Chapter-6

DISCUSSION

Stress is a cause for concern due to its potential affect on human testicular function and fertility. In the testis, stress induces the damage of the supportive cells called Sertoli cells,
leading to Fas death receptor-dependent apoptotic elimination of germ cells. Therefore, the aim of the present in vitro work with GC-2 cells was to functionally characterize the relationships between Fas, DR5, p53 and c-FLIP in germ cell apoptosis. GC-2 cells were originally created to provide an in vitro model of germ cell differentiation. GC-2 cells were created from spermatocytes that had been immortalized with the SV40 virus’s large T antigen and co-transformed with a mutant p53 protein. The p53 protein is activated by proper folding and nuclear localization at the lower temperatures in GC-2 cells, where it binds the large T antigen and suppresses its proliferative ability. This enables the cells to undergo differentiation. (120)The cells were therefore deemed unsuitable for further studies. However, here these cells shows their differential p53 activity based on the temperature they are maintained at. Moreover, GC-2 cells were found to express Fas, DR5 and c-FLIP (L) as shown by western blot analyses.(121)

Compromised DNA damage under genotoxic insult will likely result in accumulation of DNA lesions and eventually severely affect the genomic integrity. Defects in balance between cell cycle arrest and damage repair processes may lead to hypersensitivity to cellular stress, susceptibility to DNA damage, and genomic defects. Activation of ATM through its auto-phosphorylation results in phosphorylation of many downstream targets that modulate numerous damage response pathways, most notably cell cycle checkpoints.(122-125)

This is supported by our qualitative screening experiments for the expression of ATM. The present study demonstrated a significant increase in ATM levels in Cold Stress treated GC-2 (Fig. 2a) cell line indicative of DSBs formation and nuclear relocalization through enhanced binding to the damaged sites. Further, the accumulation of γ-H2AX foci (at Ser139) in the treated cells (Fig. 2b) fosters the concept that γ-H2AX is required for the retention of checkpoint proteins and DSB repair factors in damage foci.(126, 127) In other words, our results signify the extent of DNA damage that has occurred due to the exposure to Cold Stress and also any alterations in these cellular events may indirectly promote genome unsteadiness. The damage caused by cold stress found repaired by the treatment of 50% MeOH extract of M. pruriens and H. Perforatum.

A study shows that after creating DNA double-strand breaks in human cells, the repair factors Rad50 and Rad51 each colocalized with phosphorylated H2AX (gamma H2AX) foci. This was estimated by western blotting. This protein is important for DNA double-strand break repair, cell cycle checkpoint activation, telomere maintenance, and meiotic recombination.
Moreover, the up-regulation of p53 activity in the cell can initiate apoptosis by inducing the expression of pro-apoptotic members involved in cell death and by transactivating the expression of death receptor-associated genes. Recently it has been demonstrated that the isocyanates are capable of inducing apoptosis in cultured human lymphocytes. (128-131) Thus, the p53-dependent apoptotic cell death eliminates cells that have acquired the damage that are too severe to be repaired or that may be oncogenic. But the treatment of M. pruriens and H. Perforatum in current study enhances the protein expression of Rad protein which might be the basic reason behind the cellular repair.

However, the most prominent features of apoptosis are DNA fragmentation and phosphatidyl serine externalization. When the apoptosis is uncontrolled the quantity of phagocytosis and macrophages are decline and thus apoptotic debris are accumulated. The treatment of M. pruriens and H. Perforatum extract (50% MeOH) prevents the accumulation of such debris by enhancing phagocytosis.

Exposure to stress causes dysfunctions in circuits connecting hippocampus and prefrontal cortex (H-PFC). Long term potentiation (LTP) induced in vivo in rats at H-PFC synapses is impaired by acute elevated platform stress in a manner that can be restored by treatment with certain antidepressants. To identify biochemical pathways in rat frontal cortex underlying this stress-mediated impairment of synaptic plasticity, we examined the phosphorylation state of receptors, signaling proteins and transcription factors implicated in neuronal plasticity. Stress exposure is known to precipitate depression in humans (132, 133) and forms the basis of several animal models of this disease (134). Acute and chronic severe stressors reduce measures of plasticity (LTP, dendritic arborization, memory function) on the key cognitive circuits connecting the H-PFC while increasing the measures of plasticity in the amygdala subserving fear and emotional memories. Pharmacological intervention against the negative effects of stressors on neuroplasticity may turn out to be beneficial for the prevention (135) and treatment of stress induced disorders (ulceration, depression and infertility).

Sildenafil citrate in Infertility: Impotence is associated with all major systemic diseases as well depression and stress. Stress is also one of the causes of infertility. Therefore, male reproduction appears to be extremely sensitive to internal and external stressors, additionally erectile dysfunction is a predictor of myocardial infarction and stroke, whereas men with regular sexual activity have lower risk of death due to coronary disease. (136)
The mechanism through which chronic stress inhibits the sexual functioning of the body has been investigated through assessing the hypothalamic-pituitary-testicular axis functioning. Chronic restraint stress decreases testosterone secretion, an effect that is associated with a decrease in plasma gonadotropin levels. Generally, in stressed rats there was a decrease in hypothalamic luteinizing hormone-releasing hormone (LHRH) content and the response on plasma gonadotropins to LHRH administration was enhanced. Thus, the inhibitory effect of chronic stress on plasma LH and FSH levels seems not to be due to a reduction in pituitary responsiveness to LHRH, but rather to a modification in LHRH secretion. It has been suggested that β-endorphin might interfere with hypothalamic LHRH secretion during stress. It has been considered that β-endorphin secreted by the pituitary may play some role in the stress-induced decrease in LHRH secretion. Since stress increases the secretion of glucocorticoids, endogenous opioids and corticotropin-releasing factor (CRF), the inhibition of the testicular axis caused by stress could be due to: (1) increased levels of glucocorticoids during chronic stress, which may interfere with testosterone or LH secretion (2) increased release of endogenous opioids, which have been reported to inhibit luteinizing hormone-releasing hormone (LHRH) or (3) enhanced secretion of CRF, since this peptide is known to decrease LHRH release.(137)

Sildenafil, a potent inhibitor of phosphodiesterase 5, has been therapeutically used for men with erectile dysfunction. (138) Sildenafil-treated mice showed a significant increased levels of total testosterone. (139) Sildenafil therapy improves erectile dysfunction caused by both psychogenic and organic factors. (140) However, the sperm production and androgen synthesis are controlled by a complex feedback loop involving the testes, hypothalamus, and pituitary gland. The pituitary controls testis function by producing follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates spermatogenesis, in part by affecting sertoli cells, while LH stimulates androgen production by interstitial cells. Pituitary production of these hormones depends on secretion of gonadotropin-releasing hormone (GnRH) by the hypothalamus. In addition to enhancing the secretion of GnRH, sildenafil can cross the blood–brain barrier and significantly improve learning/memory by modulating glutamate–NO–cGMP signal transduction pathway. Sildenafil has been shown to increase neurogenesis, functional recovery and decrease the neurological deficits in rats after stroke. (141) Sildenafil have shown to prevent oxidative stress by increasing intracellular cAMP and cGMP(142). In addition to its cognitive enhancing ability recent studies have shown that sildenafil possess neuroprotective properties. (141)
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Most studies have confirmed the safety of *H. perforatum* as a treatment for mild to moderate depression and as a nutritional supplement as it is devoid of side effects such as sedation, anticholinergic reactions, gastrointestinal disturbances. The efficacy of extract of *H. Perforatum* delays the ejaculation with rapid onset of action, ease of use and improved safety makes the *Hypericum* extract, an attractive option for men to increase the ejaculation time during sexual intercourse. One of the most interesting findings of this study which increases the sexual efficiency in stressed animals. Hydroalcoholic extract of *H. Perforatum* significantly delays the ejaculation time in normal and stressed animals. In addition, no side effect was observed with the hydroalcoholic extract of *H. perforatum in-vivo* and *in-vitro*.(143)

Traditionally, the seeds of *M. Prurien*s have been used for treating male sexual dysfunction in Unani Medicine, the traditional system of medicine of Indo-Pakistan subcontinent. It is also used in ayurvedic medicine. *M. prurien*s has been shown to improve sexual function in rats which contains L-DOPA, a precursor to the neurotransmitter dopamine. The hydroalcoholic extract of *M. Prurien*s increases L-DOPA content where L-DOPA is converted into dopamine, an important brain chemical involved in mood, sexuality, movement and regulation of stress.(144, 145) According to the Indian Systems of Medicine, *M. prurien*s Linn. was used for treating male sexual disorders since ancient times. In this study, the effects of hydroalcoholic extracts of the *M. prurien*s Linn. seed on general mating behaviour and significantly enhance the libido and potency in normal and stressed wistar rats were investigated which was compared with the standard reference drug, Sildenafil citrate. The differential effect of stress on the release of gastric somatostatin, prostaglandin E and gastrin was assessed in the rat and found an excessive rise in the release of gut hormones during stress. (146)

Gastrin is an important polypeptide hormone in the regulation of several gastrointestinal functions, especially in the stimulation of HCl-secretion in the stomach. During stress, the release of gastrin is increase significantly(147) which tends to cause ulceration, in addition the stress induces the release of histamine which proliferates the formation of ulcers, hence produces synergistic action in combination with gastrin which causes a heavy damage to the mucosal membrane. (148) Apparently, one of the basic cause of stress induced ulceration is due to the excessive release of gastric somatostatin. (146)

Dysregulation of the brain noradrenergic system may be a factor in determining vulnerability to stress-related pathology, or in the interaction of genetic vulnerability and
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environmental sensitization as well as increased vulnerability to stress-induced gastric ulcers and exaggerated activation of the hypothalamic-pituitary-adrenal (HPA) stress axis. Data regarding effects of H. perforatum on the gastric mucosa are controversial. Yesilada and Gürbüz reported a potent antiulcer activity of H. perforatum ethanol extract on the ethanol-induced gastric lesions in mice (80) while another study showed that H. perforatum inhibits gastric acid secretion in pyloric-ligated rats, but increases indomethacin-induced gastric mucosal lesions in a dose dependent manner. (149) Novel research has revealed that moderate doses of H. perforatum extracts heal hypothermic restraint stress-induced gastric ulcer in rats. (80) Studies proved the presence of hyperforins and its analogues as well as of flavonoids, quercetin, but the absence of the naphthodianthrones (hypericin), (150, 151) although Isacchiet al claimed only the presence of phloroglucinol derivatives. (152) The anti-ulcer property of M. pruriens has not yet been reported and hence the hydroalcoholic extract of M. Pruriens was assessed and observed a significant inhibition in release of gastrin and gastric somatostatin, which confirms its potential anti-ulcer efficacy.

However a standard antidepressant, epinephrine has many functions in the body, regulating heart rate, blood vessel and air passage diameters, and metabolic shifts where the release of epinephrine is a crucial component of the fight-or-flight response of the sympathetic nervous system aroused during exposure of stressors. (153) During stress response, the epinephrine pathway become activated within seconds, when the sympathetic branches of the autonomic nervous system carry the alarm signal from the hypothalamus of the limbic system to the adrenal medulla (the inner portion of the adrenal gland), which releases epinephrine. Epinephrine is one of two catecholamines released by the activation of the sympathetic nervous system. The other is norepinephrine (NE) which is released due to the activation of the brain noradrenergic system under the influence of acute stress. The post-synaptic effects of NE, exerted at a cellular or neural circuit level, have been described as modulatory in nature, as NE facilitates responses evoked in target cells by both excitatory and inhibitory afferent input.

It has been observed that the acute immobilization stress activates NE release in a number of stress-related limbic forebrain target regions, such as the central and medial amygdala, lateral bed nucleus of the stria terminalis, medial prefrontal cortex, and lateral septum. This stress-induced release of NE facilitates a number of anxiety-like behavioral responses that are mediated in these regions, including stress-induced reduction of open-arm
exploration on the elevated plus-maze and stress-induced reduction of social interaction behaviour.

A better understanding of the role of NE in adaptive responses to acute stress, the pathological consequences of prolonged, repeated or severe stress, and the mechanisms of action of drugs used to treat stress-related diseases, may contribute to the future development of more effective strategies for the treatment or even prevention of such disorders.(154) The effects of stress on dopamine, GABA and acetylcholine content and its turnover in the brain are less well defined but the brain dopamine turnover have been reported as increased, unchanged or decreased. (11, 155, 156) The fact that increases in 5-HT in the dorsal nuclei of brain are observed after some stressors (saline injection) but not others (handling, forced swimming), which seems to indicate that the release of 5-HT in this nucleus is dependent on how aversive events are perceived by the animals. In contrast, the output of 5-HT in the median nuclei of brain appears to increase as a response to all the stressors. This indicates that the 5HT respond to diverse novel or threatening stimuli in a non specific manner. (157)

Treatment of depression with imipramine induced an increase in cortical noradrenaline output (150% over basal values) whereas acute administration of imipramine dose-dependently enhanced cortical noradrenergic transmission by increasing the extracellular concentration of norepinephrine in brain area. (158) Similarly, Hypericum is therapeutically equivalent to imipramine, but is better tolerated by patients and produce less toxic effects. (159) Recent overview and other comparative trials provide compelling evidence that hypericum is therapeutically equivalent to the standard antidepressants but the toxicity data is not addressed. In view of the mounting evidence as well as data received with 50% MeOH extract of H. perforatum with comparable efficacy to a standard antidepressant, imipramine. Considering efficacy and its safety record, hypericum should be considered for first line treatment in mild to moderate depression, especially in the primary care setting. Whereas, psycho-pharmacological investigations involved acute and chronic treatment (14 days) of M. pruriens in forced swim test (FST), tail suspension test (TST) in mice revealed the initial anti-depressant-like effect of M. pruriens. (160) Investigation of anti-depressant action of hydroalcoholic (50% MeOH) extract of M. pruriens seed was carried out using forced swim test (FST) and tail suspension test (TST) in animals. Other studies also provided evidence of an antidepressant action of M. pruriens due to its effect on monoaminergic systems. (160)
In present study, the level of neurotransmitters were measured in whole brain of animals revealed a significant change in the levels were increased and decreased after treatment of both hydroalcoholic extracts of M. pruriens and H. Perforatum respectively which may be mediated via interaction with the noradrenergic and serotonergic systems.

It has been observed that the stress and depression are associated with increased circulating concentrations of cytokines and hyperactivity of the HPA-axis which has been proved clinically and experimentally. It has also been reported that immunological activation induces “stress-like” behavioral and neurochemical changes in laboratory animals. Although for many years it has been suggested that stress acts a predisposing factor to depressive illness,' the precise mechanisms by which stress-induced depressive symptoms occur are not fully understood. Nevertheless, behavioural changes due to stress have often been explained in terms of changes in neurotransmitter function in the brain. It has been suggested that cytokine hypersecretion may be involved in the aetiology of depressive disorders. (161)

P-glycoprotein (Pgp), an ATP-dependent membrane transporter is found in epithelial tissues of the liver, kidneys, intestine and blood-brain barrier. In tumor cells, Pgp is often over-expressed and confers multidrug resistance toward cancer chemotherapeutics. It has been previously shown in rats that induction of an inflammatory response evokes a decrease in hepatic expression of P-GP. Inflammatory and pro-inflammatory cytokines (interleukin (IL)-6, IL-1beta and tumor necrosis factor (TNF)-alpha) significantly reduces the hepatic expression of Pgp in mice. A significant reduction in the hepatic expression in Pgp genes were seen with exposure of endotoxin and similarly, IL-6-treated mice displayed a 70% reduction in protein expression and a 40-70% reduction in the mRNA levels of all Pgp which indicate that release of cytokines during stress plays a principal role in the downregulation of Pgp. (162) Hence, an increased cytokine secretion is implicated as a mechanism whereby stress can induce depression.

Apparently, the Pgp knockout mice have been shown to accumulate more P-gp substrates (e.g., ivermectin, vinblastine, nelfinavir) within testicular tissue than wild-type controls. As Pgp is also expressed in leydig cells, testicularmacrophages and Sertoli cells, although it does not appear to be expressed in germ cell populations. Thus, Pgp is likely to be involved in protection of somatic cells from xenobiotics within the testes and may also influence the microenvironment of the seminiferoustubules through transport of testicular
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steroids. Pgp is present in the blood-testis barrier and prevents the penetration of xenobiotics, thus providing protection for the testis and play a major role in preventing infertility.(163)

Pgp, also known as multidrug resistance protein 1 (MDR1) or ABC subfamily B member 1 (ABCB1), is a large transmembrane glycoprotein of approximately 170 kDa which is encoded by the ABCB1 gene and belongs to a large superfamily of highly conserved ABC transporters.(164) In humans, Pgp is expressed on the luminal surface of the intestinal epithelium, renal proximal tubule, bile canalicular membrane of hepatocytes, placenta and blood–brain barrier, which acts as a defense mechanism against harmful xenobiotics.(165) A high concentration of Pgp is found on the gastrointestinal (GI) tract epithelium which actively pumps out potentially cytotoxic substances. The expression of Pgp in the upper gastrointestinal tract is reduced in peptic ulcers which may be directly associated with H. pylori infection in the stomach.(166, 167)

There is an evidence that Wnt signalling, particularly via the canonical pathway plays a role in vascular endothelial survival and proliferation. (168) Wnt ligands and Wnt ligand receptors have been identified in different types of vascular endothelial cells. (169). Certainly, the Wnt signalling pathways are now of interest in providing possible new targets suitable for therapeutic modification of angiogenesis. The idea that Wnt signalling pathway may also influence the barrier properties of blood vessels has not yet been addressed. It is interesting to note, however that Wnt signalling in blood vessels in the brain during development(170) appears with the same time frame as appearance of the efflux transporter, P-gp in the brain vasculature. (171)

It is interesting too, to note that manipulations to the Wnt/β-catenin signalling pathway produced similar changes in expression in endothelial cells of both rat and of human origin. Hence recent results collectively suggest very strongly that the alteration in Wnt/β-catenin signalling is responsible for the changes seen in Pgp expression. However, Pgp plays an important role at the blood–brain barrier and blood-testis barrier in preventing access of unwanted substances to the brain(172) as well as testicular tissue(173) and prevent the stress induced disorders like ulceration, depression and sexual deficiency.

The effects of M. pruriens and H. Perforatum extract (50% MeOH) on human brain endothelial and sertoli cells takes the initial steps towards determining the influence of the Wnt/β-catenin canonical pathway on expression of Pgp by analysing the effects of activation downstream i.e. β-catenin signalling on Pgp expression which is widely expressed in gastric
mucosa, brain endothelial and sertoli cells. Recent pharmacological intervention for the involvement of Wnt/β-catenin pathway in expression of Pgp as well as the effects of hydroalcoholic extract of H. perforatum and M. pruriens that inhibit GSK-3β activity (key inhibitor of Wnt/β-catenin pathway) which enhance the level of β-catenin. This reveals that P-gp can be positively regulated in these cells via Wnt/β-catenin pathway activation after the treatment. Expression of this ATP-binding cassette (ABC) transporter through the exposure of hydroalcoholic extract of H. perforatum and M. pruriens is assessed first time which produces significant increase in the expression of efflux transporters (Pgp and Lrp-1) in immortalized human brain endothelial cells. In addition, the effect of H. perforatum and M. pruriens extract was also assessed inline with LiCl, a well known Gsk-3β inhibitor. This comparative study ensures the involvement of Wnt/β-catenin pathway in regulation of Pgp as well as indicates the potential role of M. pruriens and H. perforatum in up-regulation of Pgp . This study is entirely novel in its kind, which unveils the role of exact mechanism function behind Pgp expression which is reduced during stress. As Pgp is an important efflux transporter expressed in wide range of tissues and plays a major role in effluxing and regulating the movement of toxins and xenobiotics. Hence, the treatment of hydroalcoholic extract of H. perforatum and M. pruriens enhance the expression of Pgp where the extract of H. perforatum upregulates the expression of Pgp significantly against LiCl which indicate that both extracts may upregulates the expression of Pgp via Wnt/β-catenin pathway. These results open new horizons in the treatment of stress induced ulceration, depression and sexual deficiency, however the involvement of Wnt/β-catenin pathway needs further investigation where the effect of H. perforatum and M. pruriens on other parameters of the pathway to be assessed.

The importance of such gene level studies cannot be over-looked since stress induced genome-wide damage can have catastrophic long-term health consequences that may range from accelerated ageing, carcinogenesis, immuno-compromisation and more importantly vertical transmission of these genetic aberrations. Essential in this regard, is the use of hydroalcoholic extract of H. perforatum and M. pruriens to identify the key biological effects on stress induced ulceration, depression and sexual deficiency. In the present study we predicted that increasing knowledge on efflux proteins expressed at the luminal surface of BBB and BTB. Cellular damage-triggered signaling pathways leading to cell death could provide new strategies for characterization of fragile sites on the cells.
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