EVALUATION OF THE PROTECTIVE EFFECTS OF LOW DOSE NALTREXONE AGAINST 2,4 TOLUENE DIISOCYANATE INDUCED TOXICITY IN ALBINO RATS

Amin D¹; El Teli³ A²

¹forensic medicine and toxicology, faculty of medicine zagazig university; ²general surgery department faculty of medicine zagazig university

ABSTRACT

Introduction: low-dose naltrexone has been discovered to be a potential therapy for multiple inflammatory conditions. Aim of the Work: This work was performed to demonstrate the inflammatory changes on the lung of adult albino rats sensitized with 2,4 toluene diisocyanate and to assess the possible immunomodulator role of low dose naltrexone supplementation against these changes. Materials and Methods: This study was carried out on 40 adult albino rats for 4 weeks treated 7 days / week. The rats were divided into 4 groups each of 10 rats; Group I (negative control), Group II (positive control): each rat was gavaged orally with naltrexone same dose in group IV. Group III (2,4 toluene diisocyanate); each rat was sensitized intranasally with 5 μL of 10% of 2,4 Toluene Diisocyanate once daily then provoked with 5% 2,4 Toluene Diisocyanate and Group IV (2,4 Toluene Diisocyanate +Naltrexone); each rat was gavaged orally with 10 mg/kg naltrexone after exposure to 2,4 Toluene Diisocyanate in the previous manner. Results: There was a significant increase in the means of the counts of blood eosinophils and neutrophils in group (III) compared with control group (I). Cytokines production in serum and spleen cell cultured in vitro were significantly increased in group (II) compared to control group (I). Upon supplementation with low dose naltrexone combined to sensitized rats there was a significant decrease in the means of counts of blood neutrophil and eosinophil, cytokines production in serum and spleen cultured cell compared with group (III). Microscopic examination of 2,4 Toluene Diisocyanate sensitized group of lung revealed prominent infiltration of airways with numerous eosinophils was observed, and goblet cells were seen in the epithelium of bronchi. Upon supplementation low dose naltrexone to 2,4 Toluene Diisocyanate sensitized rats showed an improvement of the airway and goblet cells were not seen in the epithelium of bronchi. Conclusion: 2,4 Toluene Diisocyanate induced inflammation and sensitization in the lung in rats and with administration of low dose naltrexone modulate the secretion of cytokines protecting lung tissues.

 Recommendation: It is recommended to increase public awareness regarding the health impact of 2,4 Toluene Diisocyanate and the protective role of low dose naltrexone as an immunomodulator.

Key Words: Cytokine; Eosinophil; Immunomodulator; low dose naltrexon; and 2,4 Toluene diisocyanate.

INTRODUCTION

2,4 Toluene diisocyanate (TDI), a low molecular-weight compound mainly used as solvent in paint thinner, contact cement and airplane glue, and used to produce polyurethane foams. Moreover it is used as a recreational inhalant (McKeown, 2015). It is known as chemical sensitizers that cause occupational asthma (Collins et al., 2017), The asthmatic rat model exposed to TDI, showed inflammatory reaction in airway and increased the cytokine profiles in serum and spleen cells (Joo-hee et al., 2017). Naltrexone hydrochloride is an opioid antagonist mainly used as a therapy of opioid and alcohol dependence (Lee et al., 2016). Naltrexone inhibits the mu and the delta opioid receptors (Weerts et al., 2008), abolishing the euphoric effects of alcohol or opioid. It has been suggested that treatment with low-dose naltrexone (LDN) is beneficial for many inflammatory conditions, including Crohn’s disease (Smith et al., 2011), multiple sclerosis (Cre et al., 2010), and fibromyalgia (Younger et al., 2009); (Younger et al., 2013). Berkson et al. (2007) described that low-dose naltrexone used in treatment of tumors.
including lymphoma and pancreatic cancer (Berkson et al., 2009).

Donahue et al. (2011) reported that naltrexone inhibits the non-canonical opioid growth factor receptor, inhibiting cell proliferation. Naltrexone also inhibits the activity of the toll-like receptor (TLR) family, mainly TLR4, in vivo and in vitro signaling in an immune context (Grace et al., 2015).

The aim of this study was to demonstrate the inflammatory changes on the lung of adult albino rats sensitized with 2,4 toluene diisocyanate and to assess the possible immunomodulator role of low dose naltrexone supplementation against these changes..

MATERIAL AND METHODS

I- Material:
1) 2,4 Toluene Diisocyanate: It was obtained from Wako Chemical Co, (Japan). It was provided in a liquid form.
2) Naltrexone hydrochloride: It was obtained from Multi-Apex Pharmaceutical Co, (Egypt). It was provided in a tablet 50mg form.
3) Distilled water: solvent for naltrexone.
4) Ethyl acetate: solvent and vehicle for TDI
5) Animals:
The study was done for 4 weeks and the rats were divided into 4 groups each of 10 rats:-
- This study was carried on 40 adult albino rats, each weighing 150 gm. The animals were obtained from Animal House of the Faculty of Veterinary Medicine, Zagazig University. The study had been designed in the Faculty of Medicine, Zagazig University.

II- Methods:
Study design:
The study was done 4 weeks and the rats were divided into 4 groups each of 10 rats:-
- Group I (negative control group): Each rat received only regular diet, distilled water and ad-libitum to determine the basic values of performance. These rats were left without intervention to measure the basic parameters.
- Group II (positive control group): Each rat gavaged orally with 10 mg/kg body weight naltrexone dissolved in 1 ml of distilled water once daily 7 days/ week.
- Group III (2,4 Toluene Diisocyanate (TDI)):
Each rat was sensitized nasally with 5 μL of 10% TDI dissolved in ethyl acetate for seven consecutive days. After a week of rest, the rats were sensitized again for another week. A week after the second course of sensitization, the rats were provoked by intranasal administration of 5 μL of 5% TDI (Zheng et al., 2001).
- Group IV (2,4 Toluene Diisocyanate +Naltrexone (TDI+NTX)): Each rat was gavaged orally with 10 mg/kg body weight naltrexone dissolved in 1 ml of distilled water once daily 7 days/ week (Rachel et al., 2017) given every day with sensitization by the same protocol mentioned before.

The airway hyperreactivity signs of rats in sensitized groups were appeared for an hour after provocation and noticed then recorded. At the end of 4th week of the study period, rats from each group were subjected to blood sample collection form the retro-orbital plexuses. The blood samples will be used for assessing the following blood (eosinophils & neutrophils) cell count and cytokine assay. Then the anesthetized rats were sacrificed & specimens from the spleen for in vitro cell culture for cytokine assay and from the lung were taken for histopathological study.

A) Total leucocytes & blood eosinophils & neutrophils count and serum preparation (Špela et al., 2009)

Blood collection. Blood samples of 250 μL were collected and microcentrifuged using EDTA containing tubes (Microtainer Brand Tubes, NJ). Samples were put in an automated blood counter (ABC Vet, ABX Diagnostics, Montpelier, France).

B) Flow cytometric analysis (Špela et al., 2009)

Blood samples were diluted (1:2) with PBS. Erythrocytes were lysed by adding 0.5 mL of (OptyLyse, CA). To determine appropriate concentrations of primary and secondary antibodies for samples, antibody titration was performed (International Reagents Corp, Japan).

D) Cytokine assay (Mansell and Jenkins, 2013):
The selected cytokine productions of IL-4, IL-6 and TNFα in serum, spleen cell cultured in vitro
were determined with commercial ELISA kit, as the manufacturer’s instructions. IL-4 and IL-6 kits were from Cosmo Bio Co., LTD. (Tokyo, Japan), and TNFα kit was from Endogen Inc. (MA, USA).

Then the anesthetized rats were sacrificed & specimens from the spleen were taken for cell culture and lung and airway specimens were taken for histopathological study.

**C) Spleen cell culture (Garbers, 2012):**

Spleen was excised and single-cell suspension was obtained by teasing the tissue through a steel mesh into ice cold RPMI 1640. The suspension was treated with 8 g/l Tris ammonium and then centrifuged for 5 min at 4 C. Cell viability was determined by trypan blue. Spleen cells were cultured in a plate for 48 h in a 5% CO2 incubator in the presence of concanavalin A (ConA) (Sigma, St. Louis, USA).

At the end, the supernatant fraction was used for cytokine analysis.

**E) Histopathological study by H & E:**

Lung and trachea were filled intratracheally with buffered formalin. The lung was removed, fixed and embedded in paraffin. 2 Um thickness section was sliced and stained by Haematoxylin and Eosin (H&E) according to Horobin and Bancroft (1998) then examined by light microscope.

**F) Statistical analysis:**

The results were analyzed by Student’s t-test in the control groups and TDI group, TDI+NTX and TDI group.

### RESULTS

**Table (1):** Total leucocytes, eosinophils and neutrophils counts in negative and positive control groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total leucocyte cells Mean ± SD</th>
<th>Eosinophils Mean ± SD</th>
<th>Neutrophils Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control Group</td>
<td>15.50 ±1.11</td>
<td>0.06 ±0.03</td>
<td>1.86 ±0.23</td>
</tr>
<tr>
<td>positive control Group</td>
<td>15.41 ±1.01#</td>
<td>0.05 ±0.02#</td>
<td>1.84 ±0.20#</td>
</tr>
</tbody>
</table>

Each of negative control and positive control groups has 10 rats/4 weeks. Data are expressed as means ± SD. #Non significantly different from negative control group values p>0.05; SD = Standard Deviation.

**Airway hyperreactivity signs:**

After provoked with 5% TDI, the TDI-sensitized rats sneezed. The exertional breathing which was similar to asthma was observed to last 10-20 min in TDI-sensitized rats. While the TDI+NTX group showed only sneezing. The control rats did not manifest airway hyperactivity signs.

**A) Laboratory results:**

The laboratory findings of both negative and positive control groups were within normal values and nonsignificantly different (Tables 1) (Fig 1,2), so we used group (I) for comparison with group (III).

**1) Total leucocytes & blood eosinophils and neutrophils count**

There no significant difference in Total leucocyte count (TLC) between different groups (Table 1,2,3,4).

There were highly significant differences in groups by (Student’s t-test) as regard, eosinophil (ESO) and neutrophils (NEU) counts.

There was highly significant elevation in (eosinophils and neutrophils) level in group (II) when compared with control group (I) (Table 2). There was no significant difference in (eosinophils and neutrophils) value between TDI+NTX group (IV) and control group (I) (Table 3). There was highly significant elevation in (eosinophils and neutrophils) level in group (III) when compared with TDI+NTX group (IV) (Table 4).
### Table (2): Total leucocytes, eosinophils and neutrophils counts in negative control and TDI sensitized groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total leucocyte cells Mean ± SD</th>
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</tr>
<tr>
<td>TDI Group</td>
<td>16.20 ±1.01#</td>
<td>0.42 ±0.11**</td>
<td>2.10 ±0.19**</td>
</tr>
</tbody>
</table>

Each of negative control and TDI-sensitized groups has 10 rats/4weeks. Data are means ± SD.

** highly significant different from negative control group values p<0.05.
# non significantly different; SD = Standard Deviation; TDI= Toluene diisocyanate

### Table (3): Total leucocytes, eosinophils and neutrophils counts in negative control and TDI+ NTX treated groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total leucocyte cells Mean ± SD</th>
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</tr>
<tr>
<td>TDI+NTX Group</td>
<td>15.10 ±1.13#</td>
<td>0.07 ±0.02#</td>
<td>1.66 ±0.20#</td>
</tr>
</tbody>
</table>

Each of negative control and TDI+NTX treated groups has 10 rats/4weeks. Data are means ± SD.

# Non significantly different from negative control values p>0.05 ; SD = Standard Deviation.
TDI= Toluene diisocyanate; NTX=Naltrexone

### Table (4): Total leucocytes, eosinophils and neutrophils counts in TDI sensitized and TDI+ NTX treated groups.

<table>
<thead>
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<th>Group</th>
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Each of TDI+NTX treated and TDI-sensitized groups has 10 rats/4weeks. Data are means ± SD.

** highly significant different from TDI+NTX treated group p<0.05; # non significantly different; SD = Standard Deviation.; TDI= Toluene diisocyanate; NTX=Naltrexone
(2) Cytokine production in serum
There were highly significant differences in groups by (Student’s t-test) as regard (IL-4, IL-6 and TNFα) values (Fig. 1).
There was highly significant elevation in (IL-4, IL-6 and TNFα) level in group (III) when compared with control group (I). There was no significant difference in (IL-4, IL-6 and TNFα) value between TDI+NTX group (IV) and control group (I) and there was highly significant elevation in (IL-4, IL-6 and TNFα) level in group (III) when compared with TDI+NTX group (IV) (Fig. 1).

(3) Cytokine production in spleen cell
There were highly significant differences in groups by (Student’s t-test) study as regard (IL-4, IL-6 and TNFα) values (Table 1).
There was highly significant elevation in (IL-4, IL-6 and TNFα) level in group (III) when compared with control group (I). There was no significant difference in (IL-4, IL-6 and TNFα) value between TDI+NTX group (IV) and control group (I) and there was highly significant elevation in (IL-4, IL-6 and TNFα) level in group (III) when compared with TDI+NTX group (IV) (Fig. 2).

Fig. (1): Serum Cytokine concentrations in group 1 (negative control), group 2 (positive control), group 3 (TDI sensitized rats) and group 4 (NTX+ TDI treated rats) for 4 weeks of treatment adult albino rats.

Fig. (2): Spleen Cytokine concentrations in group 1 (negative control), group 2 (positive control), group 3 (TDI sensitized rats) and group 4 (NTX+ TDI treated rats) for 4 weeks of treatment adult albino rats.
(B) Histopathological study:

(1) Histopathological changes of the lung:
Light microscopic examination of H&E stained sections from the lung of the control groups (groups I & II) were nearly similar and revealed that; the lung showed no pathologic change was found in airways (Fig. 3).
Examination of H&E stained sections of the lung specimens of the rat (group II) showed a prominent infiltration of central airway and peripheral airway with numerous eosinophils were observed, and goblet cells were seen in the epithelium of the central bronchi (Fig. 4). Examination of H&E stained sections of the lung specimens of the rat (group IV) showed improvement of the changes that occurred after TDI sensitization with little infiltration of airways with little eosinophils was observed, and no goblet cells were seen in the epithelium of the central bronchi (Fig. 5).

Fig. (3): A photomicrograph of a section in the lung of adult albino rat from the negative control group showing normal lung section. Normal alveolar spaces (A) or terminal bronchile (TB) are seen. Stained with hematoxylin-eosin, light microscopy; a) magnification × 100.

Fig. (4): A photomicrograph of a section in the lung of adult albino rat from the TDI-sensitized showing small airways with epithelial hyperplasia and mucous plug (black rectagle) prominent smooth muscle hyperplasia (asterisks), thickened basement membrane (arrowheads) and both central airways and peripheral airways are infiltrated by eosinophils (large arrows) in a TDI-sensitized rat. A large number of goblet cells (arrows) are seen in the epithelium of central bronchi of TDI-sensitized rat. Stained with hematoxylin-eosin, light microscopy; a) magnification × 100. B) magnification × 400.
DISCUSSION
Toluene diisocyanate is a potent sensitizer that causes occupational bronchial asthma in exposed individuals through inflammatory mechanism (Lemons et al., 2014). Toll like receptors (TLR) are powerful agents within the immune system. Intracellular TLRs have been targets for the therapy of inflammatory diseases and tumor (Rahmani and Rezaei, 2016). Naltrexone inhibit TLR producing potential immunomodulator against inflammatory disease (Jiang et al., 2013).

In the present study, there was a significant increase in blood cells (ESO and NEU) mean values in TDI sensitized group as compared to their corresponding values in control groups. These results confirmed by notifiable histopathological changes in the general architecture of the lung stained with H&E associated accumulation of eosinophils was in central airways and peripheral airways.

These results are in agreement with liang et al. (2015) who stated that the local inflammatory effect of TDI on bronchi is the main cause of allergic asthma in exposed patients. Also reported in their experimental study on rats increasing in the number of circulating eosinophils and neutrophils, which also indicated an inflammatory reaction in the sensitized rats.

Carreras et al. (2015) reported that airway inflammation plays an important role in the pathogenesis of asthma is a type of cell-mediated immune response in which specialized populations of activated lymphocytes interact with other inflammatory cells through cytokines.

Eosinophil infiltration is damaging to bronchial mucosa and the main histopathologic feature of asthma. Studies have demonstrated that the most of these infiltrated eosinophils are activated which secret eosinophil cationic protein (ECP) and major basic protein (MBP) leaving underlying smooth muscle more vulnerable to nonspecific contractile mediators, or perhaps by contributing to the airway remodeling and narrowing (Plager et al., 2009).

In the present study, IL-4 and IL-6 and TNF were predominately secreted in serum, in vivo and in vitro of cultured spleen cell in TDI-sensitized rats.

These results were in agreement with the findings reported by Ogawa et al. (2006) cytokines play an important role in airway inflammation and in immune reaction in animals exposed to TDI. IL-4 is required for the starting of Th2 cells promoting the production of IgE antibodies which producing hypersensitivity reactions associated with respiratory allergy.

IL-6 has been reported activate T-cell activation and immunoglobulin production by B cells, and thus it enhances IL-4-dependent IgE synthesis (Kim et al., 2011).

Ye et al. (2016) reported that T helper cell (Th2) type associated cytokines IL-4 and IL-6.
were preferentially produced in lymph node cells.

To the contrary, in a study conducted by Sato et al. (2009) the production of IL-2, IL-4, IL-6 in serum between TDI-sensitized and control mice were not different as shown in his study.

In the present study, TDI induced a prominent infiltration of central airway and peripheral airway with numerous eosinophils and goblet cells were seen in the epithelium of the central bronchi.

These results were in agreement with Andrea et al. (2016) who reported that TDI sensitization increased eosinophil, goblet cells in the respiratory airways and lung tissue by histological examination.

In the present study, upon the administration of low dose naltrexone in group (IV) result in improvement of the blood cells counts (NUT and EOS) and inhibit the production of cytokines in serum and cultured spleen cell a significant improvement in histopathological changes; almost normal lung section when compared with TDI sensitized treated group (III).

These results also reported by Hutchinson et al. (2008) who stated that monocytes activated in vitro with ligands for the intracellular receptors TLR7, TLR8, and TLR9 and treated with LDN showed that naltrexone inhibited IL-6 production stimulated by TLR7/8 ligands and TNFα production in response to TLR9 ligand. No cell damage and it was not the cause of decrease in cytokine secretion as no significant effects on cell numbers or expression of apoptotic markers was observed.

Naltrexone inhibits TLR4 activity in vitro in microglial cells focusing on production of IL-6, a main cytokine produced following TLR stimulation that inhibit IL-6 production following stimulation with ligands for TLR7/8 and TLR9 via the MyD88 pathway that inhibited by naltrexone (Kanzler et al., 2007).

The effect on TLR activity justify the use of naltrexone for the treatment of inflammatory conditions as in psoriasis (Pinto et al., 2011). Members of the TLR family, including TLR9, are expressed in tumors (Sandholm et al. 2012). Similarly, expression of TLR9 has been correlated with the invasive and metastatic potential of pancreatic carcinoma (Wu et al., 2011).

Naltrexone inhibits TLR-mediated inflammatory effects in other cell types such as mucosal epithelial cells (Gribar et al., 2008), and whether exposure to naltrexone results in upregulation of TLR in a similar manner to that seen for its opioid receptor targets (McLaughlin et al., 2012) and (Bigliardi et al., 2007). It is important to note that the inflammatory diseases and cancer have adopted a LDN regime as opposed to the dosages used in the treatment of opioid and alcohol dependency. Nanomolar doses of naltrexone were previously seen in studies by Liu et al. (2016) to result in upregulation of pro-apoptotic genes, rendering tumor cells more susceptible to chemotherapy.

CONCLUSIONS

Improvement of health education programs for the purpose of increasing public awareness regarding the health impact of TDI Administration of LDN may be of immense prophylactic and therapeutic values in exposed individuals. More research is needed before the treatment approach can be widely recommended. We expect the active immune modulator to be area of interest in future researches.

REFERENCES


Evaluation of The Protective Effects of Low......

Title: Evaluation of The Protective Effects of Low Dose Diazepam on the Protective Effect Against the First Shocking Organisms of the Narceusis

Abstract: This study aimed to evaluate the protective effects of low-dose diazepam on the protective effect against the first shocking organisms of the Narceusis. The study was conducted on 45 adult male rats, divided into three groups: Group A received low-dose diazepam, Group B received the standard dose, and Group C received no treatment. The results showed that low-dose diazepam significantly increased the protective effect against the first shocking organisms of the Narceusis compared to the standard dose and the control group. The study concluded that low-dose diazepam is a promising candidate for further research in the field of neuroprotection.

Key words: Diazepam, Protective effect, First shocking organisms, Narceusis.