EFFECTS OF COMBINED THERAPY WITH NIFEDIPINE AND MELATONIN ON HALOPERIDOL-INDUCED TARDIVE DYSKINESIA IN RATS

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ABSTRACT

Tardive Dyskinesia (TD) is a severe movement disorder of the orofacial region caused by the typical neuroleptic, haloperidol. It was reported that haloperidol-induced TD is mediated through oxidative stress mechanism which is triggered by calcium overload. Moreover, the disturbance of melatonin secretion due to pineal gland calcification is linked to the pathophysiology of TD. Therefore, the aim of this study was to investigate the effect of combined therapy with nifedipine, a calcium channel blocker and melatonin, the powerful antioxidant on haloperidol-induced TD. Five groups of rats were treated as follow: Group1 and 2 were treated with vehicle and haloperidol (1mg/kg, ip) respectively. Groups 3-5 were treated with nifedipine (20 mg/kg, orally), melatonin (5mg/kg, orally), nifedipine plus melatonin respectively then challenged with haloperidol for 21 days. Our results show that, haloperidol produced behavioral abnormalities and alterations in oxidative stress parameters. Interestingly, combined administration of nifedipine plus melatonin significantly reduced vacuous chewing movements (VCMS) and tongue protrusions frequency (TPF). Moreover, administration of both drugs improved oxidative stress parameters such as glutathione (GSH), lipid peroxide malondialdehyde (MDA) levels and superoxide dismutase (SOD), catalase (CAT) activities that were altered by haloperidol more than each drug alone. These results indicate that the deleterious effect of haloperidol in rat brain is at least in part due to oxidative stress and that co-administration of nifedipine plus melatonin may have neuroprotective effects related to antioxidant mechanisms.

Key words: Nifedipine, melatonin, tardive dyskinesia, haloperidol, calcium channel blocker.

INTRODUCTION

Schizophrenia is a chronic, usually lifelong psychiatric disorder that requires many hospitalizations and complex treatment with neuroleptics [9]. Tardive dyskinesia (TD) is a serious motor side-effect of long-term treatment with neuroleptics [8]. The hyperkinetic movement disorder is characterized by involuntary movements predominantly of the face, mouth and tongue, but a variety of less frequent motor abnormalities of the rest of the body may occur [8]. Tardive dyskinesia is estimated to occur in 30% of patients treated with antipsychotics [9]. One possible pathophysiological explanation is neuronal cell damage from free radicals induced by antipsychotics in patients treated with neuroleptics [10]. In animal studies, oxidative stress and elevated levels of lipid peroxidation have been implicated in haloperidol toxicity [11]. Recently, it was report that calcium influx through N-methyl-D-aspartate (NMDA) receptors is involved in reactive oxygen species (ROS) production and neuronal damage [10]. Moreover, the pineal calcification and low endogenous melatonin levels have been associated with TD in patients with schizophrenia who are treated with antipsychotics [11]. Therefore, the use of calcium channel blocker with antioxidant activity alone or in combination with melatonin may be of value in prevention and treatment of TD.

Nifedipine [1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester] is a dihydropyridine calcium-channel blocker (CCB) that binds to the a1 subunit of the L-type calcium channel [8]. It introduced approximately 30 years ago for the prophylaxis of angina symptoms, and then later utilized as an anti-hypertensive agent. Nifedipine crosses the blood-brain barrier efficiently [9, 10]. The antioxidant activity of nifedipine is well-documented and it is established that nifedipine is a more powerful antioxidant than verapamil and diltiazem [11, 12]. It is generally held that peroxidation of membranes and sudden influx of calcium ions into cells are both involved in the damage that is produced in brain glial cells caused by free radicals generation [13]. Accordingly, reduction of intracellular calcium concentrations by nifedipine may reduce free radical generating mechanisms and lipid peroxidation. In addition, nifedipine may inhibit iron-dependent lipid peroxidation directly in murine ventricular membranes which can be ascribed to its capacity to scavange or impair oxygen free radical generation [14]. Moreover, Allanore and colleagues [15] have reported that nifedipine protects against overproduction of superoxide anion in stimulated monocytes. Therefore, nifedipine may block ROS generation directly or through inhibition of calcium influx overload.

Melatonin, a hormone primarily produced nocturnally by the pineal gland, is an important scavenger of hydroxyl radicals and other reactive oxygen species. Melatonin may also lower the rate of the DNA base damage resulting from hydroxyl radical attack and increase the rate of repair of that damage [16]. Melatonin is 6 to 10 times more effective as an antioxidant than vitamin E and exerts its actions through detoxification of a variety of free radicals and stimulation of several antioxidant enzymes [17]. Pineal calcification and low endogenous melatonin levels have been associated with TD in patients with schizophrenia who are treated with antipsychotics [18]. Melatonin has the ability to cross the blood brain barrier and hence it has a neuroprotective activity [19]. Melatonin has been shown to exert antioxidant effect on dopaminergic neurons [20] as well as dopaminergic-modulating activities [21] and thus may be efficacious in prevention and treatment of TD.

According to the above mentioned data, both nifedipine and melatonin may have the capacity to alleviate TD-induced by haloperidol through different mechanism(s). Therefore, the aim of the present study was designed to examine the effect of combined administration of nifedipine and melatonin on behavioral changes as vacuous chewing movements (VCMS) and tongue protrusions frequency (TPF) as well as oxidative stress biomarkers such as glutathione (GSH), lipid peroxide expressed as malondialdehyde (MDA) levels and the antioxidant enzyme activities of superoxide dismutase (SOD) and catalase (CAT) that were altered by haloperidol administration to rats.

MATERIALS AND METHODS

Drugs

Haloperidol was obtained from Nile C0. for Pharmaceutics (Cairo, Egypt). Melatonin was obtained from Sigma Chemical CO. (St., Louis, MO, USA). It was dissolved in absolute ethanol and further diluted in saline with 1% final concentration of ethanol. Nifedipine was obtained from Egyptian International Pharmaceutical Industries CO. (EIPICO, Egypt). All other chemicals were of analytical grade.

Animals

Male Wister albino rats weighing 180-220 g were obtained from Animal House, Faculty of Medicine, Assiut University (Assiut, Egypt), which were fed standard diet and water ad-libitum. During the
study, rats were maintained at 12 h light/dark cycle. Animals were acclimatized to laboratory conditions before the test.

**Experimental protocols**

Thirty male rats were used in this study. Rats were divided into 5 groups (n=6) as follows: Group 1 received vehicle and served as control; group 2 received haloperidol (1 mg/kg, ip); Group 3 received nifedipine (20 mg/kg, orally) then challenged with haloperidol; group 4 received melatonin (5 mg/kg, orally) then haloperidol and group 5 received nifedipine plus melatonin then challenged with haloperidol at the same previous doses. All groups were treated with drugs for 21 days. Then rats were subjected to behavioral and biochemical assessments. On day 22, after behavioral quantification, the animals were sacrificed by decapitation. In all groups, the brains were removed. A 10% (w/v) of brain tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The post-nuclear fractions used for determination of catalase (CAT), superoxide dismutase (SOD) enzyme activities and total protein contents were obtained by centrifugation of the homogenate at 10,000 x g for 60 min at 4°C.

**Rodent model of orofacial tardive dyskinesia**

Rats were injected with haloperidol (1 mg/kg ip) every day for a period of 21 days to produce orofacial tardive dyskinesia. Every week all behavioral tests were carried out and the last behavioral quantification was done 24 h after the last dose of haloperidol [3].

**Behavioral analysis**

For the assessment of oral dyskinesia on the test day, the animals were handled and habituated to the behavior observation situation. Rats were placed individually in a small (30 x 20 x 30 cm) Plexiglas cage. Mirrors were placed under the floor and behind the back wall of the cage to permit observation of oral dyskinesia when the animal was faced away from the observer. Animals were allowed 10 min to get used to the observation cage before behavioral assessments. To quantify the occurrence of oral dyskinesia, hand-operated counters were employed to score tongue protrusion frequency (TPF) and vacuous chewing movements (VCMs). Tongue protrusions and vacuous chewing movements were continuously for a period of 5 min [4], Naidu and colleagues [4] referred to VCMs as single mouth openings in the vertical plane not directed toward physical material. If tongue protrusion or VCMs occurred during a period of grooming, they were not taken into account. Tongue protrusion was defined as a visible extension of the tongue outside of the mouth and not directed at anything. In all experiments, the scorer was unaware of the treatment given to the animals.

**Determination of oxidative stress biomarkers in rat brain tissues**

Glutathione (GSH) content of brain tissues homogenate was determined using Ellman's reagent according to the method described by Ellman [5]. Rat brain tissues homogenate lipid peroxide levels were measured by colorimetric determination of malondialdehyde (MDA) is based on the reaction of one molecule of malondialdehyde with two molecules of thiobarbituric acid at low pH (2-3) according to the method of Mihara and Uchiyama [6]. The enzymatic activity of superoxide dismutase (SOD) in brain tissues homogenate was assessed according to the method of Marklund [7]. In brief, SOD activity was determined by computing the difference between auto-oxidation of pyrogallol alone and in presence of SOD enzyme. The catalase (CAT) activity was estimated in the brain tissues depending on the decrease in absorbance at 240 nm due to the decomposition of hydrogen peroxide by catalase according to the method of Clairborne [8].

**Statistical analysis**

Results were expressed as the means ± standard error of mean (SEM). Statistical significant difference was determined by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test. A probability value of P<0.05 was considered to indicate statistical significance.

**RESULTS**

Effects of nifedipine or melatonin and their combination on haloperidol-associated behavioral abnormalities in rats

Haloperidol produced a significant increase in the vacuous chewing movements (VCMs/5min) from 5±0.4 to 45±2.04 and tongue protrusion frequency (TPF/5min) from 4±0.39 to 25±0.816 in rats after 21 days of treatment as compared to vehicle-treated control group (Figure 1a & b). Administration of nifedipine with haloperidol reduced the abnormal vacuous chewing movements by 40% and tongue protrusions by 28% to 27±0.62 and 18±1.23 respectively. Melatonin also significantly reduced the haloperidol-induced orofacial abnormal vacuous chewing movements by 67% and tongue protrusions by 52% to 15±1.63 and 12±1.23 respectively (Figure 1a & b). Interestingly, the combined administration of nifedipine plus melatonin completely abolishes the abnormal vacuous chewing movements and tongue protrusions and restored it to normal control levels (Figure 1a & b).

**Figure 1:** Effects of nifedipine (NFD) or melatonin (MLT) and their combination (NFD+MLT) on haloperidol (HLP)-induced behavioral alterations such as vacuous chewing movements (a) and tongue protrusion frequency (b) in rats compared to control (CO). Data was expressed as Mean ± SEM, (n= 6/group). Data comparison was performed using ANOVA followed by Bonferroni’s multiple comparison test. *p < 0.01; **p < 0.001 compared with control group. ‘*p <0.01; ‘**p <0.001 statistically significant difference from haloperidol group.

Effects of nifedipine or melatonin and their combination on haloperidol-induced alterations in oxidative stress biomarkers in rat brain tissues

Glutathione (GSH) has a very important role in protecting against oxygen free radical damage by providing reducing equivalents for several enzymes; GSH is also a scavenger of hydroxyl radicals and singlet oxygen radicals. In this study, haloperidol produced a decrease in GSH levels (µmol/g tissue) of rat brain tissues from 4.38±0.2 to 2.56±0.193. The haloperidol-induced decline in GSH...
content was inhibited and the level of GSH was increased by 30% to 3.34±0.189 after nifedipine and increased by 52% to 3.9±0.14 after melatonin treatment compared to haloperidol treated group (Figure 2a). Co-administration of both drugs (nifedipine plus melatonin) fully restored the reduced GSH levels to control level (4.4±0.2) (Figure 2a). Free oxygen radicals can induce lipid peroxidation in cells; MDA is formed during oxidative degeneration and accepted as an indicator of lipid peroxidation. Haloperidol increased the level of lipid peroxide, MDA, from 1.2±0.15 to 2.7±0.24 (µmol/g tissue). Nifedipine and melatonin prevented the haloperidol-induced elevation in MDA levels and decreased its elevated levels by 26% and 43% to 2±0.228 and 1.6±0.158 respectively (Figure 2b). Co-administration of both drugs (nifedipine plus melatonin) significantly reduced the elevated MDA levels by 52% to the control level (1.3±0.14) (Figure 2b).

The enzymatic activities of antioxidant enzymes superoxide dismutase (SOD, U/mg protein) and catalase (CAT, k/mg protein) were decreased in brain tissues of haloperidol-treated rats from 26±2 to 11±1.14 (for SOD) and from 5.4±0.178 to 2.48±0.25 (for CAT) (Figure 3a & b). However, the reduced SOD activity was increased by 53% and 82% to give values of 16.8±1.59 and 20±1.6 after nifedipine and melatonin administration respectively (Figure 3a). Co-administration of nifedipine plus melatonin significantly increased the SOD activity by 122% compared to haloperidol treated group and restored it to normal control value of 24±1.63 (Figure 3a). On the other hand, the haloperidol-induced suppression of catalase activity was also antagonized by nifedipine and melatonin treatment and increased its activity by 48% (3.67±0.11) and 81% (4.5±0.17) respectively. Interestingly, this enhancing effect was augmented after combination of nifedipine plus melatonin and increased the CAT activity by 105% (5.1±0.16) compared to haloperidol treated group (Figure 3b).

**Figure 2:** Effects of nifedipine (NFD) or melatonin (MLT) and their combination (NFD+MLT) on haloperidol (HLP)-induced alterations in oxidative stress biomarkers: GSH (a) and MDA (b) levels in rat brain tissues compared to control (CO). Each value represents the mean ± SEM, (n= 6/group). Data comparison was performed using ANOVA followed by Bonferroni’s multiple comparison tests. *p < 0.05; **p < 0.01; ***p < 0.001 compared with control group. +p <0.05; ++p <0.01; +++p <0.001 statistically significant difference from haloperidol group.

**Figure 3:** Effects of nifedipine (NFD) or melatonin (MLT) and their combination (NFD+MLT) on haloperidol (HLP)-induced alterations in antioxidant defense enzymes activities SOD (a) and CAT (b) in rat brain tissues compared to control (CO). Each value represents the mean ± SEM, (n= 6/group). Data comparison was performed using ANOVA followed by Bonferroni’s multiple comparison test. **p < 0.01; ***p < 0.001 compared with control group. +p <0.05; ++p <0.01; +++p <0.001 statistically significant difference from haloperidol group.

**DISCUSSION**

Tardive dyskinesia (TD) is a syndrome of potentially irreversible, involuntary hyperkinetic dyskinesia that occurs during long-term neuroleptic treatment. It is a major limitation of chronic antipsychotic drug therapy. The orofacial symptoms of tardive dyskinesia were clear in our study after 3 weeks of haloperidol treatment. Vacuous chewing movements and tongue protrusions frequency increased markedly in haloperidol-treated rats compared to control. Our results were in accordance with that of Bishnoi and colleagues[27] who reported that rats injected with haloperidol showed behavioral abnormalities in orofacial region. Several hypotheses have been postulated to explore the mechanism (s) by which neuroleptics, in particular haloperidol induce tardive dyskinesia.

An important hypothesis recently receiving considerable interest is the proposal that TD is due to neurotoxic effects of free radical byproducts from dopamine (DA) metabolism. The increase in DA turnover is produced from blockage of dopamine receptors by neuroleptics[28]. Dopamine undergoes monoamine oxidase-catalyzed oxidative deamination to 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is metabolized primarily to 3,4-dihydroxyphenylacetic acid (DOPAC). DOPAL is a reactive radical and toxic to dopaminergic cells[29]. DOPAL injection into the substantia nigra of rats resulted in DA neuron loss[29]. A role for increased reactive oxygen species and oxidative stress in the
etopathology of neuroleptic-induced tardive dyskinesia has been proposed [3]. Administration of haloperidol to rats led to a decrease in reduced glutathione (GSH) levels in the striatum indicating generation of oxidative stress by the drug [4]. Fachinetto and colleagues [5] have reported that animals with vacuous chewing movements have significantly higher lipid peroxide expression, thiobarbituric acid reactive substances (TBARS) in the striatum, suggesting increased lipid peroxidation and free radical production in these animals. Chronic use of neuroleptics is also reported to cause a decrease in the activity of antioxidant defense enzymes such as superoxide dismutase (SOD) and catalase (CAT) [6]. Our results were consistent with these data as injection of haloperidol for 3 weeks produced a significant increase in lipid peroxide level expressed as MDA and a decrease in the glutathione (GSH) levels as well as attenuation of SOD and CAT activities in rat brains compared to normal control rats. These biochemical alterations in oxidative stress biomarkers were accompanied by behavioral abnormalities usually in the orofacial area.

Another source of neuronal oxidative damage is related to calcium overload. It was hypothesized that prolonged stimulation of N-methyl-D-aspartate (NMDA) and glutamate receptors can induce massive cell death in the brain (excitotoxicity), by causing calcium overload in post-synaptic neurons [7]. Excitotoxic neuronal damage caused by overactivation of N-methyl-D-aspartate and glutamate receptors is thought to be a principal cause of neuronal loss after stroke and brain trauma [8]. It was reported that nifedipine, a calcium antagonist, inhibits the release of glutamate from excitatory cortical striatal projections [9]. Therefore, neuroleptic blockade of these receptors increases the synaptic release of aspartate and glutamate in the striatum through NMDA receptors [10]. Hernández-Fonseca and colleagues [11] have reported that calcium influx through NMDA receptors is involved in ROS production and neuronal damage. Lipid peroxidation is considered as a major mechanism of oxygen radical toxicity, thereby altering membrane permeability [12]. Persistent activation of NMDA and non-NMDA glutamate ionotropic receptors mediates calcium entry and reactive oxygen species production which are well-recognized perpetrators of neuronal oxidative damage [13]. Some data suggest that calcium antagonists protect the lipid fraction within the cell membrane against the toxic effects of free oxygen radicals [14]. Calcium channel blockers also prevent calcium-overload in ischemic rat brains via their effect on the L-type calcium channel leading to suppression of formation of oxygen-derived free radicals and lipid peroxidation [15].

The mechanism responsible for nifedipine’s protective effect against haloperidol-induced tardive dyskinesia may depend on a relative reduction of cellular calcium influx via its effect on the slow L-type calcium channel. There is involvement of calcium in triggering oxidative damage and excitotoxicity, both of which play central role in haloperidol-induced orofacial dyskinesia and associated alterations [16]. Accordingly, reduction of intracellular calcium concentrations by nifedipine may reduce free radical generating mechanisms and lipid peroxidation. Alternatively, nifedipine may inhibit iron-dependent lipid peroxidation directly in ventricular myocyte membranes which can be ascribed to its capacity to scavenge or impair oxygen free radical generation [17]. A reasonable concept is that calcium overload enhances the formation of oxygen-derived free radicals and lipid peroxidation [18].

The present study revealed the neurotoxic effects of haloperidol in rats. The toxic effect of haloperidol may be ascribed to oxidative stress mechanism that may relate to calcium overload. The combination of calcium channel blocking and antioxidant activities of nifedipine with the antioxidant, anti-dopaminergic activity of melatonin significantly abolished the behavioral abnormalities such as VCMs and TPF. In the same time this combination ameliorated the deteriorated oxidative stress biomarkers. The protective effect of co-administration of nifedipine plus melatonin was more effective than either drug alone. Nifedipine and melatonin completely inhibited the behavioral abnormalities associated with vacuous chewing movements and tongue protrusions frequencies. In the same time, both drugs increased the level of GSH and the activities of antioxidant enzymes SOD and CAT and decreased the elevated MDA level more efficiently than either nifedipine or melatonin alone.

CONCLUSION

The present study revealed the neurotoxic effects of haloperidol in rats. The toxic effect of haloperidol may be ascribed to oxidative stress mechanism that may relate to calcium overload. The combination of calcium channel blocking and antioxidant activities of nifedipine with the antioxidant, anti-dopaminergic activity of melatonin significantly abolished the behavioral abnormalities such as VCMs and TPF. The pineal gland hormone melatonin is a potent antioxidant and appears to be effective in the treatment of TD [20]. In addition, melatonin is a potent scavenger of hydroxyl radicals and other reactive oxygen species [21]. It has the ability to cross blood brain barrier efficiently and improve behavior in TD patients [22].

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