

## Immunomodulatory Changes Induced By High Doses Of Dextromethorphan In Male Rats

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### ABSTRACT

#### Background:

Dextromethorphan (DXM) is a synthetic opioid analogue, similar to codeine that has antitussive effect but no opiate-like analgesic activity. It is widely used as an over-the-counter cough suppressant available in various cough and cold preparations; it is one of the often overlooked types of substance abuse by adolescents and young adults in the United States and around the world. So, the present study aims to investigate the side effects of the DXM abuse (sub-chronic and lethal doses) on the immune functions in rats.

#### Material and Methods:

The rats were divided into three equal groups, the first one served as control, and the second and third were treated. Treated groups received oral doses of DXM which increasing per 10 days (double dose) for a month.

#### Results

We examined the sub-chronic and lethal effects of DXM administration on the cellular immune responses in rats. T cell stimulator, Phytohemagglutinin showed a significant suppress on lymphocytes of peripheral blood proliferation and a highly significant decrease on phagocytic and killing of *S. aureus* by PMN and macrophage cells. Moreover, it induced a significant decrease in serum IL-6, and IFN- $\gamma$  levels, but, it exhibited a highly significant increase in serum IL-10 level throughout the period of experiment. In addition, it induced also a significant decrease in the production of cortisol during the experimental time except the last period of treatment in the 3<sup>rd</sup> group, where, serum cortisol level gradually return to normal level.

#### Conclusion:

These results suggest that sub-chronic and lethal doses of DXM administration in rats disturbed cellular immune responses, exhibited potent anti-inflammatory actions, suppressed of leukocytes dependent production of cytokines such as IL-6 and IFN- $\gamma$ . Moreover, it has some effects on serum cortisol concentration presumably via blockade of NMDA receptors.

**Key words:** Dextromethorphan, IFN- $\gamma$ , IL-10, IL-6, Phagocytosis, Lymphocytes transformation, Cortisol, Albino rat.

### Introduction

Dextromethorphan (DXM) is an over-the-counter (OTC) cough suppressant commonly found in cold medications <sup>(1)</sup>. DXM is often abused in high doses by adolescents to generate euphoria and visual and auditory hallucinations <sup>(1,2)</sup>. DXM is an antitussive that has been proposed to have potential utility in the treatment of various neurological disorders <sup>(3)</sup>. Moreover, it has been suggested to be useful in attenuating tolerance to opioids, reducing opioid withdrawal and inhibiting the reinforcing properties of a variety of abused drugs <sup>(4)</sup>. Aside from its effects, DXM is essentially devoid of other opioid-type effects <sup>(5)</sup>.

However, it interacts at non-opioid sites and possesses other pharmacological activity. For example, DXM is both a substrate and an inhibitor of CYP450 2D6 <sup>(6)</sup>. It is metabolized to an active metabolite dextrorphan, which, along with DXM, antagonizes the N-methyl-D-aspartate receptors (NMDA), producing phencyclidine (PCP) - like effects, inhibiting serotonin reuptake, competing for serotonergic receptors, and eliciting adrenergic effects by inhibiting peripheral and central catecholamine uptake <sup>(7)</sup>. It is well known that the central nervous system and behavior can influence immune function <sup>(8)</sup>.

DXM is commonly formulated with other agents, such as antihistamines, analgesics and decongestants that can complicate and worsen drug effects, especially in overdose. Symptoms of mild to moderate toxicity include hallucinogenic and dissociative effects, agitation, somnolence, ataxia, mydriasis, diaphoresis, and tachycardia; severe intoxication can lead to seizures and death <sup>(1)</sup>. DXM is formulated as a hydrobromide salt which can result in bromide toxicity, especially with chronic abuse <sup>(2)</sup>. The risk for serotonin syndrome can also occur with concomitant use of competitive inhibitors of CYP450 2D6, such as selective serotonin reuptake inhibitors (the most potent being paroxetine) and amphetamine analogues, including methyl-enedioxy-methamphetamine <sup>(9)</sup>.

In rats and monkeys, DXM produces discriminate stimuli that generalize to PCP, ketamine and other noncompetitive NMDA receptor antagonists <sup>(10)</sup>. Clinically, high doses of DXM can induce psychotropic effects/euphoria <sup>(11)</sup>. DXM also binds with high affinity to other sites, including sigma binding sites <sup>(9)</sup>, nicotinic receptors and calcium channels <sup>(12)</sup>. It has been suggested that the discriminative stimulus effects DXM more closely resemble the effects of drugs that interact at sigma receptors compared with other sites <sup>(10)</sup>.

The sympathetic nervous system has been implicated in the modulation of splenic natural killer (NK) cell activity. In some cases, psychoactive compounds suppressed splenic NK cell activity and mitogen-induced lymphocyte proliferative responses <sup>(13)</sup>. There is a critical need for current information regarding the scope of DXM abuse in order to understand the significance of the problem. In addition, little has been reported in the literature on effects of higher doses of DXM on immune system in rodents. So, the present study aim to investigate oral administration of the DXM abuse side effects on the immune function after exposure to its sub- chronic and lethal doses in rats.

## **MATERIAL AND METHODS**

### **Animals**

Male albino rats of the Sprague-Dawley, 60 days old and 150 to 180 g in weight were used as experimental animals throughout the present work. They were supplied from

Theodore Billharz Research Institute (TBRI). Upon arrival, the rats were individually caged in a colony room, where a reversed day-night (12 hr) cycle was maintained through artificial illumination. Rats received access free to both food pellets (protein 21% and energy 2950 K.C.) and water throughout the experiments.

### **Drug**

TUSSILAR (Dextromethorphan hydrobromide tablets) (DXM), produced by Kahira Pharma. & Chem. IND. Co. Cairo, Egypt.

### **Experimental design and DXM treatment:**

The animals (63) were divided into three equal groups (n=21). The first one served as control. While, the second and third were treated. Control received 0.1 ml saline for the same period of treatment. Treated groups received daily oral doses of DXM which increasing per 10 days (double dose) for a month, In the 2<sup>nd</sup> group, the rats received daily oral doses of DXM, starting with the dose 12.5 mg/kg/day and ending with 50 mg/kg/day. In the 3<sup>rd</sup> group, the rats were treated with DXM, starting with the dose 25 mg/kg/day and ending with 100 mg/kg/day. Seven animals from each group were sacrificed per 10 days. These daily doses were calculated for rats according to Paget and Barnes, <sup>(14)</sup>.

### **Measurement of body weight**

Each rat was weighted on 10, 20, 30 days to study the changes rate in body weight throughout the experimental period.

### **Serum IFN- $\gamma$ , IL-10 and IL-6**

To investigate the effect of DXM administration on T-helper (Th) cell population of lymphocytes [Th1 cytokine (IFN- $\gamma$ )/Th2 cytokine (IL-10)] cell function and on this response, immunoassay kits {Abnova (cat # KA0274.V.01), PBL Biomedical Laboratories (product # 43500-1, V.1.3)} were used to determine serum concentration of IFN- $\gamma$  <sup>(15)</sup> and IL- 10 <sup>(16)</sup> as an indicator of Th1 and Th2 cell function, respectively. IL-6 level, Max-Discovery<sup>TM</sup> ratIL-6 ELISA test kit (catalog # 2203 reference #2202-01) is designed for quantitative determination for the concentration of ratIL-6 in serum, plasma, and cell culture supernatant. Sensitivity: 60 pg/ml.

### **Cortisol level:**

The calbiotech, Inc. (CBI) cortisol ELISA kit is intended for the quantitative

measurement of cortisol in serum or plasma (Catalog No. C0103S) <sup>(17)</sup>.

#### **Phagocytosis and killing assay**

Blood collected in heparin-coated universal bottles from experimental animals were used to prepare leukocytes for bacterial phagocytosis. The heparinized blood samples were treated with 0.83% ammonium chloride to lyse red blood cells, washed three times with cold phosphate buffer solution (PBS) at PH 7.2 and resuspended in minimal essential medium (MEM) with 0.5% inactivated fetal calf serum to give a final concentration of  $10^8$  viable polymorphonuclear leukocytes (PMN) and macrophage cells per ml <sup>(18)</sup>. A strain of coagulase-positive *Staphylococcus aureus* (*S. aureus*) isolated in the laboratory was used. The bacteria were washed in PBS and resuspended in (MEM) to give a final concentration of  $5 \times 10^8$  bacteria per ml.

#### **Assay of phagocytosis**

The mixtures of bacteria (*S. aureus*) and leukocytes were incubated at 37°C for two hours with regular stirring and then the mixtures were centrifuged at 200xg for 5 min. at 4°C. The supernatants were used to estimate the percentage of bacteria phagocytosed using the formula:

Phagocytosis % = (CFU before incubation - CFU after incubation)/CFU before incubation

#### **Assay of bacterial killing**

Samples of MEM containing bacteria (*S. aureus*) alone and containing mixtures of bacteria (*S. aureus*) and leukocytes were incubated at 37°C for two hours. Then the samples were treated with one cycle of freezing with liquid nitrogen and thawing. The number of colony forming units (CFU) was then estimated by the method of Woldehiwet and Rowan <sup>(18)</sup>. The percentage of bacteria killed was estimated according to the formula:

Bacterial killing % = CFU in sample containing bacteria - CFU in sample containing bacteria & leukocyte mixture / CFU in sample containing bacteria alone.

#### **Splenocytes preparation**

The spleen of rats were individually removed and placed in cold culture media (RPMI-1640) containing 100 U/ml penicillin, 100µg/ml, streptomycin, 200 mM Glutamine/100 mM Na pyruvate. Each spleen was gently teased

loose and passed through a stainless steel mesh (40µm pores) to remove cell aggregates and connective tissue. Each suspension was centrifuged at + 4°C for 10 min at 400xg. To lysis the RBCs, the pellet was resuspended in 1ml sterile dist. water (hypotonic solution) for 30 sec, then 40 ml of RPMI medium were immediately added and the suspension was centrifuged at +4°C for 5 min at 400xg. Cells were then washed twice with ice-cold RPMI-1640 and resuspended for counting. Counting and assessment of viability were performed by trypan blue exclusion method <sup>(19)</sup>.

#### **Lymphocytes transformation test using MTT:**

MTT (3, 4, 5- dimethylthiazoyl -2 , 5 - diphenyl tetrazolium bromide) colorimetric analysis was used to measure the proliferation rate of lymphocytes. The effect of the drug on lymphocyte transformation was performed according to Rai-Elblhaa *et al.*, <sup>(20)</sup>. Briefly, heparinized blood samples were taken from all rat groups and layered carefully on the surface of Ficol-hypaque solution in a ratio of 2/1 and centrifuged at 400xg at 4°C for 30 min. to give packed blood cells with granulocytes, interface layer (monocytes and lymphocytes) and upper plasma layer. The interface layer containing monocytes and lymphocytes was carefully aspirated using sterile glass Pasteur pipette, and then placed in sterile tubes containing 2ml RPMI-1640 media. Cells were washed 3 times with RPMI-1640 media by centrifugation at 1800 r.p.m. at 4°C. After the last wash the sediment lymphocytes were resuspended in one ml of RPMI-1640 containing 10% FCS. Lymphocytes were setting in 96 well TC plate (150µl/well) the first row was left free as blank, where in the other wells number of lymphocytes was adjusted to  $1 \times 10^6$ /well. Each well received 50 µl PHA (5µg/ml) as mitogen. The plate was incubated at 37°C, 5% CO<sub>2</sub> for 72h. In humid incubator, MTT solution was added in a ratio of 1/10 of total sample then incubated at 37 °C, 5% CO<sub>2</sub> for 4h. After incubation, lysis buffer was added (50µl/well) over night in incubator then the absorbance was recorded at 470nm. Stimulation index (SI) = mean cpm of triplicate test cultures / mean cpm of corresponding replicate of control rat.

### Statistical analysis:

Data were presented as mean  $\pm$  SE. The statistically significant differences of treated and control animals were evaluated by Student's *t*-test or one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered statistically significant <sup>(21)</sup>.

### RESULTS

#### Effect of DXM administration on body weight

Daily administration of DXM doses for 30 days in the 2<sup>nd</sup> and 3<sup>rd</sup> treated groups in rats caused a non significant difference on body weight during the experimental period when compared to the control group (Fig.1).

#### Effects on cellularity of spleen

Spleen cellularity of both DXM treated groups showed a significant effect ( $P \leq 0.05$ ) versus control rats. It started by decreasing the number of splenocytes after 10 and 20 days in the 2<sup>nd</sup> and 3<sup>rd</sup> treated groups then increasing after 30 days of both treated groups (Fig. 2 ).

#### Effects on mitogen stimulation

The results of PHA (5 $\mu$ g/ml) stimulated lymphocytes of peripheral blood appeared a highly significant suppress ( $P \leq 0.001$ ) of both DXM treated groups in a dose dependence throughout treatment period as compared to control group (Fig. 3).

#### Effects of phagocytosis and killing of *S. aureus* induced polymorphonuclear and macrophage cells in the peripheral blood.

As shown in Figs. (4,5), the results of both DXM treated rats showed a highly significant decrease ( $P \leq 0.01$ ) on phagocytic and killing of *S. aureus* by PMN and macrophage cells versus control animals.

#### Effects on serum IFN- $\gamma$ , IL-10 and IL-6

The results of IL-6 production in serum exhibited a significant decrease ( $P \leq 0.01$ ) throughout the period of administration of both the 2<sup>nd</sup> and 3<sup>rd</sup> treated group as compared to control rats Fig. (6). Moreover, as shown in Fig. (7), the production of IFN- $\gamma$  in serum exhibited a highly significant suppress ( $P \leq 0.01$ ) with increasing doses of both DXM treated rats. While, the administration of DXM induced a highly significant increase ( $P \leq 0.01$ ) in the production of serum IL-10 during the period of treatment (Fig.8).

### The assessment of cortisol production in serum

Daily administration of both DXM treated groups induced a significant decrease ( $P \leq 0.01$ ) in the production of cortisol during the experimental time except the last period of treatment in the 3<sup>rd</sup> group, where, the level of cortisol production gradually return to normal level as the control group (Fig.9).

### DISCUSSION

In the present study, the results showed the effects of DXM on cellular immune response in rats. T cell stimulator, phytohemagglutinin induced suppression effect on lymphocyte proliferation of peripheral blood of rats in the treated groups. These results are in agreement with the findings of other investigators who reported that high doses of DXM can induce psychotropic effects/euphoria <sup>(22)</sup>. Furthermore, psychoactive compounds suppressed splenic NK cell activity and mitogen-induced lymphocyte proliferative responses <sup>(13)</sup>. But other investigators <sup>(23)</sup> who studied that the chronic effect of dextromethorphan (DXM) on the cellular immune responses in mice. T cell stimulator, phytohemagglutinin did not show significant effect on lymphocyte proliferation.

The results of the current study revealed that the administration of DXM with increasing its doses in two treatment groups for 30 days induced suppression in the activity level of phagocytosis and killing of the PMN cells and macrophage cells of blood induced by *S. aureus* bacteria *in vitro*. These results are in line with the findings of other investigators <sup>(13)</sup> who documented that DXM attenuated production of superoxide and intracellular reactive oxygen species in Kupffer cells and neutrophils. Real-time RT-PCR analysis <sup>(24)</sup> revealed also that DXM administration suppressed the expression of a variety of inflammation-related genes such as macrophage inflammatory protein-2, CXC chemokine, thrombospondin-1, intercellular adhesion molecular-1, interleukin-6, the production of tumor necrosis factor-alpha, monocyte chemo-attractant protein-1, and superoxide in macrophage cell culture after stimulation. DXM also decreased the expression of genes related to cell-death pathways, such as the DNA damage protein genes *GADD45* and *GADD153* <sup>(25)</sup>. Moreover, these effects on

cytokines production in dose dependent pattern and duration of the drug administration showed also suppression of mitogen - induced lymphocyte proliferative responses, total number of splenocytes and the activity level of phagocytosis and killing of the PMN cells and macrophage cells of blood induced by *S. aureus* bacteria *in vitro*.

In the present study, treatment of normal rats with two different doses of DXM caused a significant reduction in the level of Th1 cytokine (IFN- $\gamma$ ) and IL-6, but increased in the level of Th2 cytokine (IL-10) in serum. From previous studies, Hu *et al.*,<sup>(26)</sup> who revealed that IFN- $\gamma$  enhances macrophage responses to other inflammatory factors such as toll like reaction (TLR) ligands. TLR activation of macrophages also induces cytokines of the IL-6/IL-12 family that regulate the transition from innate to acquired immunity. While, IL-10 is produced as part of the homeostatic response to inflammation and played a critical role in limiting the duration and intensity of immune and inflammatory reactions<sup>(27)</sup>.

Furthermore, in rats and monkeys, DXM produced discriminate stimuli that generalize to PCP and ketamine as noncompetitive NMDA receptor antagonists<sup>(10)</sup>. Aside, in our previous studied<sup>(28)</sup> treatment of normal rats with ketamine in subanesthetic and lethal doses for 15 days induced significant reduction in the level of Th1 cytokine (IFN- $\gamma$ ) and a non significant change in the level of Th2 (IL-10) in serum in dose dependence pattern.

In harmony with previous studies, DXM produced similar effects as noncompetitive NMDA receptor antagonists. The results of the current study revealed that treatment of normal rats with two different doses of DXM displayed a significant reduction in the level of cortisol in serum. These results are in agreement with other investigators who studied that NMDA receptors are involved in general glutamatergic excitatory action. What makes DXM so interesting is that the behavioral changes caused by varying doses of the drug can be traced directly to proportional amounts of receptor occupation. Sigma<sub>1</sub> receptors are also located throughout the body. Research suggests that sigma<sub>1</sub> receptors can inhibit tumor growth as well as inhibit the immune system<sup>(29)</sup>.

Furthermore, many investigators reported that noncompetitive NMDA antagonists such as PCP, ketamine and DXM produced profound behavioral effects and stimulate the hypothalamo- pituitary-adrenal (HPA) axis in the rat. This response is centrally mediated and not due to direct stimulatory effects at the level of the pituitary or adrenal gland<sup>(9)</sup>.

Moreover, another investigator<sup>(30)</sup> reported that the effect of ketamine (KET) and cocaine (COC) interaction on corticosterone secretion can be explained, in part, by indirect effect of KET. It significantly prevented the stimulatory effect of COC on serum corticosterone concentration presumably via blockade of NMDA receptors. In addition, the NMDA receptor stimulation is essential for corticosterone release induced by COC treatment in rats. Reduction of serum corticosterone concentrations by KET correlates with reversal of COC-induced effects on leucocytes counts, serum IgM, and IL-10 concentrations.

On the other hand, some investigators reported that DXM and dextropran stimulate the HPA axis in the rat; however, acute dose of DXM induced a significant increase in plasma ACTH and cortisol levels after 30 min of the administration of the drug, but both of them non significant change in the plasma after 120 min of acute single dose when compared to control group<sup>(29)</sup>. Some studies are disagreement with the current study. When, in an earlier study, he<sup>(31)</sup> did not find any change in the plasma level of corticosterone after administration dextropran, but the dose used in their study (1 mg/kg) was far lower than the dose that caused a significant increase in the Pechnick and Poland experiment<sup>(29)</sup> (i.e., 30 mg/kg). To our knowledge the present study is the first report to show its effects on the rat, (i.e., 12.5 to 100 mg/kg throughout 30 days).

From the aforementioned studies, it could be concluded that the DXM abuse perturbed cellular immune responses, exhibited potent anti-inflammatory actions, suppressed of leukocytes dependent production of cytokines such as IL-6 and IFN- $\gamma$ . Moreover, its effect on serum cortisol concentration presumably via blockade of NMDA receptors that this is similar to the immunosuppressive effects caused by

ketamine and phencyclidine. Physicians need to be aware of the abuse potential of DXM, as well as the availability of drug information on the internet. Adolescents can download instructions on making illicit substances, as well as purchase the ingredients necessary to create these dangerous products. Finally, physicians need to remember that DXM found as a component of a large number of nonprescription cough and cold remedies, usually in combination with decongestants, antihistamines, and acetaminophen. These other compounds have toxic properties that are independent of dextromethorphan and may require specific treatment.

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