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Role of antioxidants in chronic fatigue syndrome in mice

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The present study was carried out using mice model of chronic fatigue syndrome (CFS) in which mice were forced to swim everyday for 7 days for a 6 min session. There was a significant increase in despair behavior (immobility period) in saline treated mice on successive days. Treatment with potent antioxidants carvedilol (5 mg/kg, ip) and melatonin (10 mg /kg, ip) produced a significant reduction in immobility period. Similar results were observed with herbal products St. John's Wort (*Hypericum perforatum.L)* (10 mg/kg, po) and GS-02 (20 mg /kg, po). Fluoxetine, a selective serotonin reuptake inhibitor produced a significant effect only on first and second day of its treatment. Biochemical analysis revealed that chronic swim test significantly increased lipid peroxidation and catalase levels in whole brains of mice. There was a decrease in the levels of super oxide dismutase (SOD) and glutathione reductase (GSH) in the brain. Administration of carvedilol, melatonin, GS-02 and St. John's Wort restored the levels of lipid peroxidation and glutathione. The enzymes SOD and catalase were also restored. Fluoxetine affected the biochemical variables not to the same extent as other treatments. The findings of the present study suggest that oxidative stress might play a significant role in the pathophysiology of CFS. Thus antioxidants and herbal products like St. Johns wort and GS-02 could be useful in the treatment of CFS.

The chronic fatigue syndrome (CFS) is a heterogeneous disorder of unknown etiology characterized by fatigue, neuropsychiatric symptoms, and various other somatic complaints[1]. Fatigue is associated with immunoligical disturbances, which is evident in two-third of the patients. Much of this depression seen may be reactive but the prevalence exceeds that in other chronic medical illnesses. The various neuroendocrine abnormalities also occur which again contribute to the impaired energy and mood. Further, it has been proposed that chronic fatigue syndrome is fundamentally a psychiatric disorder and various neuroendocrine and immune disturbances arise secondarily to it[2].

There is no definite diagnosis and treatment for this syndrome. Various drugs have been proposed for its treatment and more recently role of antioxidants in diet have been considered useful in this syndrome[3]. Carvedilol is a lipophilic nonselective β-adrenoreceptor antagonist with strong antioxidant properties[4]. Carvedilol is approximately 10-fold more potent as an antioxidant than vitamin E[5]. Melatonin synthesized exclusively in the pineal gland, plays an important role in establishing circadian rhythms. It has been recently reported to have potent free radical scavenging property and is capable of stimulating endogenous anti-oxidants activity superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)[6]. *Hypericum perforatum.L* (St. Johns wort) contains a wide variety of active constituents including flavanoids, phuloroguginols and essential oils. *H. Perforatum* extracts have repeatedly shown significant antidepressive effects in various animal models of depression but the mechanisms responsible are as yet incompletely understood[7].

The method of chronic 7-day exposure of mouse to forced swim test has been successfully validated as an animal model of CFS[2]. Using this model in the present study the role of fluoxetine, carvedilol, *H. perforatum*, GS-02 and melatonin was explored in CFS. Biochemical estimates were also carried out to establish whether the antioxidant activity of these compounds was responsible for their effective action.

Material and Methods

Animals—Male laka mice (20-25 g) bred in the Central Animal House (CAH) of Panjab University were used for the study. The animals were housed under standard laboratory conditions and were supplied with food and water *ad libitum*. The animals were maintained at 12 hr day/night cycle and were acclimatized to the laboratory conditions prior to ex-

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perimentation. All the experiments were carried out between 10 00 hrs and 17 00 hrs. The animals were drawn at random for the study. The institutional animal ethical committee approved the experimental protocols.

Experimental procedure—The animals were forced to swim individually in a glass jar (25×12×25 cm) containing water at room temperature (22°C±3°C)[8]. The height of water level was adjusted to 15 cm and kept constant throughout the experiments. After an initial period of vigorous activity each animals assumed a typical immobile posture. The total duration of immobility was measured during a total period of 6 min[2]. The mice were judged immobile, when they ceased struggling and made minimal movement of their limbs to keep the head above water level. This procedure was followed for 7 days. This chronic forced swimming produced depression and fatigue resembling chronic fatigue syndrome (CFS).

Dissection and homogenization—On the 8th day of study, the animals were sacrificed by decapitation. The brains removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared in 0.1 *M* phosphate buffer (*p*H 7.4). The post nuclear fraction of catalase assay was obtained by centrifugation of the homogenate at 1000 *g* for 20 min, at 4°C and for other enzyme assay the homogenate was centrifuged at 12000 *g* for 60 min, at 4°C

Lipid peroxidation—The quantitative measurement of lipid peroxidation in the whole brain was measured according to the method of Wills[9]. The amount of malondialdehyde (MDA) formed was measured by the reaction with thiobarbituric acid at 532 nm using Perkin Elmer lambda 20 spectrophotometer. The results were expressed as nmol of malondialdehyde/mg protein using the molar extinction coefficient of chromophore ($1.56\times10\ M^{-1}\ cm^{-1}$).

Estimation of reduced glutathione—Reduced glutathione in the forebrain was estimated according to the method of Ellman[10]. A, 0.75 ml of homogenate was precipitated with 0.75 ml of 4% sulfosalicylic acid. The samples were centrifuged at 1200 *g* for 15 min at 4°C. The assay mixture contained 0.5 ml of supernatant and 4.5 ml of 0.01M DTNB. The yellow color developed was read immediately at 412 nm using Perkin Elmer lambda 20 spectrophotometer. The results were expressed as nmol GSH/mg protein.

Enzyme assays

Superoxide dismutase—Superoxide dismutase activity was assayed according to the method of Kono[11], wherein the reduction of nitrazoblue tetrazolium (NBT) was inhibited by superoxide dismutase is measured at 560 nm using Perkin Elmer lambda 20 spectrophotometer. Briefly, the reaction was initiated by the addition to the reaction mixture containing NBT and post nuclear fraction of the homogenate. The results were expressed as units per mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of a reaction by 50%.

Catalase activity—Catalase activity was assayed by method of Luck[12], wherein the breakdown of H_2O_2 being measured at 240 nm. Briefly the assay mixture consisted of 3 ml of H_2O_2 phosphate buffer (1.25×10^{-2} H_2O_2 moles) and 0.05 ml of supernatant of tissue homogenate (10%) and the change in the absorbance was recorded at 240 nm using Perkin Elmer lambda 20 spectrophotometer. The results were expressed as K/min/mg protein.

Protein estimation—The protein content was measured according to Lowry[13] using bovine serum albumin as standard.

Drugs, sources and treatment—Fluoxetine (Dr Reddy laboratories), GS-02 (Himalaya Drug company, Banglore India), melatonin (Dabur India Limited, Delhi India), carvedilol (Sun Pharma Mumbai India), and *Hypericum perforatum.L* (St. Johns wort) (Panacea Biotech Ltd. Lalru Punjab) were used. GS-02, carvedilol and St. Johns Wort were suspended in 0.5% w/v carboxymethylcellulose and administered orally (po). Melatonin was dissolved in minimal quantity of DMSO and the volume was made up with distilled water and administered intraperitoneally (ip), fluoxetine was dissolved in saline and administered intraperitoneally. All the test drugs were administered half an hour before the test.

Statistical analysis—The data were expressed as mean ± SE, immobility duration and compared with control group. The data were analyzed by using analysis of variance (ANOVA- one way) followed by Dunnet's test. In all the tests, the criterion for statistical significance was $P<0.05$.

Results

Effect of fluoxetine, carvedilol and melatonin on mean immobility period during 7 days of chronic swimming—Chronic forced swimming for 6 min session per day for 7 days produced depression and fatigue in control animals resembling chronic fatigue syndrome. As indicated by increase in the mean im-

mobility time, treatment with fluoxetine (10 mg/kg, ip) once daily prior to the exposure to forced swimming (30 min before) significantly reduced the immobility period on first and second day, but the antidepressant effect disappeared on other days with a little effect on 7th day. Carvedilol (5 mg/kg, ip), melatonin (10 mg/kg, ip) when administered, once daily prior to the exposure to forced swimming (30 min before) significantly reduced the immobility period from 2nd day to 7th day of the study (Fig. 1a).

Effect of St. Johns wort and GS-02 on immobility period during 7 days of chronic swimming—St. Johns wort (10 mg/kg, po) and GS-02 (20 mg/kg, po), herbal preparations were given once daily prior to the exposure to forced swimming (30 min before) significantly reduced the immobility period on all days of the treatment (Fig.1 b).

Effect of fluoxetine, carvedilol, melatonin GS-02, and St. Johns wort on whole brain enzyme levels in fatigued mice—Chronic forced swimming for 6 min session per day for 7 days induced oxidative stress as indicated by a significant raise in the whole brain MDA and catalase levels, and a decrease in GSH and SOD levels, control animals as compared to untreated group not subjected to chronic forced swimming. Carvedilol (5 mg/kg, ip), melatonin (5 mg/kg, ip) St. john's wort (10 mg/kg, po) and GS-02 (20 mg /kg, po) Given once daily 30 min prior to the test significantly reversed the extent of oxidative stress as compared to control groups, indicated by a decrease in MDA, and catalase levels and a significant increase in GSH and SOD levels. (n=6 $P<0.5$) Fluoxetine (10 mg/kg, ip) did not show significant changes in the above enzyme levels. (Table 1).

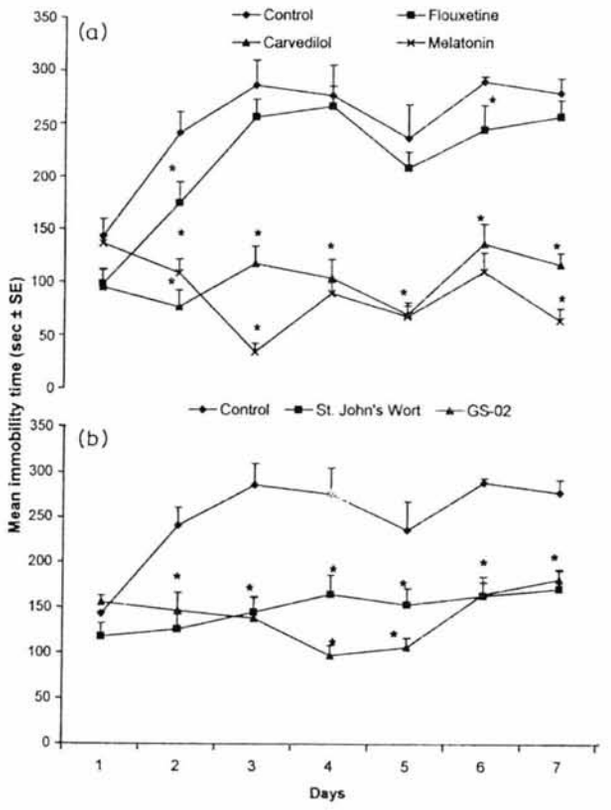

Fig. 1—Effect of fluoxetine (10mg/kg, ip), carvedilol (5 mg/kg, ip) melatonin (10 mg/kg, ip), St. Johns Wort (10 mg/kg, po) and GS-02 (20mg/kg, po) on mean immobility time on days 1-7. *p<0.0.5.(ANOVA followed by Dunnet' test) as compared to the control group, n =(6 for each group)

Discussion

The role of oxidative stress in CFS is an important area for current and future research as it suggests the use of antioxidants in the management of CFS[14]. In the present study forced swimming-induced immobility test model was used. Porsolt[8] first described this model and has been extensively validated for studying the antidepressant profile of new drugs. The model measures behavioral depression. In this, mice when exposed to aversive situation from which there is no possibility to escape, eventually stops struggling and assume a typical immobile posture indicative of behavioral depression[2].

In the present study there was a significant increase in the immobility time each day, when mice were subjected to chronic swim test for 7 days with the maximum effect on 6th and 7th day of the study. There was a considerable increase in the lipid peroxidation and decrease in the glutathione levels in brains of the mice subjected to chronic swim test as compared to normal mice not subjected to chronic swim test. These results were also well supported by the enzymatic estimation of SOD and catalase. These results clearly indicate an increased oxidative stress in brains of mice subjected to chronic swim test and thus indicate the possible involvement of oxidative stress in the pathogenesis of CFS.

Pretreatment with already well established antioxidants carvedilol, melatonin, 30 min before the test on each day not only reduced the immobility time but also decreased lipid peroxidation and increased the glutathione levels, again well supported by the enzymatic essays of SOD and catalase. These drugs were effective on all the days of the treatment. There are multiple mechanisms through which carvedilol and melatonin act. The antioxidant action of carvedilol

Table 1—Effect of fluoxetine, melatonin, carvedilol, GS-02 and St. johns wort on MDA (malonaldihyde), GSH (reduced glutathione), SOD (super oxide dismutase) and catalase levels

[Values are mean ± SE from 6 observations in each group]

Treatment	MDA (n mol MDA/ mg protein)	GSH (n mol/mg protein)	SOD (Units/ mg protein $\times 10^{-3}$)	Catalase (K/min10^{-3})
Normal [@]	303 ± 7.7	0.78 ± 0.01	250 ± 2	13 ± 0.3
Control[#]	362 ± 8.9[a]	0.56 ± 0.01[a]	140 ± 3[a]	21 ± 0.2[a]
Fluoxetine (10mg/kg ip)	344 ± 9.9	0.67 ± 0.01*	160 ± 13*	18 ± 0.1
Melatonin (10 mg/kg ip)	320 ± 7.8*	1.0 ± 0.02*	373 ± 13*	68 ± 0.1*
Carvedilol (5 mg/kg ip)	277 ± 17.4*	0.99 ± 0.02*	389 ± 33*	70 ± 0.3*
GS-02 (20 mg/kg po)	315 ± 4.8*	1.1 ± 0.02*	340 ± 12*	65 ± 0.3*
St. Johns Wort 10 mg/kg po)	266 ± 5.3*	1.2 ± 0.06 *	380 ± 11*	59 ± 0.1*

Normal [@] group comprises saline treated animals who were not subjected to chronic swimming. Control[#] refers to vehicle treated group subjected to chronic swim test. [a]$P<0.05$ (T-test umpaired) as compared to the normal group. *$P<0.05$(ANOVA followed by Dunnet's test) as compared to the vehicle treated control[#] group

may be due to the inhibition of direct cytotoxic action of free radicals or prevention of oxygen free radicals from activating transcription factors such as NF-χB or protection and replenishing the endogenous antioxidant defense mechanisms or their combinations. It has also been reported that carvedilol inhibits lipid peroxidation by scavenging free radicals[5]. Melatonin is reported to prevent the oxidative stress by free radical scavenging[6], and by activating antioxidant defense enzymes[15]. It is also reported to scavenge peroxynitrate anions and also inhibits nitric oxide synthase (NOS) a known pro-oxidant enzyme[16]. Accordingly, one of the mechanisms of carvedilol and melatonin may contribute to their protective effect towards oxidative stress in CFS.

Pretreatment with GS-02 a multiherbal psychotropic preparation (Himalay Drug Co) and St. Johns wort 30 min prior to the test produced a significant decrease in the immobility time and also decreased the lipid peroxidation, increased glutathione levels, again well supported by enzymatic estimation of SOD and catalase. Studies of St. John's wort extracts have repeatedly shown significant antidepressive effects in various animal models of depression[7]. The mechanisms responsible are as yet incompletely understood. St. John's wort is a weak MAO inhibitor *in vitro*[17]. The suggestion, however, that the antidepressant action of *H. perforatum.L* (St. Johns wort) is due to monoamine oxidases (MAO) inhibition is no longer credited. One of the important constituent of *H. perforatum* is hypericin[7] and more recently it has been reported that hypericin scavenges the free radicals in both cell free and human vascular tissue[18] Another important constituent of *H. perforatum* is quercetin[7], a well-known potent antioxidant[19]. Quercetin has free radical scavenging properties; the antioxidant property may result from direct scavenging of free radicals and other oxidizing intermediates, or from chelation of iron or copper ions and from the inhibition of the oxidases[20]. Thus *H. perforatum* exhibit a significant antioxidant property. GS-02 is an unknown compound, but it also showed a significant antioxidant property.

Fluoxetine, well-known antidepressant drug had a variable effect in the preset study and also the antioxidant profile of the drug was not comparable with the other drugs used in the study. Fluoxetine is a selective serotonin reuptake inhibitor commonly prescribed for the treatment of depression. Case reports and uncontrolled studies suggest that fluoxetine is beneficial in CFS. But there are contradicting reports in two studies, which reported serotonergic hypersensitivity in CFS, and from that perspective it may not prove beneficial[21].

In conclusions the result of the present study suggest the role of oxidative stress in the pathophysio-

logy of chronic fatigue syndrome thus suggesting the use of antioxidants like carvedilol and melatonin in the management or treatment of the syndrome. Herbal products like St. Johns wort, and GS-02 due to their antioxidant profile may also provide an additional benefit in the syndrome. Since synthetic compounds carvedilol and melatonin showed better results than the natural herbal compounds *H. perforatum* and GS-02, the synthetic compounds may be preferred over the natural ones in the treatment of CFS.

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