Acute Toxicity and Dependence of Tramadol in Albino Rats: Relationship of Nestin and Notch 1 as Stem Cell Markers

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Abstract: Tramadol, a synthetic, centrally acting analgesic is used worldwide. It is devoid of many serious adverse effects of traditional opioids. However, recently, abuse and dependence as well as toxicity and tramadol-related deaths have been increasingly reported. The researches that focus on the potential applications of stem cells (SCs), in drug screening and toxicology tests attract our attention to assess the expression of both Nestin and Notch 1 as stem cell markers in response to acute toxicity and dependence of tramadol in brain, lung and liver of albino rats. Our study includes sixty adult male albino rats were divided into three groups. Group I (control), group II (tramadol acute toxicity) and group III (tramadol dependent). Observed behavioral changes and manifestations were recorded. Surviving rats were sacrificed and autopsy was performed for all rats. Immunohistochemical (IHC) staining of both Nestin and Notch 1 in lung, brain and liver were carried out. **Results:** A significant direct correlation was observed between Nestin and Notch 1 scoring and localization in different studied groups (r = +0.95, *P* value <0.001). Both stem cell markers showed higher significant values in dependant group than the acute toxicity one (*P*<0.001). Additionally, highly significant relationship between expression of both Nestin and Notch 1 and the histopathological changes in brain, lung and liver whereas they are higher in dependence group related histopathologic changes than the acute toxicity group related histopathological changes. Apoptotic index in brain showed an inverse correlation with both Nestin H score and Notch 1 H score (r=-0.94, r=-0.88 respectively and *P*<0.001 for both). Therefore we conclude that stem cell markers are the main modulators of life saving as they re-expressed early in response to cell injury by toxicity and late in maintenance of cellular regeneration by playing crucial roles throughout the journey. Activation of Nestin and Notch 1 signaling in both acute and chronic tramadol toxicity groups might provide a molecular basis for potential protective and treatment strategies.
[Rehab M. Samaka, Naira Fahmi Girgis and Tahany M. Shams. **Acute Toxicity and Dependence of Tramadol in Albino Rats: Relationship of Nestin and Notch 1 as Stem Cell Markers.** J Am Sci 2012;8(6):313-327]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 39

Key words: Tramadol, stem cells, acute toxicity, LD_{50}, dependence, Nestin, Notch 1, IHC

1. Introduction

Nowadays addiction is an ever-increasing problem in the world and despite all efforts to prevent and control it, it continues to be a tremendous public health issue. Analgesics are among the most popular drugs which are being abused. [(1)] Tramadol is a synthetic, centrally acting analgesic, available in Europe since 1977 and in the United States since 1995 for the treatment of pain syndromes previously amenable only to the opiate analogues. [(2)] It has dual mode of action. Its analgesic efficacy is attributed to its partial affinity for the mu-opiate receptor and its inhibition of nor-epinephrine and serotonin reuptake. [(3)] Tramadol is considered a safe drug devoid of many serious adverse effects of traditional opioids. However, recently, abuse and dependence of tramadol as well as toxicity and tramadol-related deaths have been increasingly reported. [(4)]

Tramadol is rapidly absorbed orally; a peak concentration is detected 2-3 hours post oral dose. It has extensive tissue distribution. Thirty per cent of the drug is excreted through the kidneys in an unchanged manner. Elimination half-life is 5–6 hours, while the remaining is metabolized in liver by N- and O-demethylation, followed by conjugation with glucuronic acid and sulphate. The active metabolite, o-desmethyl tramadol shows higher affinity for the m-opioid receptors and has twice the analgesic potency of the parent drug. [(5)]

Stem cells (SCs) have unique properties such as self-renewal, plasticity to generate various cell types and availability of cells of human origin. The characteristics are attentive in the toxicity screening against chemical toxicants and to predict and avoid toxicity in humans. [(6)] Recently the researches focus on the potential applications of SCs, in drug screening and toxicology tests. [(7)] Several stem and progenitor cell populations have already been described as valuable tools for developing therapeutic strategies in regenerative medicine. [(7)]

NESTIN, a class VI intermediate filament, was originally described as a neuronal stem/progenitor cell marker during CNS development, [(8)] shows a wider range of expression than previously thought. [(9)] The expression of Nestin is generally ceased in mature cells, but resumes following injuries. [(10)] Regarding its functions the re-expression of Nestin appears to be

responsible for the promotion of cell proliferation during the post-injury phase. [11]

Initially Notch 1 discovered for yielding a 'notched' wing phenotype in Drosophila due to a partial loss of function. [12] Notch 1 signaling coordinates a wide range of fundamental processes and cellular programs including proliferation, apoptosis, migration, growth, and differentiation depending on the cellular context, as Notch 1 pathway including gene expression and cross talk with other signaling system. [13]

Research discoveries in the fields of stem cell biology and regenerative medicine are beginning to advance and refine an understanding of injury and repair. [14]

Aim of the current study was to demonstrate the role of Nestin and Notch 1 as stem cell markers in response to acute toxicity and dependence of tramadol in brain, lung and liver of albino rats and also study the interrelationship between them.

2. Material and Methods:

Drug:

Commercially available Tramal capsules. Each capsule contains 50 mg tramadol hydrochloride. (Tramal®, manufactured by Minapharm, Hilupollis, Cairo, Egypt under licence of German Grunenthal Company).

Animals and Experimental Design:

Sixty adult male albino rats of an average weight (150-200 grams) were selected randomly. They were kept under good hygienic conditions. They were fed on ordinary food with free access water and housed under standard laboratory conditions. The general condition and behavior of rats were noticed.

Animal experimentations were carried out in an ethical manner following guide lines set by the Ethical Committee of Menoufyia University. The animals were divided into three main groups of twenty rats for each group as follows:

Group I: control group "A" and "B"

Control group A: (10 rats), each animal received 1ml/day normal saline (0.9%) orally by gavage and were kept for the same period as the rats of group II.

Control group B: (10 rats) each animal received 1ml/day normal saline 0.9% orally by gavage. They were kept throughout the experiment under the same conditions for one month as the rats of group III.

Group II (Tramadol acute toxicity group 20 rats): Each animal received a single dose of LD_{50} of tramadol hydrochloride orally by gavage. It equals 300mg/kg b.wt.. [15] The rats were observed for acute toxicity manifestations as well as for LD_{50} deaths.

Group III (Tramadol Dependent group 20 rats): Each animal received tramadol hydrochloride in gradually increasing doses until it reached the dependent dose in one month. Dependence was induced by giving the therapeutic dose of tramadol hydrochloride which was calculated according to Paget's equation. [16] The therapeutic dose for rat weighting 200 gm = 18/1000 x adult human therapeutic daily dose (400 mg) [5] = 07.2 mg. Then the dose was gradually increased by adding the initial calculated therapeutic dose every three days till the end of the month.

The calculated tramadol hydrochloride doses were delivered in normal saline and given orally to each animal by a curved needle –like oral tube that was introduced directly into stomach (a gavage process). At the end of the experiment, surviving animals of the all groups were sacrificed by cervical dislocation at 24 hours after a single dose for group II (acute toxicity group) and their control group IA and the last dose at the end of the month for group III (dependent group) and their control group IB. After death, autopsy was carried out for all animals of the all groups. The brain, lung and liver were fixed in 10% formalin solution.

Histopathological Examination:

The selected organs for each mouse were received at the Pathology Department, Faculty of Medicine; Menoufyia University preserved in 10% formalin solution and dehydrated in a graded alcohol series. After xylene treatment, the specimens were embedded in paraffin blocks. Five-micron thick sections were cut and stained with hematoxylin and eosin (H&E).

H&E stained sections were examined to study the histopathological changes. Microscopic examinations were carried out in all selected organs of tramadol acute and dependant groups. Unintentional bias was prevented by coding rats' tissue samples.

Lung sections were assessed for the followings; a) ***Interstitial changes*** including; congestion, edema, hemorrhage, fibrin deposition, fibrosis, inflammatory cellular infiltrate and its type, b) ***Alveolar changes*** including: intra-alveolar edema, diffuse alveolar damage, hyaline membrane, alveolar wall thickness, alveolar wall destruction and collapse.

Brain sections were assessed for the followings: a) ***Neuoral changes*** including; red neuron, neuronal degeneration, b) ***Glial changes*** including; gliosis, microglial proliferation, oligodendrocyte proliferation, Rosenthal fiber, c) ***Others;*** congestion, edema, inflammatory cellular infiltrates and apoptotic count. [17]

Liver sections were assessed for the followings; a) ***Paranchymal changes*** including steatosis, feathery degeneration, necrosis, congestion, haemorrhage, interface hepatitis, Kupffer cell hyperplasia and sinusoidal dilation, b) ***Portal changes*** including inflammatory cellular infiltrates, fibrosis and bile duct proliferation.

Immunohistochemical (IHC) staining and examination:

IHC staining was performed on formalin fixed, paraffin embedded material that were sectioned at 5 µm thickness and placed onto positive charged slides. Nestin; rabbit polyclonal (aa254-270) antibody,

concentrated one (1 mg/ml) diluted by phosphate buffer saline (PBS) in a dilution of 1:100 (Catalogue no. LS-C40764, Lifespan, Biosciences, USA) and Notch 1, rabbit polyclonal antibody (Catalogue No. LS-B1930, Lifespan, Biosciences, USA) 0.2 mg/ml IHC staining were performed using the Universal Dako cytomation Labelled stretavidin–Biotin_2 system, Horseradish Peroxidase (LSAB_2 System, HRP Kit, Catalogue No. k0679). All slides were de-paraffinized using xylene and then dehydrated in decreasing concentrations of ethanol. Antigen retrieval using microwave heating (20 min; 10 mmol/citrate buffer, pH 6.0) after inhibition of endogenous peroxidase activity (0.3) hydrogen peroxidase for 15 min) were used. The primary antibodies were applied to the slides. The slides were incubated overnight with the primary antibody at room temperature, and washed by using phosphate buffered solution (PBS) then incubated with secondary antibody for 15 min followed by PBS wash. Finally the detection of bound antibody was accomplished using a modified labeled avidin–biotin (LAB) reagent for 20 min then PBS wash. A 0.1% solution of diaminobenzidine (DAB) was used for 5 min as a chromogen. Slides were counterstained with Mayer's hematoxylin for 5– 10 min.

Immunostaining Interpretation:

Positive Nestin expression was considered when cytoplasmic immune-localization was detected at any number of cells. [(18)] For Notch 1, cytoplasmic staining for any number of cells is required to assign positivity. [(19)] Nestin and Notch 1 expression was further evaluated by the reproducible H score system. Mild staining is assigned as 1+, moderate staining: 2+ and strong staining: 3+. In H score (histo-score) system, both the intensity and percentage are considered. The score was calculated as follows: H-score= (%3+cells x3)+(%2+cells x2)+(%1+cells x1). [(20)]

Unintentional bias was prevented by coding tissue samples so that, IHC study was done without knowledge of the used component characteristics. Assessment of slides was done by the authors separately. The inter-observers variation of this scoring system was shown to be 0.99 for both Nestin and Notch 1. Excellent concordance between the instances of scores was reached (0.99 for both stem cell markers).

Statistical Analysis:

Data were collected, tabulated and statistically analyzed by using SPSS "statistical package for the social science" program for windows, version 17, SPSS, INC., Chicago, Illinois, USA. To test whether these variables differed according to clinico-pathological parameters and biological markers, the Fisher's exact (FE) and x^2 tests, Mann-Whitney test, Student t test, Kruskal-Wallis test and Pearson correlation (r) were used. All P-values were two-sided, P values of ≤ 0.05 were considered statistically significant and highly significant when P value ≤ 0.01. [(21)]

3. Results:

Observed behavioral changes and manifestations:

Group I (control A and B): sustained normal behavior.

Group II (Tramadol acute toxicity group):

The main symptoms of intoxication were characterized by restlessness, unsteady gait, reduced spontaneous activity, tremors, convulsions, slight cyanosis then they entered into drowsiness, and coma with difficult noisy breath. This was followed by death of 10 rats within 8-12 hours from the onset of receiving the LD_{50} dose, as a result of respiratory arrest in combination with severe convulsions. Surviving animals showed slight recovery, within a short time.

Group III (Tramadol Dependent group):

The animals of this group were observed daily. During the first week they behaved normally except for sedation and calmness. During the second week, the dose reached up to 36 mg/kg b.wt., the rats became restless, irritable and some had tremors one to two hours before the designed dose, after which calmness, laziness and anorexia had developed. As the dose increased (43.2 – 57.6mg/kg b.wt) the rats became more irritable, some of them developed aggressiveness, yet the manifestations alleviated shortly after the dose. Then after another episode of altered behavioral parameters developed (pronounced defensive behavior, increased fearfulness), salivation, and spasms were also noted on most of the rats. Nearly almost all rats developed convulsions. During the last week the dose reached up to 72 mg/kg b.wt., the main symptom was convulsions. Additionally, other symptoms like tremors, irritability and salivation were also noted. Anorexia and loss of body weight were more exaggerated at the end of the experiment.

Histopathological examination:

Group I (control A and B)**:**

Lung, brain and liver tissue sections were free of pathological changes.

Group II & Group III:

Pathology of acute toxicity was extensively observed in group II as well as pathology of chronic toxicity was clearly detected in group III. The following histopathological changes showed highly statistical significant difference between group II and group III (P <0.001 for all).

Regarding lung tissue, the interstitial changes including marked pulmonary congestion, hemorrhage, fibrin deposition, mixed inflammatory cellular infiltrates (90% for all), marked inflammatory cellular infiltrates (95%) and edema (80%) were detected in group II, whereas, the fibrosis (95%), chronic inflammatory cellular infiltrates (90%) and mild inflammatory cellular infiltrates (100%) were detected in group III. Alveolar changes; diffuse alveolar damage

(DAD), marked alveolar wall thickening (90% for all). Together with marked alveolar wall destruction (emphysema) was also observed in all examined sections of all rats of group II whereas 95% of group III showed mild emphysematous changes. However, intra-alveolar edema was detected in all cases of group III (Figure 1 A, B, C).

Regarding the brain tissues, neuronal degeneration was detected in (95%) of rate brain sections of group III. Glial changes; gliosis, microglial proliferation, oligodendrocyte proliferation and Rosenthal fibers were observed in all cases of group III. Others; marked brain congestion, edema and inflammatory cellular infiltrates were detected in all members of group II, whereas, mild congestion (100%), mild edema (10%) and inflammatory cellular infiltrates were detected in (5%) in group III. Additionally, mean±SD of apoptotic count was 33.9±4.03 and 9.45±2.66 in group II and III respectively (Figure 1 D, E, F).

Regarding the liver tissue; parenchymal changes in form of marked steatosis, sinusoidal dilation (85% for both), focal necrosis, marked congestion and haemorrhage (90% for all) were seen in group II, whereas, mild congestion, interface hepatitis and Kupffer cell hyperplasia were observed in all liver specimens of group III. Portal changes; marked inflammatory cellular infiltrate (95%), mild fibrosis (85%) and bile duct proliferation was noticed in all cases of group III (Figure 1 G, H, I).

Nestin & Notch 1 Immunohistochemistry examination:

Nestin immunoreactivity in group I (control A and B): In the selected organs showed negative expression except the choroid plexus lining cells, subventricular zone (SVZ) and endothelial cells in brain together with only endothelial lining of blood vessels in liver and lung.

Notch 1 immunoreactivity in group I (control A and B): in selected organs showed positive expression in choroid plexus lining cells, SVZ and endothelial cells in blood vessels in brain. Whereas, normal liver showed Notch 1 positivity in hepatocytes, bile duct cells and endothelial cells as well as in normal lung displayed focal mild positivity in bronchial lining and alveoli.

Nestin and Notch 1 immunreactivity were assessed by intensity and H score for group II and group III. Each tool of assessment for both stem cell markers in the selected organs showed highly statistical significant difference between tramadol acute toxicity group and tramadol dependant group (P<0.001 for all) (Table 1). Whereas, the higher intensity and H score values for both markers were noticed in tramadol dependant group than the acute toxicity one. Additionally, a direct significant correlation between Nestin and Notch 1 scoring and localization was observed (r = +0.95, P value <0.001) (Figure 2).

Relationship of H scores in both Nestin and Notch 1 and the histopathological parameters in lung tissue

Highly statistical significant association were observed between H scores in both Nestin and Notch 1 immunostaining and the majority of histopathological parameters in lung tissue specimens (P<0.001 for all) except congestion. The higher values of H scores of both stem cell markers were associated with the histopathological parameters of dependent toxicity group (III) as mild edema, absence of hemorrhage, mild fibrin deposition, presence of fibrosis, chronic inflammatory cells, mild inflammatory cellular infiltrates, marked intra-alveolar edema, mild alveolar wall thickness as well as damage and absence of diffuse alveolar damage (Table 2 & Figure 3).

Relationship of H scores in both Nestin and Notch 1 and the histopathological parameters in brain tissue

In brain tissue specimens, high statistical significant association was observed between H scores in both Nestin and Notch 1 immunostaining and the histopathological parameters (P<0.001 for all). The higher values of H scores of both stem cell markers were associated with the histopathological parameters of dependent toxicity group (III) as red neuron, congestion, edema and inflammatory cellular infiltrate as well as presence of marked gliosis, microglial proliferation, oligodendrocyte proliferation, and Rosenthal fibers (Table 3 & Figures 4 and 5).

Relationship of H scores in both Nestin and Notch 1 and the histopathological parameters in liver tissue

Regarding the liver tissue specimens, high significant relationship was obtained between H scores in both Nestin and Notch 1 immunostaining and the histopathological parameters (P<0.001 for all). As higher values of H score for both Nestin and Notch 1 immunoreactivity tend to be associated with the histopathological parameters of dependent toxicity group (III), as mild steatosis, congestion, sinusoidal dilatation, marked feathery degeneration, marked Kupffer cell hyperplasia, marked inflammatory cellular infiltrates presence of interface hepatitis, presence of mild fibrosis and bile duct proliferation as well as absence of focal necrosis and hemorrhage (Table 4 & Figure 6).

Pearson correlation between apoptotic index and H scores for both Nestin & Notch1 in the brain tissue in group II and group III.

In brain tissue specimens, apoptotic index showed an inverse significant correlation with both Nestin H score (r=-0.94 and P<0.001) and Notch 1 H score (r=-0.88 and P<0.001) (Figure 7).

Table 1: Statistical comparison of tramadol acute toxicity group and tramadol dependant group as regards to Nestin and Notch 1 expression:

Parameters	*Acute No (%)*	*Chronic No (%)*	*Test of significance*	*P value*
		Lung tissue		
Nestin intensity Mild Moderate	17 (85) 3 (15)	3 (15) 17 (85)	X^2 test= 19.60	<0.001
Nestin H score Mean ± SD	95.25±4.06	239.80±14.49	t-test=42.93	<0.001
Notch1 intensity Mild Moderate Marked	19 (95) 1 (5) 0 (0)	3 (15) 0 (0) 17 (85)	X^2 test= 29.63	<0.001
Notch 1 H score Mean ± SD	143.85±3.18	265.05±32.72	t-test=16.48	<0.001
		Brain tissue		
Nestin intensity Mild Moderate	18 (90) 2 (10)	3 (15) 17 (85)	X^2 test= 22.55	<0.001
Nestin H score Mean ± SD	95.25±4.06	239.80±14.49	t-test=42.93	<0.001
Notch 1 intensity Mild Moderate Marked	18 (90) 2 (10) 0 (0)	2 (10) 0 (0) 18 (90)	X^2 test= 32.80	<0.001
Notch 1 H score Mean ± SD	143.85±3.18	266.25±32.48	t-test=16.76	<0.001
		Liver tissue		
Nestin intensity Mild Moderate	18 (90) 2 (10)	3 (15) 17 (85)	X^2 test= 22.55	<0.001
Nestin H score Mean ± SD	93.30±2.97	194.15±8.02	t-test=52.67	<0.001
Notch 1 intensity Mild Moderate Marked	18 (90) 2 (10) 0 (0)	2 (10) 0 (0) 18 (90)	X^2 test= 32.80	<0.001
Notch 1 H score Mean ± SD	137.45±12.01	267.35±30.98	t-test=17.47	<0.001

Table 2: Statistical comparison of H scores for both Nestin & Notch 1 with the histopathological parameters in lung tissue

Lung parameters	Nestin H score			Notch 1 H score		
	Nestin score Mean ± SD	Test of significance	*P* value	Notch score Mean ± SD	Test of significance	*P* value
		Interstitial changes				
Congestion Mild Marked	183.6±85.85 165.22±73.22	U=0.12	0.90	205.0±71.05 204.37±65.80	t=0.59	0.55
Edema Mild Marked	215.86±56.59 102.11±31.92	t=8.06	<0.001	243.21±54.77 152.0±35.44	t=6.38	<0.001
Heamorrhage Absent Mild Marked	235.58±10.96 197.4±90.82 94.94± 4.16	K=25.82	<0.001	261.64±34.43 230.0±74.51 143.33±2.86	K=28.67	<0.001
Fibrin deposition Mild Marked	224.47± 46.97 104.57±37.80	U=3.97	<0.001	252.38 ±47.80 151.47 ±33.46	t=7.79	<0.001
Fibrosis Absent Present	101.57±29.20 227.19±45.0	t=10.34	<0.001	144.94 ±6.72 258.28 ±43.86	t=11.68	<0.001
Inflammatory cell Chronic Mixed	223.10±44.31 111.95±52.51	U=4.05	<0.001	251.15±47.37 157.75±44.45	t=6.42	<0.001
Inflammatory cellular infiltrate Mild Marked	233.0±34.21 95.15± 4.15	t=18.31	<0.001	259.23±41.55 143.89±3.26	t=12.67	<0.001
		Alveolar changes				
Intra- alveolar edema Absent Mild Marked	95.25 ±4.06 237.0±12.20 267.0± 7.07	K=30.57	<0.001	143.85±3.18 262.55±33.62 287.5±2.12	K=29.96	<0.001
Diffuse alveolar damage Absent Present	227.0±43.66 94.83±4.07	t=14.12	<0.001	254.04±47.30 143.83±3.34	t=10.89	<0.001
Alveolar wall thickness Mild Marked	226.23±46.89 102.63±30.61	t=9.75	<0.001	253.23±48.45 150.52±29.10	t=8.21	<0.001
Alveolar wall destruction Mild Marked	238.73±14.07 103.09±36.16	U=4.95	<0.001	263.78±33.11 150.76±31.82	t=10.98	<0.001

Table 3: Statistical comparison of H scores for both Nestin & Notch 1 with the histopathological parameters in brain tissue

Brain parameters	Nestin H score			Notch 1 H score		
	Nestin score Mean ± SD	Test of significance	*P* value	Notch score Mean ± SD	Test of significance	*P* value
Neuronal changes						
Red neuron Absent Present	 95.25± 4.06 239.80±14.49	 t=42.93	 <0.001	 143.85±3.18 266.25±32.48	 t=16.76	 <0.001
Neuronal degeneration Absent Present	 102.90±35.30 238.94±14.37	 U=4.98	 <0.001	 150.14±29.0 265.73±33.29	 t=11.73	 <0.001
Glial changes						
Gliosis Mild Marked	 95.25± 4.06 239.80±14.49	 t=42.93	 <0.001	 143.85±3.18 266.25±32.48	 t=16.76	 <0.001
Microglial proliferation Absent Present	 95.25± 4.06 239.80±14.49	 t=42.93	 <0.001	 143.85±3.18 266.25±32.48	 t=16.76	 <0.001
Oligodendrocyte proliferation Absent Present	 95.25± 4.06 239.80±14.49	 t=42.93	 <0.001	 143.85±3.18 266.25±32.48	 t=16.76	 <0.001
Rosenthal fibers Absent Present	 95.25± 4.06 239.80±14.49	 t=42.93	 <0.001	 143.85±3.18 266.25±32.48	 t=16.76	 <0.001
Others						
Congestion Mild Marked	 239.80±14.49 95.25± 4.06	 t=42.93	 <0.001	 266.25±32.48 143.85±3.18	 t=16.76	 <0.001
Edema Absent Mild Marked	 241.77±13.90 107.65±39.30 98.0±1.41	 K=28.88	 <0.001	 276.38±9.40 146.50±10.24 148.50±2.12	 K=29.92	 <0.001
Inflammatory cellular infiltrate Absent Present	 240.36±14.66 101.61±29.45	 t=18.54	 <0.001	 265.15±33.0 150.66±31.39	 t=11.24	 <0.001

Table 4: Statistical comparison of both Nestin score & Notch 1 score with different measured parameters in liver tissue

Liver parameters	Nestin H score			Notch 1 H score		
	Nestin score Mean ± SD	Test of significance	*P* value	Notch 1 score Mean ±SD	Test of significance	*P* Value
Parenchymal changes						
Steatosis Mild Marked	 178.90±38.30 108.55± 36.79	 t=3.47	 0.001	 247.05±54.98 157.75±52.58	 t=5.24	 <0.001
Feathery degeneration Mild Marked	 99.22±24.09 180.13±36.78	 t=8.02	 <0.001	 145.77±37.87 248.72±53.63	 t=7.09	 <0.001
Focal necrosis Absent Present	 183.50±31.93 103.95±32.79	 t=7.77	 <0.001	 253.90±48.41 150.90±45.36	 t=6.94	 <0.001
Congestion Mild Marked	 185.22± 29.87 93.0± 2.99	 t=14.39	 <0.001	 256.36±46.19 136.44±12.21	 t=11.68	 <0.001
Heamorrhage Absent Present	 185.22± 29.87 93.0± 2.99	 t=14.39	 <0.001	 256.36±46.19 136.44±12.21	 t=11.68	 <0.001
Interface hepatitis Absent Present	 92.92 ± 2.81 171.07±43.55	 U=4.24	 <0.001	 134.64±13.34 138.88±59.50	 U=4.52	 <0.001
Kupffer cell hyperplasia Mild Marked	 93.30± 2.97 194.15±8.02	 t=52.67	 <0.001	 137.45±12.01 267.35±30.98	 t=17.47	 <0.001
Sinusoidal dilatation Mild Marked	 179.95±36.80 103.68 ±31.61	 t=6.99	 <0.001	 249.38±52.59 150.47±45.23	 t=6.34	 <0.001
Portal changes						
Inflammatory cellular infiltrate Mild Marked	 98.42±23.80 184.71±30.51	 t=9.89	 <0.001	 143.78±34.14 255.42±47.12	 t=8.63	 <0.001
Mild fibrosis Absent Present	 106.82±35.84 193.64±8.50	 U=4.27	 <0.001	 156.26±50.92 264.82±33.04	 t=7.66	 <0.001
Bile duct proliferation Absent Present	 93.30± 2.97 194.15±8.02	 t=52.67	 <0.001	 137.45±12.01 167.35±30.98	 t=17.47	 <0.001

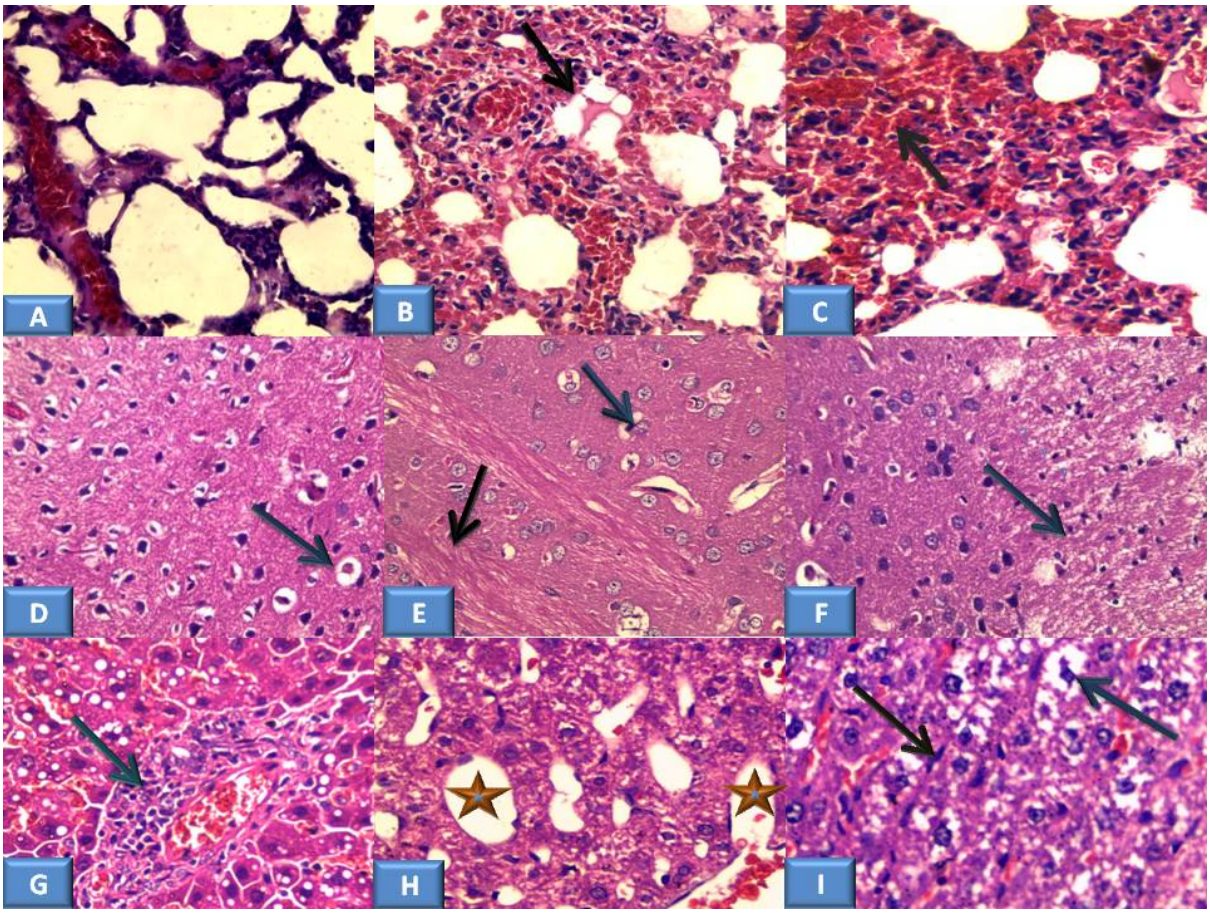

Figure 1: Rat lung tissue. **A**, Massive alveolar wall damage. **B,** Alveolar wall thickening and intra-alveolar edema (arrow). **C,** Haemorrhage (arrow) and inflammatory infiltrates in necrotic area. **Rat brain tissue. D,** Red neurons (arrow). **E,** Gliosis (black arrow) and apoptosis (blue arrow). **F,** inflammatory cellular infiltrates (blue arrow). **Rat liver tissue. G,** Steatosis and spotty necrosis (arrow). **H,** Marked sinusoidal dilation (asterisk). **I,** Kupffer cell hyperplasia (black arrow) and feathery degeneration of hepatocytes (blue arrow) (H&E X 400).

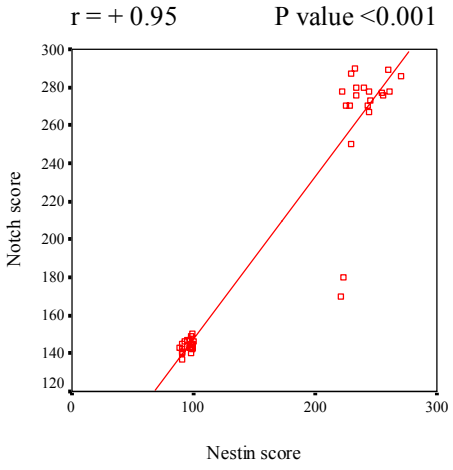

Figure 2: Direct correlation of Nestin and Notch 1 expression and localization in the selected organs of different groups.

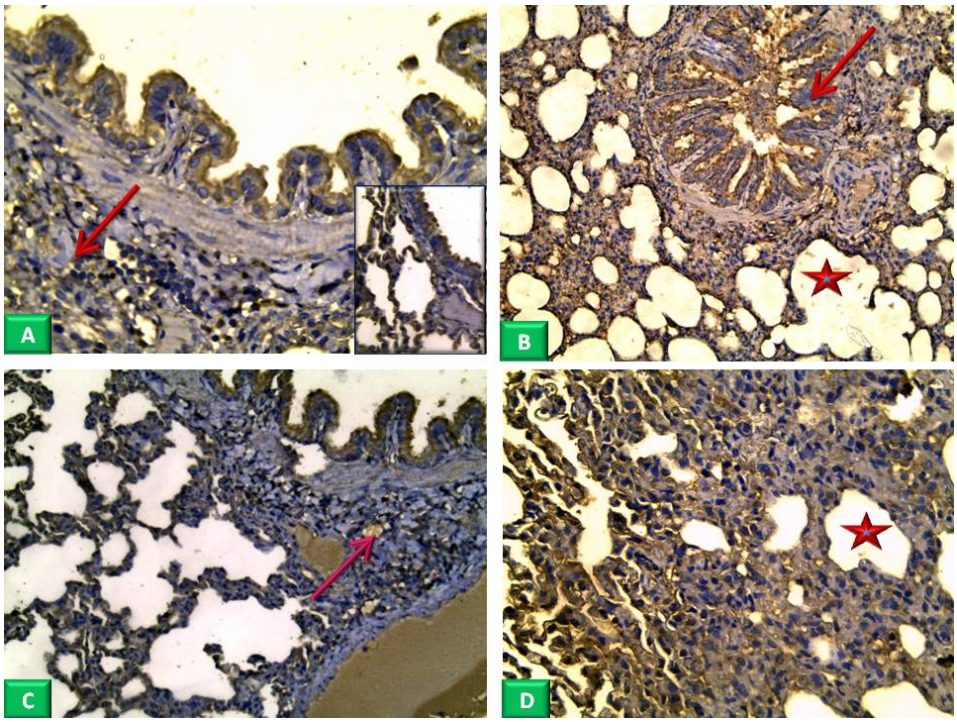

Figure 3: Nestin and Notch 1 expression in rate lung tissues. **A,** mild intensity of Nestin immunostaining in bronchial lining epithelium and the thickened alveolar wall (arrow) in group II in comparing with group I (inset). **B,** moderate intensity of Nestin staining in bronchial wall (arrow) and thickened and destructed alveolar wall (asterisk). **C,** mild intensity of Notch 1 staining in bronchial lining cells and thickened alveolar wall (arrow) in group II. **D,** moderate intensity of Notch 1 immunoreactivity in thickened destructed alveolar wall (asterisk) in group III. (Immunoperoxidase X400 "A, C, D" and X 200 "B").

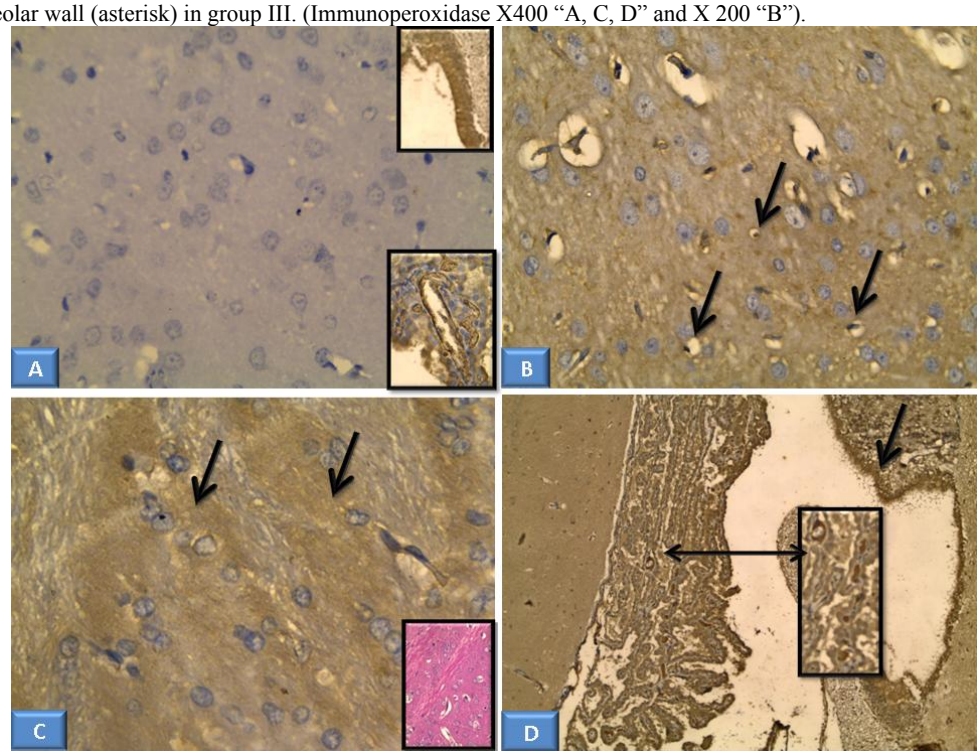

Figure 4: Nestin expression in rate brain tissues. **A,** Negative expression in group I in all brain sections except the choroid plexus lining cells and blood vessels (lower Inset) as well as subventricular area (upper inset). **B,** Mild expression in group II with frequent apoptosis "arrows". **C,** Moderate expression in group III with its prominence at areas of gliosis "arrows" inset; its H&E stained counterpart. **D,** Moderate expression in brain tissues and choroid plexus (double head arrow) (inset; high power view displaying blood vessels) with subventricular accentuation of Nestin stain (arrow) (group III). (Immunoperoxidase X 400 "A, B, C" X 200 "D").

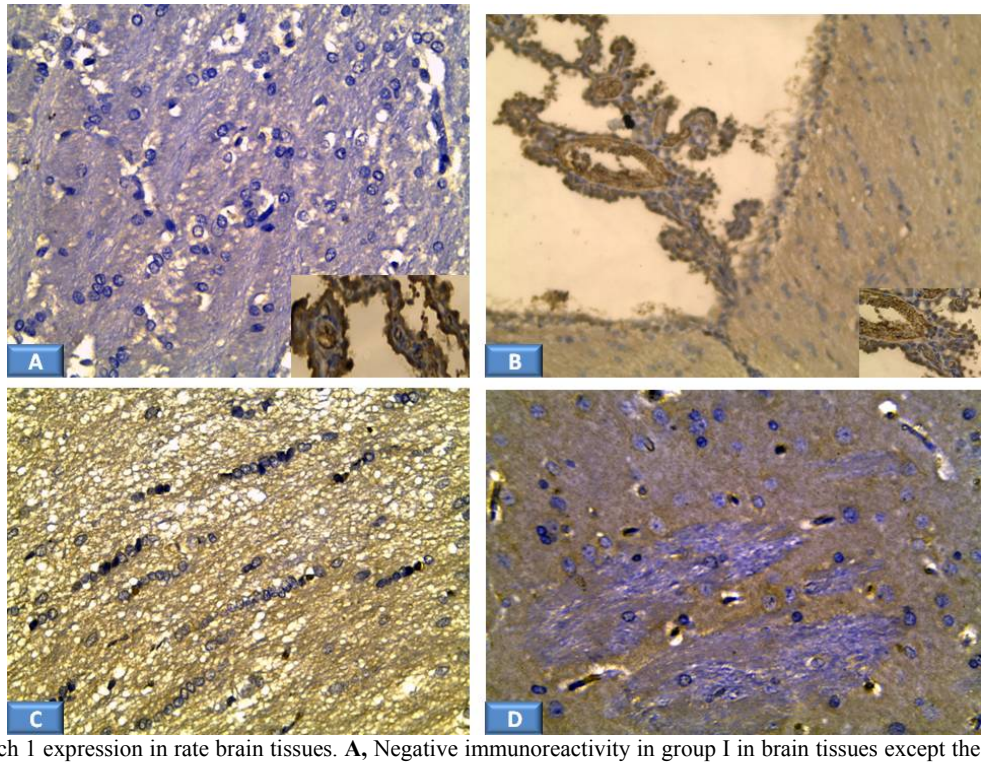

Figure 5: Notch 1 expression in rate brain tissues. **A,** Negative immunoreactivity in group I in brain tissues except the choroid plexus lining cells and blood vessels (inset). **B,** mild staining in group II with accentuation at choroid plexus (inset; high power view of choroid plexus). **C,** mild staining in group II with prominent edema and degenerative changes. **D,** moderate immunostaining in group III (Immunoperoxidase X 400 "A, C, D" X 200 "B").

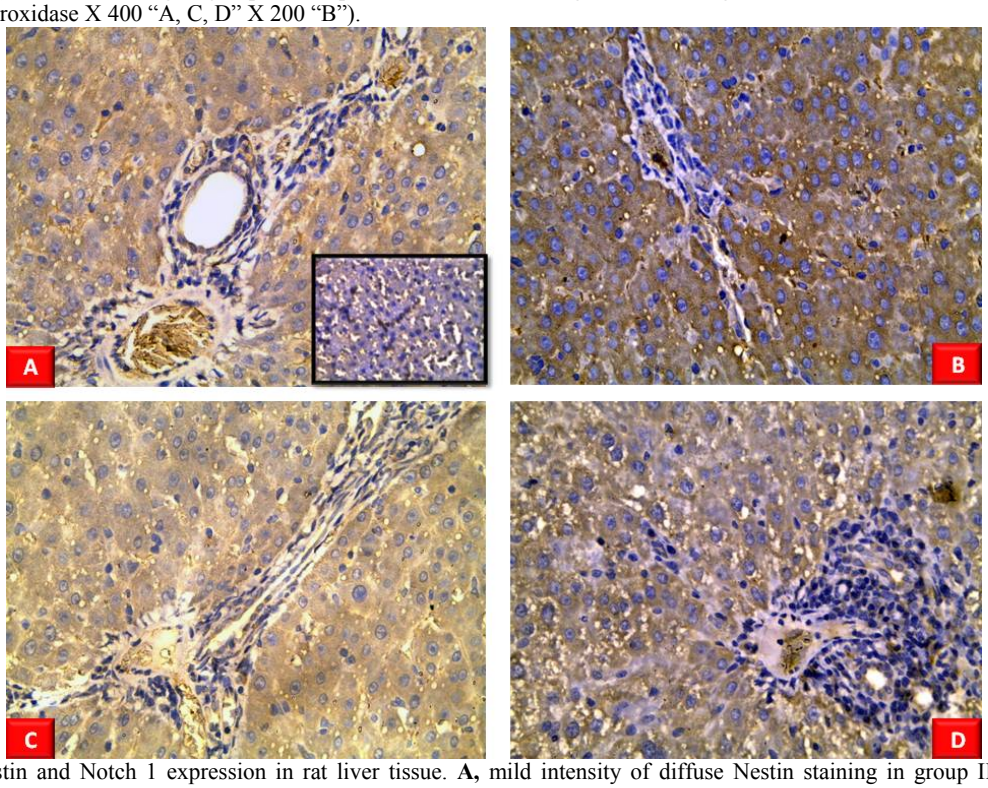

Figure 6: Nestin and Notch 1 expression in rat liver tissue. **A,** mild intensity of diffuse Nestin staining in group II. **B,** moderate intensity of diffuse Nestin staining in group III in contrast with normal liver group I (inset) that shows absence of nestin expression in hepatocytes and its restricted immune-localization to Kuffer cells. **C,** mild intensity of diffuse Notch 1 staining in group II. **D,** moderate intensity of diffuse Notch 1 staining in group III in contrast with normal liver group I (inset) (Immunperoxidase X 400).

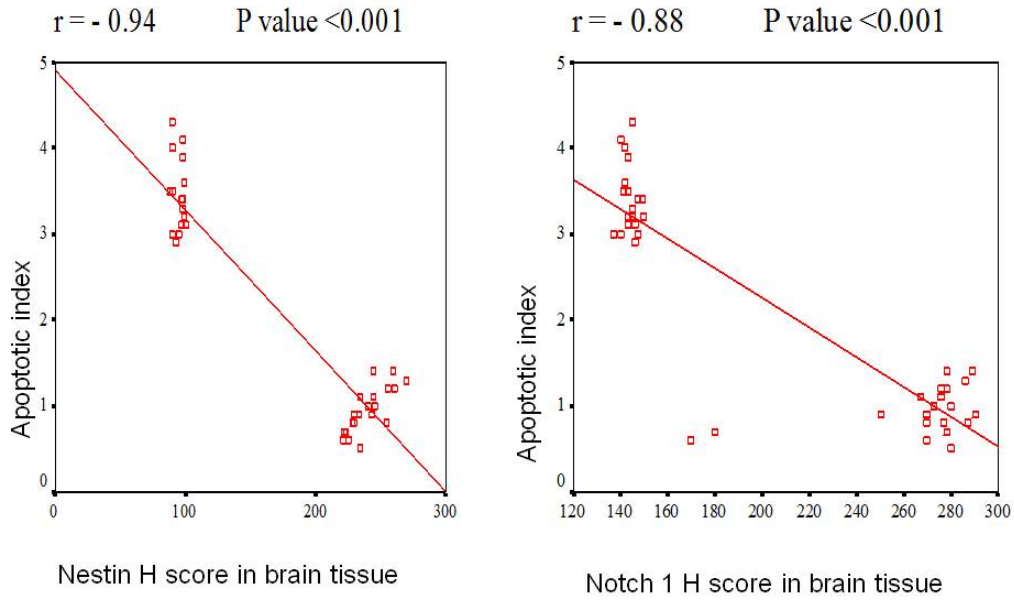

Figure 7: Inverse correlation of Nestin and Notch 1 immunostaining profile with apoptosis in brain tissue.

4. Discussion

Analgesics are the most commonly consumed over- the-counter preparation all over the world. Tramadol, a synthetic analogue of codeine, is a centrally acting analgesic drug with a dual mechanism of action. [1] After its introduction in the 70s, tramadol gained great interest because of its low abuse potential. [22]

In acute tramadol toxicity group, we observed deaths of rats that may be explained by reports, [23,24] as serious complications may occur in case of intoxication and tramadol related fatalities have been reported.

Within the context of acute tramadol toxicity, tremors, convulsion, slight cyanosis, coma and respiratory arrest were explained by association with serotonin. [25] Additionally **Vizcaychipi *et al.*** (2007) [26] recorded that serotonin syndrome triggered by tramadol administration by increasing cerebral serotonin activity via partial inhibition of its uptake. In the current study, the clinical data that noticed during could be appropriately explained by multiple organs failure as respiratory depression, acute respiratory syndrome and hepatic dysfunction,[25] that seem to be exhausted by the sustained augmented activity in response to partial inhibition of reuptake of serotonin and norepinephrin, [1] as well as supported by H&E histopathological findings of the selected organs; lung, brain and liver.

In the current study, restlessness, unsteady gait, reduced spontaneous activity, tremors and convulsion were observed in acute tramadol toxicity could be explained by the histopathological changes of brain tissues as neural, glial and other changes including congestion, edema, inflammatory cellular infiltrates and apoptosis. Moreover, **El-Naggar *et al.* (2009)** [27] concluded that tramadol administration for short term caused a significant elevation of serotonin level in the cerebral cortical tissues of rats that might explain the clinical observation and the histopathological changes in the brain tissues.

Similarly difficult noisy breathing up to drowsiness and coma might be linked to hypoxia and cellular damage that is confirmed by the histopathological changes of lung as marked pulmonary congestion, hemorrhage, edema, fibrin deposition, marked mixed inflammatory

infiltrates and diffuse alveolar damage (DAD) together with marked alveolar wall thickening. Cardiopulmonary arrest was found to be the cause of death in cases who had ingested more than 5000 mg tramadol in a study conducted in Iran [3] on a total of 114 cases of intentional tramadol intoxications. Some of the observed clinical data in the same group as tremors, restlessness and coma could be also linked to the histopathological changes of hepatic tissues as congestion, haemorrhage and necrosis. **Loughrey and his associates (2003)** [28] reported that acute liver failure in acute tramadol toxicity due to hepatic necrosis.

Regarding the observation of rats of tramadol dependent group in the present study, behavioral alterations, essentially restlessness, hyperactivity, or increased excitability and convulsion by increasing the dose were in accordance to the work of others [29,1] who stated that tramadol is associated with the development of physical dependence with greater abuse potential than originally believed and a severe withdrawal syndrome that could be explained by hepatic biotransformation of tramadol to an active metabolite is necessary to produce significant mu-agonist effects. An accumulation of the active metabolite which has a significant affinity for the mu-receptor likely leads to CNS adaptations (**Lanier *et al.* 2010**).[30]

The observed manifestations in dependant group could be explained by the histopathologic changes of brain tissues that seem to be the results of neural exhaustion by the sustained augmented activity in response to continuous administration of tramadol.[31] The observed manifestations and histopathologic changes could be also explained by serotonin syndrome triggering by tramadol via partial inhibition of its uptake. [26] Also **El-Naggar *et al.* (2009)** [27] concluded that tramadol administration for long term caused a significant elevation of serotonin level in the cerebral cortical tissues of rats that directly correlated with the detected histopathological findings of dependant group.

In the same context, the clinical observations might be linked to hypoxia due to the histopathological changes of lung that consistent with **Todorović *et al.* (2011)** [32] that documented the dominant pathomorphological changes in the lung tissue and concluded the histopatological study is necessary to determinate a cause of death when a deceased person has the history of dependence or abuse of psychoactive drugs with negative toxicological results.

Atici and his associates (2005) [33] reported that severe centrolobular congestion and focal necrosis in the rat liver of chronic tramadol group which pointed out the risk of increased hepatic damage due to long term use of tramadol that are concordant with our findings.

Recently the researches focus on the potential applications of stem cells (SCs), in drug screening and toxicology tests. [7] Several stem and progenitor cell populations have already been described as valuable tools for developing therapeutic strategies in regenerative medicine.[7]

To our knowledge, the present study is the 1st one to explore the relationship between Nestin and Notch 1 in response to tramadol toxicity. We detect a significant direct correlation between Nestin and Notch 1 scoring and localization in different studied groups where that they cooperate with each other in maintain homeostasis, rapid and sustained injury and also in regeneration including maintenance of stem cell, specification of cell fate, differentiation and apoptosis

Nestin and Notch1 negativity are the common finding in the brain in the control group, however, positive expression was demonstrated in the choroid plexus lining cells and subventricular zone (SVZ) as well as endothelial cells. These findings are agreed with others. In normal adult CNS, both nestin and notch 1 are markers for neural stem cells lining the ventricular wall [34, 35], choroid plexuses [36] and also expressed by endothelial cells. [37]

Furthermore, Nestin immunoreactivity in group I in liver and lung showed positivity in endothelial cells that concordant with other report. [38] However, Notch 1 immunoreactivity in group I in liver and lung showed positive expression in hepatocytes, bile duct cells and endothelial cells as well as focal mild positivity in bronchial lining and alveoli that concordant with other reports. [39,40]

Moreover, the expression of both Nestin and Notch 1 showed highly statistical significant differences between acute toxicity and dependence groups. As the higher values of expression of both stem cell markers were detected in chronic toxicity group. Nestin expression was shown to be upregulated in progenitor cells of various tissues. **Wiese and his associates (2004)** [9] suggesting that Nestin may be a common marker of multi-lineage progenitor cells. Moreover, the demonstration of Nestin expression in adult tissues after injury suggests that Nestin-expressing cells may be directly implicated in tissue regeneration. [10]

In a context, Notch 1 pathway has only recently been associated with the maintenance of adult tissue. [13] Notch 1 signaling coordinates a wide range of fundamental processes and cellular programs including proliferation, apoptosis, migration, growth, and differentiation, [13] depending on cellular context, Notch pathway including gene expression and cross talk with other signaling system. The most prominent targets of the Notch pathway include a set of basic helix–loop–helix factors of the hairy and enhancer of split (Hes) and Hes-related repressor protein (Hey) families. [41] These transcription factors execute Notch signaling functions, including maintenance of stem cells, specification of cell fate, differentiation, proliferation, and apoptosis. [13]

Additionally, our study reveals significant relationship between expression of both Nestin and Notch 1 and the histopathological changes in brain, lung and liver. Both stem cell markers expression are higher in dependence group related histopathologic changes than the acute toxicity group related histopathological changes.

Other studies that investigated Nestin expression in adult brain demonstrated its specific role in neurogenesis and gliogenesis. [42] Similarly activated Notch signaling pathway was partially related to injury-induced neurogenesis mechanisms. [43]

Regarding Nestin and Notch 1 localization and expression in lung tissues in both acute tramadol toxicity and dependence groups, Notch 1 signaling plays an important role in the ontogeny of airway epithelial cells, but its contributions to recruitment, expansion or differentiation of resident progenitor/stem cells, and repair and re-establishment of the normal composition of airway epithelium following injury is by stimulation of Hes5 and paired-box-containing gene 6 (Pax6) genes. [44] Nestin expression after injury in lung was only investigated in one article. [45]

Regarding dual expression of Nestin and Notch 1 in liver tissues in group II and group III, we were fortunate to detect both stem cell markers in proliferated kupffer cells and in addition in hepatocytes and proliferated bile ductal epithelium.

Hepatic stellate cells (HSCs) are located in the perisinusoidal space of Disse were first described by **Kupffer in 1876**. [46] Activated HSCs proliferate vigorously and secret a large amount of extra-cellular matrix which contributes to hepatic fibrosis in response to injury. [47]

Castilho-Fernandes *et al.* (2011) [48] demonstrate that Nestin is one of the activated HSC markers and re-expression of Nestin appears to be responsible for the promotion of cell proliferation during the post-injury phase. [11] In the same context, Notch 1 signaling pathway is activated during liver regeneration and is potentially contributing to signals affecting cell growth and differentiation. [39]

Concerning apoptotic index in brain tissue specimens, it showed a significant inverse correlation with Nestin and Notch 1 immunoreactivity in both acute tramadol toxicity and tramadol dependant groups.

The molecular mechanisms of tramadol induced apoptosis have not been established yet. The mechanism of tramadol induced brain damage by decrement in rat brain activities of Na +/K +, Mg2+ and Ca2+ dependent ATPases with subsequent decrease in ATP turnover and energy metabolism as well as loss of mitochondrial membrane transport functions. [49]

The accumulation of DNA strand breaks is a well established stimulus for p53 activation. The activation of p53 is a key factor involved in neuronal death. It can cause apoptosis by inducing Bax that causes mitochondrial membrane permeablization and propagate the apoptotic pathway. [50]

Nestin is important for the proper survival and self-renewal of neural stem cells (NSCs), that is consistent with in vivo observation, NSC cultures derived from Nestin knockout embryos show dramatically reduced self-renewal ability that is associated with elevated apoptosis. [51]

Notch 1 activates the cellular survival pathways and inhibits apoptosis through many mechanisms. [52] Notch 1 can inhibit p53, [53] up-regulate NF-□B that regulates the expression of many antiapoptotic proteins like survivin [54] and interfere with JNK activation [55] and degradation of X-linked inhibitor of apoptosis protein (XIAP). [56] Notch can also promote cellular survival by up-regulation of Bcl-2 and Mcl-1 [57, 58] and protect malignant cells from chemotherapy-induced apoptosis by up-regulation of p21 (WAF/Cip). [59]

Moreover, recently, **Wickremasinghe and associates** (2011) [60] identified the Notch 1 pathway as a candidate p53-induced antiapoptotic mechanism and the pharmacological inhibition of the Notch 1 signaling pathway in leukaemia will augment apoptosis induction by cytotoxic agents. Therefore, Notch 1 signaling may protect neurons from apoptotic cell death.

The Notch 1 signaling pathway is a significant down regulator of serotonin production in GI carcinoid tumors. [61] Therefore, the previous fascinating idea could open the gate of new treatment strategies.

Therefore we conclude that stem cell markers are the main modulators of life saving as they re-expressed early in response to cell injury by tramadol toxicity and late in maintenance of cellular regeneration by playing crucial roles throughout the journey. The presence of the stem cell markers directs our attention to their role in both acute toxicity and dependence that opens the gate for new therapeutic modalities of enhancing regenerative stem cell aggregations confronting acute and chronic tramadol toxicity.

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5/8/2012