUS induced cardiac dysfunction.

4.8. Restoration potential of α7AchR Agonist (PNU-282987) in CUS Induced Cardiac Dysfunction

PNU-282987 treatment in CUS rats reduced the CUS induced increase in serum proinflammatory cytokines levels along with the expression of cardiac IL-6 and TNF-α. Moreover, treatment with PNU-282987 post CUS exposure prevented the CUS induced rise in T amplitude, heart rate and ST height, as well as the CUS induced decrease in RR interval and S amplitude.

The PNU treated CUS rats also showed reduced cardiac histopathological features observed in CUS rats such as disarrangement of cardiac myofibrils and interstitial edema between myocytes, along with few pyknotic nuclei. Moreover, Masson trichrome staining revealed that CUS rats treated with PNU exhibited less collagen deposition compared to stressed rats. Similar to our previous studies, immunohistochemistry analysis revealed elevated expression of TGF-β in the cardiac tissues of CUS-Veh animals, which was suppressed by PNU-282987 treatment. Moreover, iNOS was also found to be elevated in cardiac tissues of CUS-Veh animals. This elevation of iNOS was not observed in cardiac tissues of PNU treated CUS rats, possibly via suppression of NF-kB (Kawahara *et al.*, 2011). cTnI was also increased in CUS-Veh animals, which was restored by PNU treatment.

Apoptosis is required for the development of the heart, but dysregulated apoptosis has long been associated to a variety of life-threatening cardiac abnormalities, including

cardiomyopathy, and heart failure (Van Empel *et al.*, 2005). The present study also indicated that PNU administration for 7 weeks restored by CUS induced increased cardiac caspase 3 expression.

Thus, PNU-282987 demonstrated protective effects against chronic stress induced functional and structural cardiac abnormalities. This effect could be attributed to the activation of CAP via α7nAChR activation.

4.9. Effect of CUS on Haematological, Serum Lipid Indices, Electrolyte Indices and Metabolomics

A decrease in haemoglobin was found in CUS animals suggesting mild anaemia in animals. Haematocrit and RBC count were also found to be decreased in CUS rats, confirming the state of mild anaemia-like condition in stressed animals. Notably, anaemia (low haemoglobin and low haematocrit) contributes to various cardiac disorders like hypertension (Mozos, 2015). Furthermore, RBC indices such as RBC count were examined to gain a thorough understanding of how stress affects RBCs. Red blood cell distribution width (RDW) is a measure of variability in the mean size of RBCs. An elevated RDW count is called anisocytosis, a condition in which RBCs of unequal sizes are formed. It is not only associated with anaemia, but is also linked with several other health illnesses, such as heart failure, hypertension, dyslipidemia, and other metabolic disorders (Lippi *et al.*, 2013; Inuzuka and Abe, 2015). Elevated RDW level is associated with increased production of proinflammatory cytokines or iron-deficiency anaemia, both of which are key pathogeneses of various illnesses such as hypertension. Our study showed increased RDW, depicting inflammation and a high risk of iron deficiency in stressed animal. WBC, also called leucocytes, are a major component of the immune system that helps the human body to combat pathogenic infections and foreign invaders, and its imbalance may prove harmful to the body. An increased WBC count is associated with several pathologies, such as depression, diabetes, hypertension, and cardiovascular diseases. Moreover, an elevated level of WBC is recognised as a biomarker of inflammation (Wirth *et al.*, 2017). Our results showed elevated WBC count, indicating increased SNS activity and inflammation in stressed animals. Moreover, the platelet count was slightly lower in stressed animals, however, these changes were not found to be statistically significant.

The serum analysis showed reduced sodium and potassium levels in CUS animals. Vasopressin, an important component of the stress system (Antoni, 2017), plays a key role in stress-induced hyponatremia. The activation of SAM axis results in potassium transport to skeletal tissues through β-2 adrenoceptors and is typically responsible for lowering potassium

levels. Hyponatremia and hypokalaemia are associated with many cardiac diseases, including hypertension (Pillai, Mandakapala and Mehta, 2012). The present study observed up-regulation of calcium levels in CUS rats. Hypercalcemia is associated with various diseases, including bone pain and hypertension (Sadiq, Naganathan and Badireddy, 2021).

Abnormalities in serum lipid profiles have a critical role in endothelial dysfunction, which is essential in the aetiology of insulin resistance and cardiac abnormalities. The findings of our study indicate a positive relationship between chronic stress, triglycerides and VLDL.

NMR provides insights into the overall metabolic trajectory and underlying metabolic mechanisms during stress conditions, ultimately assisting in understanding physiological alterations associated with chronic stress conditions (Shen *et al.*, 2016). We observed that concentrations of myo-Inositol, threonine, glycine, glutamine, methionine, formate and leucine were lowered in CUS rats with increased FAR and FGR compared to control rats. These metabolite abnormalities are linked to significantly reduced glycolysis and abnormal amino acid metabolism, implying CUS induced systemic inflammation, oxidative stress, and tissue/cell damage which ultimately results in the progression of cardiac abnormalities. Moreover, our study highlighted that these metabolites could be used as biomarkers for early identification of chronic stress associated abnormalities based on metabolite alterations in serum.

5. Conclusion

Chronic stress is one of the significant risk factors for cardiac abnormalities. Our findings suggest long-term CUS can trigger cardiac dysfunction. We further found elevated proinflammatory cytokines in CUS rats suggesting proinflammatory involvement in cardiac dysfunctions induced by prolonged stress. This study shed light on the potential mechanism of CUS induced cardiac dysfunction, where inflammation plays a key role. CUS caused a significant increase in heart rate, decreased vagal tone, and altered sinus rhythm. Moreover, chronic stress caused an increase in serum cTnI, myocardial iNOS, and caspase-3 levels. In addition, chronic stress caused altered cardiac morphology, hypertrophy with fibrosis and increased TGF-β expression in the myocardium. Moreover, treatment with diacerein, tofisopam, PNU-282987 and taVNS, due to their inhibitory effects on proinflammatory cytokines, attenuated chronic stress induced cardiac dysfunction, which could be used as preventive therapies for such conditions. The current study identifies potential therapeutic targets for drug intervention in stress associated cardiac disorders. These therapeutic targets may be used for developing novel and safe therapeutic agents. Moreover, vagus nerve

stimulation, a novel non-invasive technique, could be exploited further to treat chronic stress-induced cardiac dysfunction as done in epilepsy. Additionally, the complete blood profile of CUS animals demonstrated mild anemia with reduced red blood cell count and increased white blood cell count. Serum analysis revealed hypercalcemia, hyponatremia, hypokalaemia and VLDL level. Metabolomics analysis demonstrated that CUS animals had decreased myo-Inositol, threonine, glycine, glutamine, methionine and formate. These abnormalities in metabolites are linked to significantly reduced glycolysis and abnormal amino acid metabolism, implying CUS induced systemic inflammation, oxidative stress, and tissue/cell damage, which ultimately results in the progression of cardiac dysfunction. Thus, this study provides valuable information about the potential mechanism that lead to chronic stress associated cardiac dysfunction and also suggests the possible strategies for prevention and treatment of the same. Moreover, our study revealed distinct haematological and metabolic abnormalities associated with chronic stress and various metabolites that could be used as potential biomarkers for early detection of chronic stress.

6. References

Abd Allah, O. M. (2017) 'Beneficial effects of diacerein on adipokines and pro-inflammatory cytokines involved in diet-induced nonalcoholic steatohepatitis in rats', *International Journal of Basic \& Clinical Pharmacology*, 6(4), pp. 811–820.

Al-Rasheed, Nawal M. *et al.* (2018) 'Possible protective mechanisms exerted by metformin or metformin and vitamin E in isoproterenol-induced cardiac injury', *Journal of Cellular Biochemistry*, 119(5), pp. 3903–3912. doi: 10.1002/jcb.26530.

Alamgeer *et al.* (2013) 'Antihypertensive activity of aqueous-methanol extract of Berberis orthobotrys Bien Ex Aitch in rats', *Tropical Journal of Pharmaceutical Research*, 12(3), pp. 393–399. doi: 10.4314/tjpr.v12i3.18.

Almezgagi, M. *et al.* (2020) 'Diacerein: Recent insight into pharmacological activities and molecular pathways', *Biomedicine and Pharmacotherapy*, 131(July), p. 110594. doi: 10.1016/j.biopha.2020.110594.

Amin, M. N. *et al.* (2020) 'Inflammatory cytokines in the pathogenesis of cardiovascular disease and cancer.', *SAGE open medicine*, 8, p. 2050312120965752. doi: 10.1177/2050312120965752.

Ans, A. H. *et al.* (2018) 'Neurohormonal Regulation of Appetite and its Relationship with Stress: A Mini Literature Review.', *Cureus*, 10(7), p. e3032. doi: 10.7759/cureus.3032.

Antoni, F. A. (2017) 'Vasopressin as a Stress Hormone', *Stress: Neuroendocrinology and*

Neurobiology, 2(October), pp. 97–108. doi: 10.1016/B978-0-12-802175-0.00009-7.

Arndt, D. L., Peterson, C. J. and Cain, M. E. (2015) 'Differential rearing alters forced swim test behavior, fluoxetine efficacy, and post-test weight gain in male rats', *PloS one*, 10(7), p. e0131709.

Bharti, R. *et al.* (2016) 'Diacerein-mediated inhibition of IL-6/IL-6R signaling induces apoptotic effects on breast cancer', *Oncogene*, 35(30), pp. 3965–3975. doi: 10.1038/onc.2015.466.

Crestani, C. C. (2016) 'Emotional stress and cardiovascular complications in animal models: A review of the influence of stress type', *Frontiers in Physiology*, 7(June). doi: 10.3389/fphys.2016.00251.

Czura, C. J., Rosas-Ballina, M. and Tracey, K. J. (2006) 'Cholinergic Regulation of Inflammation', *Psychoneuroimmunology*, pp. 85–96. doi: 10.1016/B978-012088576-3/50007-1.

Dubey, D. *et al.* (2021) 'NMR-Based Metabolomics Revealed the Underlying Inflammatory Pathology in Reactive Arthritis Synovial Joints.', *Journal of proteome research*, 20(11), pp. 5088–5102. doi: 10.1021/acs.jproteome.1c00620.

El-Fattah, A. *et al.* (2018) 'Resveratrol and dimethyl fumarate ameliorate depression-like behaviour in a rat model of chronic unpredictable mild stress', *Brain Research*, 1701. doi: 10.1016/j.brainres.2018.09.027.

Van Empel, V. P. M. *et al.* (2005) 'Myocyte apoptosis in heart failure', *Cardiovascular Research*, 67(1), pp. 21–29. doi: 10.1016/j.cardiores.2005.04.012.

Gabriel, A. S. *et al.* (2004) 'IL-6 levels in acute and post myocardial infarction: Their relation to CRP levels, infarction size, left ventricular systolic function, and heart failure', *European Journal of Internal Medicine*, 15(8), pp. 523–528. doi: 10.1016/j.ejim.2004.07.013.

Gautam, A. K. *et al.* (2022) 'Preclinical Evaluation of Dimethyl Itaconate Against Hepatocellular Carcinoma via Activation of the e/iNOS-Mediated NF-κB–Dependent Apoptotic Pathway', *Frontiers in Pharmacology*, 12(January). doi: 10.3389/fphar.2021.823285.

Go, Y. Y. *et al.* (2022) 'Different Transcutaneous Auricular Vagus Nerve Stimulation Parameters Modulate the Anti-Inflammatory Effects on Lipopolysaccharide-Induced Acute Inflammation in Mice', *Biomedicines*, 10(2). doi: 10.3390/biomedicines10020247.

Hamann, J. J. *et al.* (2013) 'Vagus nerve stimulation improves left ventricular function in a canine model of chronic heart failure', *European Journal of Heart Failure*, 15(12), pp. 1319–1326. doi: 10.1093/eurjhf/hft118.

Hattiangady, B. *et al.* (2014) 'Object location and object recognition memory impairments, motivation deficits and depression in a model of Gulf War illness', *Frontiers in Behavioral Neuroscience*, 8(MAR). doi: 10.3389/fnbeh.2014.00078.

Helms, S. A. *et al.* (2010) 'Smaller cardiac cell size and reduced extra-cellular collagen might be beneficial for hearts of Ames dwarf mice', *International Journal of Biological Sciences*, 6(5), pp. 475–490. doi: 10.7150/ijbs.6.475.

Herman, J. P. and Tasker, J. G. (2016) 'Paraventricular hypothalamic mechanisms of chronic stress adaptation', *Frontiers in Endocrinology*. doi: 10.3389/fendo.2016.00137.

Inuzuka, R. and Abe, J. (2015) 'Red blood cell distribution width as a link between ineffective erythropoiesis and chronic inflammation in heart failure.', *Circulation journal : official journal of the Japanese Circulation Society*. Japan, pp. 974–975. doi: 10.1253/circj.CJ-15-0254.

Ismail, H. T. H. (2022) 'The Impact of Iodine Exposure in Excess on Hormonal Aspects and Hemato-Biochemical Profile in Rats', *Biological Trace Element Research*, 200(2), pp. 706–719. doi: 10.1007/s12011-021-02681-7.

Johnston, G. R. and Webster, N. R. (2009) 'Cytokines and the immunomodulatory function of the vagus nerve', *BJA: British Journal of Anaesthesia*, 102(4), pp. 453–462. doi: 10.1093/bja/aep037.

Kalashnikov, S. V, Kalashnikova, E. A. and Kokarovtseva, S. N. (2002) 'Immunomodulating effects of tofizopam (Grandaxin) and diazepam in vitro.', *Mediators of inflammation*, 11(1), pp. 53–59. doi: 10.1080/09629350210309.

Kania, A. M. *et al.* (2021) 'Activation of the cholinergic antiinflammatory reflex by occipitoatlantal decompression and transcutaneous auricular vagus nerve stimulation', *Journal of Osteopathic Medicine*, 121(4), pp. 401–415. doi: 10.1515/jom-2020-0071.

Kanthe, P. S. *et al.* (2012) 'Atherogenic index as a predictor of cardiovascular risk among women with different grades of obesity', *International Journal of Collaborative Research on Internal Medicine and Public Health*, 4(10), pp. 1767–1774.

Kapadia, S. *et al.* (1998) 'The role of cytokines in the failing human heart.', *Cardiology clinics*, 16(4), pp. 645–56, viii. doi: 10.1016/s0733-8651(05)70041-2.

Kawahara, R. *et al.* (2011) 'Activation of α7 nicotinic acetylcholine receptors ameliorates indomethacin-induced small intestinal ulceration in mice.', *European journal of pharmacology*, 650(1), pp. 411–417. doi: 10.1016/j.ejphar.2010.10.031.

Koopman, F. A. *et al.* (2016) 'Vagus nerve stimulation inhibits cytokine production and attenuates disease severity in rheumatoid arthritis', *Proceedings of the National Academy of*

Sciences, 113(29), pp. 8284–8289. doi: 10.1073/pnas.1605635113.

Leonard, B. E. (2010) 'The concept of depression as a dysfunction of the immune system.', *Current immunology reviews*, 6(3), pp. 205–212. doi: 10.2174/157339510791823835.

Levine, G. N. (2022) 'Psychological Stress and Heart Disease: Fact or Folklore?', *The American Journal of Medicine*, pp. 1–9. doi: 10.1016/j.amjmed.2022.01.053.

Li, H., Zuo, J. and Tang, W. (2018) 'Phosphodiesterase-4 Inhibitors for the Treatment of Inflammatory Diseases.', *Frontiers in pharmacology*, 9, p. 1048. doi: 10.3389/fphar.2018.01048.

Lippi, G. *et al.* (2013) 'Association of red blood cell distribution width with plasma lipids in a general population of unselected outpatients.', *Kardiologia polska*, 71(9), pp. 931–936. doi: 10.5603/KP.2013.0228.

Liu, C. H. *et al.* (2020) 'Neural networks and the anti-inflammatory effect of transcutaneous auricular vagus nerve stimulation in depression', *Journal of Neuroinflammation*, 17(1), pp. 1–11. doi: 10.1186/s12974-020-01732-5.

Liu, D. (2018) 'Effects of procyanidin on cardiomyocyte apoptosis after myocardial ischemia reperfusion in rats', *BMC Cardiovascular Disorders*, 18(1), pp. 1–7. doi: 10.1186/s12872-018-0772-x.

Liu, M. Y. *et al.* (2017) 'Association between psychosocial stress and hypertension: a systematic review and meta-analysis', *Neurological Research*, 39(6), pp. 573–580. doi: 10.1080/01616412.2017.1317904.

Liu, Y.-Z., Wang, Y.-X. and Jiang, C.-L. (2017) 'Inflammation: The Common Pathway of Stress-Related Diseases', *Frontiers in Human Neuroscience*, 11(June), pp. 1–11. doi: 10.3389/fnhum.2017.00316.

Ma, Z.-G. *et al.* (2016) 'Protection against cardiac hypertrophy by geniposide involves the GLP-1 receptor / AMPKα signalling pathway.', *British journal of pharmacology*, 173(9), pp. 1502–1516. doi: 10.1111/bph.13449.

Martel Pelletier, J. and Pelletier, J. P. (2010) 'Effects of diacerein at the molecular level in the osteoarthritis disease process', *Therapeutic Advances in Musculoskeletal Disease*, pp. 95–104. doi: 10.1177/1759720X09359104.

Maydych, V. (2019) 'The interplay between stress, inflammation, and emotional attention: Relevance for depression', *Frontiers in Neuroscience*, 13(APR), pp. 1–8. doi: 10.3389/fnins.2019.00384.

Mayyas, F., Alsaheb, A. and Alzoubi, K. H. (2019) 'The role of fish oil in attenuating cardiac oxidative stress, inflammation and fibrosis in rat model of thyrotoxicosis', *Heliyon*, 5(12), p.

e02976. doi: 10.1016/j.heliyon.2019.e02976.

Meléndez, G. C. *et al.* (2010) 'Interleukin 6 mediates myocardial fibrosis, concentric hypertrophy, and diastolic dysfunction in rats', *Hypertension*, 56(2), pp. 225–231. doi: 10.1161/HYPERTENSIONAHA.109.148635.

Miller, A. H., Maletic, V. and Raison, C. L. (2009) 'Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression', *Biological Psychiatry*, pp. 732–741. doi: 10.1016/j.biopsych.2008.11.029.

Mishra, R. K. *et al.* (2016) 'Palonosetron attenuates 1,2-dimethyl hydrazine induced preneoplastic colon damage through downregulating acetylcholinesterase expression and up-regulating synaptic acetylcholine concentration', *RSC Advances*, 6(46), pp. 40527–40538. doi: 10.1039/c6ra04614b.

Monteiro, S. *et al.* (2015) 'An efficient chronic unpredictable stress protocol to induce stress-related responses in C57BL/6 mice', *Frontiers in Psychiatry*, 6(FEB). doi: 10.3389/fpsyt.2015.00006.

Morris, G. M. *et al.* (1998) 'Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function', *Journal of Computational Chemistry*, 19(14), pp. 1639–1662. doi: 10.1002/(SICI)1096-987X(19981115)19:14<1639::AID-JCC10>3.0.CO;2-B.

Mozos, I. (2015) 'Mechanisms Linking Red Blood Cell Disorders and Cardiovascular Diseases', *BioMed Research International*. Edited by G. Bosman, 2015, p. 682054. doi: 10.1155/2015/682054.

Mucci, N. *et al.* (2016) 'Anxiety, stress-related factors, and blood pressure in young adults', *Frontiers in Psychology*, 7(October), pp. 1–10. doi: 10.3389/fpsyg.2016.01682.

Palladino, M. A. *et al.* (2003) 'Anti-TNF-α therapies: the next generation', *Nature reviews. Drug discovery*, 2(9), pp. 736–746. doi: 10.1038/nrd1175.

Park, J. E. *et al.* (2021) 'Cardiac troponin I and the risk of cardiovascular or non-cardiovascular death in patients visiting the emergency department', *Scientific Reports*, 11(1), pp. 1–8. doi: 10.1038/s41598-021-96951-y.

Pavelka, K. *et al.* (2016) 'Diacerein: Benefits, Risks and Place in the Management of Osteoarthritis. An Opinion-Based Report from the ESCEO', *Drugs and Aging*, 33(2), pp. 75–85. doi: 10.1007/s40266-016-0347-4.

Pavlov, V. A. and Tracey, K. J. (2012) 'The vagus nerve and the inflammatory reflex - Linking immunity and metabolism', *Nature Reviews Endocrinology*, pp. 743–754. doi: 10.1038/nrendo.2012.189.

Perera, A., Soos, C. and Machin, K. (2022) 'Identification of Metabolomic Biomarkers of Long-Term Stress Using NMR Spectroscopy in a Diving Duck', *Metabolites*, 12(4). doi: 10.3390/metabo12040353.

Pillai, U., Mandakapala, C. and Mehta, K. (2012) 'Resistant hyponatremia and hypokalemia treated successfully with nephrectomy', *Clinical Kidney Journal*, 5(1), pp. 68–69. doi: 10.1093/ndtplus/sfr138.

Ramappa, V. K. *et al.* (2023) 'Fabrication of mulberry leaf extract (MLE)- and tasar pupal oil (TPO)-loaded silk fibroin (SF) hydrogels and their antimicrobial properties', *3 Biotech*, 13(2), p. 37. doi: 10.1007/s13205-022-03443-5.

Rau, A. R. *et al.* (2015) 'Increased basolateral amygdala pyramidal cell excitability may contribute to the anxiogenic phenotype induced by chronic early-life stress', *Journal of Neuroscience*, 35(26), pp. 9730–9740. doi: 10.1523/JNEUROSCI.0384-15.2015.

Rawat, J. K. *et al.* (2019) 'Transcutaneous vagus nerve stimulation regulates the cholinergic anti-inflammatory pathway to counteract 1, 2-dimethylhydrazine induced colon carcinogenesis in albino wistar rats', *Frontiers in Pharmacology*, 10(MAY), pp. 1–13. doi: 10.3389/fphar.2019.00353.

Rosengren, A. *et al.* (2004) 'Association of psychosocial risk factors with risk of acute myocardial infarction in 11 119 cases and 13 648 controls from 52 countries (the INTERHEART study): Case-control study', *Lancet*, 364(9438), pp. 953–962. doi: 10.1016/S0140-6736(04)17019-0.

Saadat, S. *et al.* (2021) 'Pivotal Role of TGF-β/Smad Signaling in Cardiac Fibrosis: Non-coding RNAs as Effectual Players', *Frontiers in Cardiovascular Medicine*, 7(January), pp. 1–18. doi: 10.3389/fcvm.2020.588347.

Sadiq, N. M., Naganathan, S. and Badireddy, M. (2021) 'Hypercalcemia.', in. Treasure Island (FL).

Santana-Krímskaya, S. E. *et al.* (2020) 'IMMUNEPOTENT CRP plus doxorubicin/cyclophosphamide chemotherapy remodel the tumor microenvironment in an air pouch triple-negative breast cancer murine model', *Biomedicine and Pharmacotherapy*, 126(September 2019), p. 110062. doi: 10.1016/j.biopha.2020.110062.

Saravanan, N. and Sarumathi, A. (2013) 'A study on the hematological parameters and brain acetylcholine esterase activity in immobilization induced stress and co-treatment with Centella asiatica leaves extract to wistar rats', *International Journal of Nutrition, Pharmacology, Neurological Diseases*, 3(2), p. 102. doi: 10.4103/2231-0738.112829.

Shaffer, F. and Ginsberg, J. P. (2017) 'An Overview of Heart Rate Variability Metrics and

Norms', *Frontiers in Public Health*. doi: 10.3389/fpubh.2017.00258.

Sharpley, C. F. (2009) 'Neurobiological Pathways between Chronic Stress and Depression: Dysregulated Adaptive Mechanisms?', *Clinical Medicine Insights: Psychiatry*, 2, p. CMPsy.S3658. doi: 10.4137/cmpsy.s3658.

Shen, G. *et al.* (2016) '1H NMR-based metabolomics study on the physiological variations during the rat pregnancy process.', *Molecular and cellular endocrinology*, 423, pp. 40–50. doi: 10.1016/j.mce.2016.01.003.

Shi, X. *et al.* (2022) 'The α7 nicotinic acetylcholine receptor agonist PNU-282987 ameliorates sepsis-induced acute kidney injury through CD4+CD25+ regulatory T cells in rats.', *Bosnian journal of basic medical sciences*, 22(6), pp. 882–893. doi: 10.17305/bjbms.2022.7111.

Singh, R. *et al.* (2012) 'HILIC quantification of Oenotheralanosterol A and B from Oenothera biennis and their suppression of IL-6 and TNF-α expression in mouse macrophages', *Journal of ethnopharmacology*, 141, pp. 357–362. doi: 10.1016/j.jep.2012.02.046.

Singh, S. *et al.* (2023) 'Pre-clinical Investigations of Therapeutic Markers Associated with Acute and Chronic Restraint Stress: A Nuclear Magnetic Resonance Based Contrast Metabolic Approach.', *Nanotheranostics*, 7(1), pp. 91–101. doi: 10.7150/ntno.76294.

Slaoui, M. and Fiette, L. (2011) 'Histopathology Procedures: From Tissue Sampling to Histopathological Evaluation', *Methods in molecular biology (Clifton, N.J.)*, 691, pp. 69–82. doi: 10.1007/978-1-60761-849-2_4.

Srivastava, P. *et al.* (2014) 'Effect of Pluchea lanceolata bioactives in LPS-induced neuroinflammation in C6 rat glial cells', *Naunyn-Schmiedeberg's Archives of Pharmacology*, 387(2), pp. 119–127. doi: 10.1007/s00210-013-0924-6.

Srivastava, S. *et al.* (2014) 'Current Update on Evidence Based Literature of Tofisopam.', *Delhi Psychiatry Journal*, 17, pp. 154–159.

Üçel, U. İ. *et al.* (2020) 'Antiamnesic effects of tofisopam against scopolamine-induced cognitive impairments in rats', *Pharmacology Biochemistry and Behavior*, 190(January). doi: 10.1016/j.pbb.2020.172858.

Vicens, P. *et al.* (2011) 'Behavioral effects of PNU-282987, an alpha7 nicotinic receptor agonist, in mice', *Behavioural brain research*, 216, pp. 341–348. doi: 10.1016/j.bbr.2010.08.015.

Wang, M. *et al.* (2020) 'Vagus Nerve Stimulation Ameliorates Renal Ischemia-Reperfusion Injury through Inhibiting NF-κB Activation and iNOS Protein Expression', *Oxidative Medicine and Cellular Longevity*, 2020, p. 7106525. doi: 10.1155/2020/7106525.

Wirth, M. *et al.* (2017) 'The Dietary Inflammatory Index is Associated with Elevated White Blood Cell Counts in the National Health and Nutrition Examination Survey', *Brain, Behavior, and Immunity*. doi: 10.1016/j.bbi.2017.12.003.

Yap, J. Y. Y. *et al.* (2020) 'Critical Review of Transcutaneous Vagus Nerve Stimulation: Challenges for Translation to Clinical Practice', *Frontiers in Neuroscience*, 14(April). doi: 10.3389/fnins.2020.00284.

Yusuf, S. *et al.* (2004) 'Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study.', *Lancet (London, England)*, 364(9438), pp. 937–952. doi: 10.1016/S0140-6736(04)17018-9.

Zhang, H. *et al.* (2022) 'Berberine attenuates sepsis-induced cardiac dysfunction by upregulating the Akt/eNOS pathway in mice.', *Experimental and therapeutic medicine*, 23(6), p. 371. doi: 10.3892/etm.2022.11298.