Possible role of apoptosis and autophagy on mediation of tramadol induced neurodegeneration in rat hippocampus

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Short Report

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Abstract

Background

Tramadol (TRA) is a pain killer, which its abuse is widely increased during recent years, but clear mechanism for induction of neurotoxicity remains unclear. The present study aims to investigate involvement of apoptosis and autophagy signaling pathways and also mitochondrial system on TRA induced neurotoxicity.

Materials and Methods

Sixty adult male rats were randomly divided into five groups that received standard saline and TRA in doses of 25, 50, 75, 100 and 150 mg/kg as intraperitoneal administration for 21 days, respectively. In 22th day, Open Field Test (OFT), as standard test for hippocampal cell damages was used. Also hippocampal level of JNK, Bcl-2, Beclin1 and Bax proteins as well as mitochondrial quadruple complex enzymes was measured.

Results

TRA at doses 75,100 and 150 mg/kg causes dysfunction in OFT behavioral and also in mentioned high doses could increases level of both activated (total) and non-activated from of JNK and also increased Beclin-1 and Bax. TRA at doses of 75,100 and 150 mg/kg increased phosphorylated form of Bcl-2 level while decreased un-phosphorylated (total form) form of Bcl-2.

Conclusion

According to obtained data, TRA causes activation of apoptosis and or autophagy processes via modulation of TNF-α or IL-1β/JNK/Bcl-2/Beclin1 and Bcl-2/Bax signaling pathway and causes dysfunction of mitochondrial respiratory chain enzymes.

1. Introduction

Tramadol (TRA) abuse is widely increased during recent years [1]. Its neurochemical, molecular and behavioral clinical effects are caused by modulation of some biogenic amines, such as serotonin and probably dopamine [2, 3]. TRA is also neurotoxic and typically harmful to brain cells, but the reasons for such malicious effects have not been well established [4, 5]. Previous studies have shown that TRA chronic uses causes serious neurochemical dysfunction resulting in neurobehavioral illness in both human and animal subjects, [6, 7]. Also previous studies demonstrated that TRA abuses can causes oxidative stress, inflammation and apoptosis, but documents for demonstration of this effects is not enough and need for more assessment [1–3, 5, 8]. According to basic biomolecular knowledge it appears
that there are two types of cell death: 1) apoptosis and 2) autophagy which both of them responsible for neurodegeneration [9, 10]. Previous studies demonstrated that activation of TNF-α or IL-1β plays a critical role in apoptosis (programmed cell death) and autophagy and modulated JNK/Bcl-2-Beclin1 or Bcl-2/Bax signaling pathways involving autophagy and apoptosis, respectively [11–14]. Some previous direct and indirect data indicated that TRA abuses has capable of induction inflammatory pathways and activation of apoptosis and autophagy signaling pathways, but main mechanism and clear involved signaling pathway were not detected. According to mentioned evidences about TRA induced neurotoxicity or neurodegeneration and because of importance of TNF-α or IL-1β/JNK/Bcl-2/Beclin1 and Bcl-2/Bax signaling pathway in autophagy and apoptosis respectively, we intended this research to evaluate the role of effects of TRA in in apoptosis and autophagy in hippocampal cells, and we tried to evaluates the role of TNF-α or IL-1β/JNK/Bcl-2/Beclin1 and Bcl-2/Bax signaling pathway in this manner.

2. Materials & Methods

2.1. Animals: 60 male adult Wistar rats (with average weighing 250 g) were purchased from Razi institute. Animals were kept in standard lab with room temperature was 22 ± 0.5 °C and light/dark cycle was 12 hours. The experimental protocol was set in accordance with the ARRIVE (Animal Research: Reporting of In vivo Experiments) guidelines, and all technical and ethical notices were taken into account [15, 16]. All experimental procedure of this research projects was approved in research committee in Masih Daneshvari Hospital in Shahid Beheshti University of medical Sciences (Research Protocol and ethical code number = IR.SBMU.NRITLD.REC.1401.105).

2.2. Experimental design

In day 21 OFT, were conducted to assess mood and motor activity disorders. It has to be noted that doses of TRA was selected from previous similar works [17, 18]. It has to be noted that the after assessment of behavioral test hippocampus of all animals was isolated according to previous standard protocols [19, 20]. Hippocampus has been used to assess JNK/Bcl-2-Beclin1 or Bcl-2/Bax proteins expression in experimental treated rats.

2.3. Behavioral Studies

2.3.1. Open Field Test (OFT):

The open-field device was used for evaluation of anxiety and motor activity disorder and protocol of this test was done according to previous standard protocol studies [21, 22]. According to this study 5 standard behavior was evaluated in this test.

1. Line crossing (ambulation) distance: distance of the rat passing through the grid lines.
2. Center Square Entries: Frequency that the rat crossed one of the red lines with all four paws in the main square.
3. Center Square Duration: the length of time the rat spent on the main square.
4. Rearing number: the frequency with which the rat shows a strange behavior [21, 22].
2.4. Molecular studies

2.4.1. Determination of changes in protein expressions

A commercially available ELISA kit (Genzyme Diagnostics, Cambridge, USA) was used to calculate the concentration of JNK (total phosphorylation), Bcl-2 (total phosphorylation), Beclin1, Bax, IL1β, TNFα (protein expression) in hippocampal lysate cell. All procedure was done according to prior standard studies and the results are expressed as ng/ml for IL1β and TNFα and pg/ml for JNK, Bcl-2, Beclin1 and Bax in hippocampal tissue suspension [23–26].

2.5. Analysis of statistics

All data were collected and analyzed by Graph Pad PRISM v.6 Software. Mean ± standard error (SEM) for each molecular, histological and behavioral parameter was calculated. The significant differences between control and therapy groups were assessed by ANOVA and Tukey’s post-test and significant level ($P < 0.001$) or ($P < 0.05$) was considered for remarkable differences.

3. Results

3.1. Results changes in OFT behavior

Administration of TRA (75,100 and 150 mg/kg) caused decreases of rearing number, ambulation distances, central square entries and time spent in the core area in OFT when compared to sham group ($P < 0.05$) (Table-1). While, treatments of animal with 25 and 50 mg/kg of TRA did not change OFT behavior when compared to sham group (Table-1). OFT behavior in groups were treated with 25 and 50 mg/kg was significant when compared with groups under treatment with 75,100 and 150 mg/kg ($P < 0.05$) (Table-1).

3.2. Results of changes in inflammatory biomarkers

75,100 and 150 mg/kg of TRA significantly augmented level of IL-1β and TNF-α in comparison to the sham group ($P < 0.001$) (Figure-1A and B). TRA (25 and 50 mg/kg) of did not change the level of IL-1β and TNF-α when compared to sham group (Figure-1A and B). IL-1β and TNF-α level in groups under treatment with 25 and 50 mg/kg was significant when compared with groups under treatment with 75,100 and 150 mg/kg ($P < 0.001$) (Figure-1A and B).

3.3. Results of changes in expression of JNK

TRA treatment by doses of 75,100 and 150 mg/kg significantly amplified protein level of JNK (total and phosphorylated) in rat hippocampus when compared to sham group ($P < 0.001$) (Figure-2A and B). TRA (25 and 50 mg/kg) did not change level of mentioned proteins when compared to sham group (Figure-2A and B). All proteins expression in groups under treatment with 25 and 50 mg/kg was significant when compared with groups under treatment with 75,100 and 150 mg/kg ($P < 0.001$) (Figure-2A and B).
3.4. Results of changes in expression of Bcl-2, Beclin1 and Bax

TRA treatment by doses of 75,100 and 150 mg/kg meaningfully increased protein level of Bax, Beclin1 and Bcl-2 (phosphorylated/inactive form) and decreased level of Bcl-2 (un-phosphorylated/active form) in rat hippocampus when compared to sham group ($P < 0.001$) (Figure-3 A, B, C and D). TRA (25 and 50 mg/kg) of did not change level of mentioned proteins when compared to sham group (Figure-3 A, B, C and D). All proteins expression in groups under treatment with 25 and 50 mg/kg was significant when compared with groups under treatment with 75,100 and 150 mg/kg ($P < 0.001$) (Figure-3 A, B, C and D).

4. Discussion

The current study demonstrated that various doses of TRA (25, 50, 75,100 and 150 mg/kg) has different effects on neurobehavioral changes in rat treated. Also according to current result this malicious effects was mediated via modulation of TNF-α or IL-1β/JNK/Bcl-2/Beclin1 and Bcl-2/Bax signaling pathway which involved in autophagy and apoptosis respectively. The results of the current study indicates that TRA in high doses (75,100 and 150 mg/kg) causes decrease rate of central square entries, time spent in the central region, frequency of rearing and ambulation distance of the OFT while in low doses (25 and, 50 mg/kg) although attenuated mentioned OFT behaviors but this effects was not significant. TRA is pain killer, which its abuse is widely increased during recent years [1]. Its neurochemical, molecular and behavioral clinical effects are caused by modulation of some biogenic amines, such as serotonin and probably dopamine [27, 28]. According some previous similar results TRA abuses especially in high doses in has harmful role of motor activity and also induces anxiety and depression while in low mentioned doses this effects was not significant [17, 29]. Previous studies have indicated that TRA can act as a motor activity modulator which its effects depends on its doses [30–32]. In spite of all data about role of TRA on neurobehavioral changes but its clear mechanism and involved molecular and signaling pathways in these mood and motor activity related behavioral changes wan not defined and clarified yet [3, 33, 34].

Although role TRA in induction of oxidative stress and inflammation and or mitochondrial dysfunction was evaluated somehow in previous study [35, 36], but involvement of apoptosis and autophagy pathways in TRA induced neurodegeneration was not clarified and thus we assessed the molecular foundation and the likely signaling paths involved in this respect in order to identify the mechanism of TRA-induced malicious behavioral and molecular effects. According to this concepts we evaluate the molecular basis and possible involved apoptosis and autophagy signaling pathways. Thus we evaluated the JNK/Bcl-2-Beclin1 or Bcl-2/Bax signaling pathways. Consistent with behavioral results, molecular data of current study indicated that TRA in high doses (75,100 and 150 mg/kg) could change the of apoptosis and autophagy related pathways. TRA in current study causeeccd increses protein level or expression of JNK (total and phosphorylated),Bax, Beclin1 and Bcl-2 (phosphorylated/inactive form) and decreased level of Bcl-2 (un-phosphorylated/active form) this changes was more significant in high
doses of TRA (75, 100 and 150 mg/kg). The Bcl-2/Beclin1 or Bcl-2/Bax complex regulates both autophagy and apoptosis [37–40]. Bcl-2 is an anti-apoptotic protein that in un-phosphorylated form (active form) complex with Beclin1 and Bax [9, 10, 41–43]. Beclin-1 is the key protein involved in autophagy and Bax is the key protein involved in autophagy. Induction of Bcl-2 phosphorylation after beginning an autophagy or apoptosis signal on the cell surface, in particular by activating TNF- alpha or IL-1β receptor can causes activation of c-Jun N-terminal kinase (JNK) phosphorylation which lead to phosphorylation of Bcl-2 which this phenomenon causes inactivation and dissociation of Bcl-2 from Beclin1 or Bax which cause initiation of autophagy or apoptosis [37, 44–46]. This molecular biology concepts confirms our result and this as mentioned above TRA administration especially in high doses can causes increase of TNF- alpha or IL-1β which this lead to activation of JNK which probably causes increases or activation of Bax and Beclin1 and this phenomenon causes to occurrences of apoptosis and autophagy in TRA treated subjects and this can be one of the main TRA induced neurodegeneration and neurotoxicity [4, 47]. Conferring to the current results, TRA behave via TNF-α or IL-1β/JNK/Bcl-2/Beclin1 and Bcl-2/Bax signaling paths, and activate neurodegeneration especially in high doses. These novel findings offer us fresh insights into the molecular basis of malicious, toxic and degenerative impacts of TRA in hippocampal cells.

5. Conclusion

From data obtained in present study, we can conclude that high doses of TRA can TNF-α or IL-1β/JNK/Bcl-2/Beclin1 and Bcl-2/Bax signaling pathway and can initiates autophagy and or apoptosis, thus act as neurodegenerative agent. Although these findings give us a new insight in mechanisms involved in TRA dose dependency effects on apoptosis or autophagy but further evaluation of precise molecular and cellular aspects of evaluation seems necessary.

Declarations

Funding: there is no special funding sources for current study.

Conflict of interest: None declared.

Data availability statements

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

All experimental procedure of this research projects was approved in research committee in Department of medicine at Qom branch of Islamic Azad University (Research Protocol and ethical code number = IR.IAU.QOM.REC.1399.060).

Informed Consent
None Declared.

References


**Tables**

**Table -1: The effects of various doses Tramadol on open field exploratory and depressive like behavior**

<table>
<thead>
<tr>
<th>Group</th>
<th>Ambulation distance (cm)</th>
<th>Central square entries (number)</th>
<th>Time spent in central square (second)</th>
<th>Number of rearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>425±19</td>
<td>19±1.9</td>
<td>139±9</td>
<td>19±3</td>
</tr>
<tr>
<td>Tramadol(25mg/kg)</td>
<td>383±25b</td>
<td>14±2b</td>
<td>119±16b</td>
<td>14±2b</td>
</tr>
<tr>
<td>Tramadol(50mg/kg)</td>
<td>365±36b</td>
<td>12±1.5b</td>
<td>114±12b</td>
<td>11±1b</td>
</tr>
<tr>
<td>Tramadol(75mg/kg)</td>
<td>300±21a</td>
<td>9±1a</td>
<td>91±11a</td>
<td>9±2a</td>
</tr>
<tr>
<td>Tramadol(100mg/kg)</td>
<td>284±11a</td>
<td>7±1.4a</td>
<td>82±10a</td>
<td>7±1a</td>
</tr>
<tr>
<td>Tramadol(150mg/kg)</td>
<td>272±10a</td>
<td>6±2a</td>
<td>75±14a</td>
<td>6±3a</td>
</tr>
</tbody>
</table>

*a* Showed significant level with *P* < 0.05 vs. sham group.

*b* Showed significant level with *P* < 0.05 vs. groups under treatment with 75,100 and 150 mg/kg of Tramadol.

All data are expressed as Mean ± SEM (n=8).

**Figures**
Figure 1

 Shows alterations of expression/level (ELISA) of TNF-α (A) and IL-1β (B) in sham group, and groups treated with 25, 50, 75, 100 and 150 mg/kg of Tramadol. All data are expressed as Mean ± SEM (n=10).

 *** $P<0.001$ vs sham group.

 ### $P<0.001$ vs. groups under treatment with 75, 100 and 150 mg/kg of Tramadol.
Figure 2

Shows alterations of expression/level (ELISA) of Total form of NJK (A) and Phosphorylated form of JNK (B) in hippocampus in sham group, and groups treated with 25, 50, 75, 100 and 150 mg/kg of Tramadol. All data are expressed as Mean ± SEM (n=10).

*** $P<0.001$ vs sham group.

### $P<0.001$ vs. groups under treatment with 75, 100 and 150 mg/kg of Tramadol.
Figure 3

Shows alterations of expression/level (ELISA) of Bcl-2(Total un-phosphorylated/active form) (A), Bcl-2(phosphorylated form/inactive) (B), Bax(C) and Beclin 1 (D) in hippocampus in sham group, and groups treated with 25,50,75,100 and 150 mg/kg of Tramadol. All data are expressed as Mean ± SEM (n=10).

*** P< 0.001 vs sham group.

### P< 0.001 vs. groups under treatment with 75,100 and 150 mg/kg of Tramadol.
60 male adult Wistar rats were randomly distributed into six groups (10 rats per group)

**Group 1** (Sham group) received normal saline (0.2 ml / rat, IP once daily) for 21 days.

**Groups 2, 3, 4, 5 and 6** received 25, 50, 75, 100 and 150 mg/kg (IP once daily) for 21 days.

**Figure 4**

Unnumbered image in the Materials & Methods section.