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Genotoxic effect of Tamiflu and Adamine on DNA content in male albino mice

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Abstract

Background Tamiflu (Oseltamivir) and Adamine (Amantadine HCl) are antiviral drugs which are used for prevention and treatment for influenza. The present study was carried out to evaluate the effect of Tamiflu and Adamine on DNA content of hepatocytes of adult male albino mice.

Results The results of comet assay showed that treatment with Tamiflu and Adamine significantly increased ($P < 0.005$) the mean of DNA damage in hepatocytes of the treated mice in time dependent manner compared to control one. However qRT-PCR analysis showed the mRNA expression of BAX in mice liver of all treated groups with Adamine was significantly increased ($P < 0.05$) after ten days of treatment while the same gene was significantly decreased in Tamiflu treated group after ten days compared to control one, While the treatment of mice with both drugs simultaneously showed a significant increase ($P < 0.05$) in expression of BCL-2 gene and a decrease in expression of BAX gene after fifteen days of treatment.

Conclusion Tamiflu and Adamine had produced deleterious impacts on DNA content of hepatocytes of treated mice. Whereas, the results of treatments with Adamine indicated pathogenic impacts more than that induced by Tamiflu.

Keywords Adamine, Comet assay, Hepatocytes, Mice, qRT-PCR, Tamiflu

Background

Two classes of antiviral agents approved for the prevention of and treatment for influenza: the M2 Inhibitors (amantadine and rimantadine) and the neuraminidase inhibitors (oseltamivir, laninamivir, peramivir and zanamivir). These agents have been proven to be safe and effective alone or in combination for the treatment of uncomplicated influenza in otherwise healthy individuals Ison [5].

Liu et al. [8] examined mice which were infected by nasal drop of 0.05 ml influenza virus strain FM1 (LD50) and treated with Oseltamivir. They found that mRNA and protein expressions of Toll-like receptor 7 (TLR7), Myeloid differentiation factor 88 (MyD88), and Nuclear factor kappa B (NF-kB) decreased in the Oseltamivir group compared with the model groups. The same authors determined mRNA and protein expressions of TLR7, MyD 88 and NF-kB in lung tissue and found that they decreased in the group of mice infected with influenza virus FM1 and treated with daily dose of 2.5 g/ml oseltamivir.

Kaefer et al. [6] examined deleterious effects on CNS induced by amantadine, in adult male CF-1 mice that were treated with a systemic injection of amantadine (15, 30 or 60 mg/kg b.wt.) 20 min before behavioral tasks on open field and inhibitory avoidance. The authors found that amantadine was not able to induce chromosomal mutagenesis or toxicity on bone marrow, as evaluated by the micronucleus assay. At the lowest dose tested, amantadine did not induce DNA damage and it was unable to impair memory, locomotion, exploration or motivation

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in mice. However, higher amantadine doses increased DNA damage in brain tissue, produced locomotor disturbances severe enough to preclude testing for learning and memory effects, and induced stereotypy, suggesting neurotoxicity.

Lan et al. [7] demonstrated that amantadine markedly inhibited the proliferation of HepG2 and SMMC-7721 cells in a dose and time-dependent manner and arrested the cell cycle at the G0/G1 phase. The levels of the cell cycle-related genes and proteins (cyclin D1, cyclin E and CDK2) were reduced by amantadine, and apoptosis was significantly induced. Amantadine treatment also reduced BCL-2 and increased the BAX protein and mRNA levels. Additionally, BCL-2/BAX ratios were lower in the two Hepatocellular carcinoma cell lines following amantadine treatment.

Methods

Experimental animals

The present study was carried out on fifty adult male Swiss albino mice of CD-1 *(Mus musculus)* with an average age of 12 weeks and body weight of ~ 25 g. The animals were obtained from Theodor Bilharz Research Institute, Imbaba, Giza, Egypt.

The animals were housed in cages and fed *ad libitum* with a standard diet and provided with free access to water, being kept under suitable laboratory conditions during the whole period of experimentation and the healthy animals were used.

The applied drugs

Tamiflu and Adamine are available in the form of capsules for oral administration. They contain 75 mg and 100 mg of the active ingredients, respectively.

In the present investigation, the experiment was designed to evaluate the effect of Tamiflu (150 mg/day/each adult human) and Adamine (200 mg/day/each adult human) on DNA content of hepatocytes of adult male albino mice.

The dose of Tamiflu (0.4 mg/each mouse, equivalent to 16 mg/kg b.wt.) and the dose of Adamine (0.5 mg/each mouse, equivalent to 20 mg/kg b.wt.) were calculated according to the equivalent therapeutic dosages of human-mouse conversion factor by Paget and Barnes [9], and were diluted in normal saline solution (0.9% NaCl). The doses were given orally either separately or concurrently way for the desired periods five, ten and fifteen days to experimental mice.

Experimental design

Fifty adult mice were allocated into ten equal groups of 5 mice each. The first group served as the control group (C group), received saline solution (0.9% NaCl) and the rest nine groups divided into T5, T10 and T15 groups; which were Tamiflu treated groups, whilst the other three groups A5, A10 and A15 groups were treated with Adamine. Beside, the last three groups (TA5, TA10 and TA15) were treated with Tamiflu concurrently with Adamine. All doses were given daily for five, ten and fifteen days.

At the end of the experiment, the mice were sacrificed by severing their neck blood vessels and immediately dissected and samples of liver were collected.

Comet assay

The technique of single cell gel electrophoresis regarding preparation of the base slides, cell isolation, electrophoresis of microgel slides and buffers preparation was performed in concordance with the premises established by Singh et al. [10].

qRT-PCR

Quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR) to detect the early changes in gene expression of:

Gene	Forward primer 5′ Reverse primer 3′
GAPDH	Forward, GAGAAACCTGCCAAGTATG Reverse, GGAGTTGCTGTTGAAGTC
BAX	Forward, CTACAGGGTTTCATCCAG Reverse, CCAGTTCATCTCCAATTCG
BCL-2	Forward, CTCGTCGCTACCGTCGTG ACTTCG Reverse, CAGATGCCGGTTCAGGTA CTCAGTC

Table 1 The Mean and standard deviation of DNA damage as revealed by comet assay of hepatocytes of the control and treated male albino mice (*Mus musculus*)

Animal group	Tail length / μm (mean& ± SD)	DNA % in tail (mean& ± SD)
C-group	0.59 ± 0.32	3.72 ± 0.7597
T5	1.61 ± 0.33*	10.08 ± 0.41*
T10	3 ± 0.77**	13.14 ± 3.63**
T15	3.84 ± 1.04**	16.41 ± 3.18**
A5	1.82 ± 0.44*	15.95 ± 5.28*
A10	2.16 ± 0.032**	11.7 ± 1.74**
A15	2.32 ± 0.35**	15.14 ± 0.74**
TA5	2.63 ± 0.48**	12.65 ± 1.59**
TA10	3.02 ± 0.841**	18.37 ± 1.75**
TA15	3.41 ± 0.94**	18.64 ± 0.56**

Level of significance:

Insignificant ($P > 0.05$)

*Significant ($P < 0.05$)

**Highly significant ($P < 0.001$)

Total RNA extraction and cDNA synthesis

Total RNA was isolated from the liver of treated mice as well as from the control group, using the (Thermo Scientific Gene JET RNA Purification Kit#K0731, #K0732), according to the manufacturer's instructions. For cDNA synthesis, one microgram of the total RNA was used according to the instructions of the (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit #K1621#K1622).

Measurement of mRNA expression levels

The relative expression levels of mRNA encoding genes were measured using the (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X)), according to manufacturer's protocol. The expression level of the target genes was normalized to GAPDH and presented as fold change relative to the control group [13].

Statistical evaluation

Statistical analysis of the data was carried out by *t*-test, SPSS statistics 17.0 V.

Results

Results of comet assay

The severity of DNA damage indicated by 2 parameters of comet tail length and DNA% in tail was illustrated in (Table 1) as mean ± SD and graphically showed at (Figs. 1 and 2).

Table 1 showed a highly significant increase ($P < 0.001$) in the mean ± SD of tail length and DNA

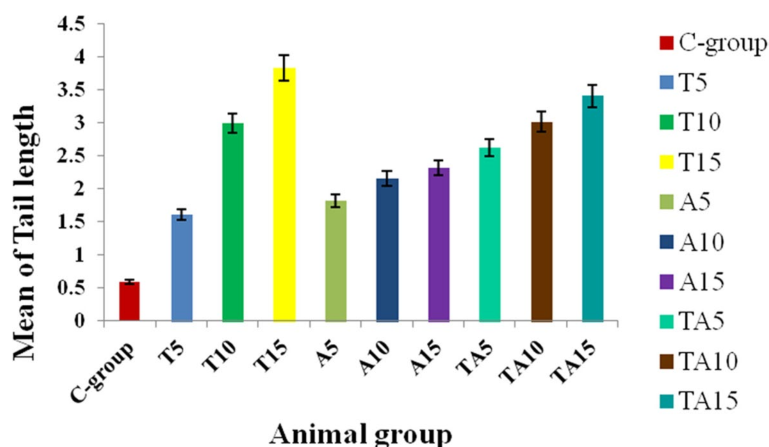

Fig. 1 Histogram represents the relationship between the mean & ± SD of DNA damage in hepatocytes of male albino mice by using tail length in control and all treated groups as calculated in Table 1

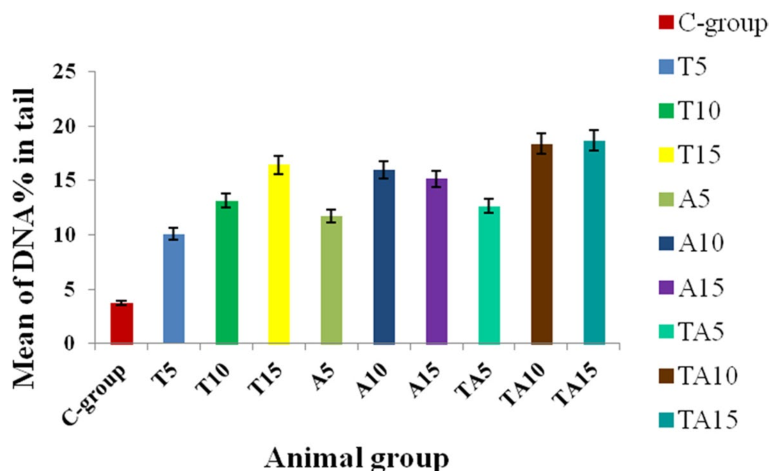

Fig. 2 Histogram represents the relationship between the mean & ± SD of DNA damage in hepatocytes of male albino mice by using DNA % in tail in control and all treated groups as calculated in Table 1

damage percentages in all treated groups except Tamiflu (16.mg/kg b.wt.) treated group for 5 days and Adamine (20 mg/kg b.wt) treated group for 5 days showed a significant increase ($P < 0.05$) as compared to the control group.

After treatment with (16 mg/kg b.wt.) of Tamiflu for five, ten and fifteen days, the mean ± SD of DNA damage in the treated mice were (10.08 ± 0.41, 13.14 ± 3.63 and 16.41 ± 3.18), respectively compared to (3.72 ± 0.7597) in the control group and the mean ± SD of tail length was (1.61 ± 0.33, 3 ± 0.77 and 3.84 ± 1.04), respectively compared to (0.59 ± 0.32) in the control group.

After treatment with (20 mg/kg b.wt.) of Adamine for five, ten and fifteen days, the mean ± SD of DNA damage in the treated mice were (15.95 ± 5.28, 11.7 ± 1.74 and 15.14 ± 0.74), respectively compared to (3.72 ± 0.7597) in the control group and the mean ± SD of tail length was (1.82 ± 0.44, 2.16 ± 0.032 and 2.32 ± 0.35), respectively compared to (0.59 ± 0.32) in the control group.

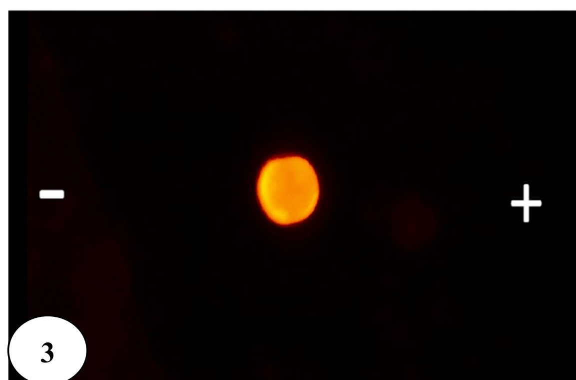

Fig. 3 Photomicrograph of comet assay of the control group showing no or minimum DNA migration. The symbols '-' and '+' represent cathode and anode, respectively, during the electrophoresis. (X: 1500)

After treatment with (16 mg/kg b.wt.) of Tamiflu concurrently with (20 mg/kg b.wt.) of Adamine for five, ten and fifteen days, the mean ± SD of DNA damage in the treated mice were (12.65 ± 1.59, 18.37 ± 1.75 and 18.64 ± 0.56), respectively compared to (3.72 ± 0.7597) in the control group and the mean ± SD of tail length was (2.63 ± 0.48, 3.02 ± 0.841 and 3.41 ± 0.94), respectively compared to (0.59 ± 0.32) in the control group.

Images of single cell gel electrophoresis were classified according to the degree of damage after migration through electrophoresis & visualized by the digital camera fitted fluorescent microscope as shown in (Figs. 3, 4, 5 and 6). It revealed an intact DNA in the control group (Fig. 3); while a high degree of DNA damage clarified by a slightly pointed end due to the migration of fragmented DNA through electrophoresis (tailed) was presented as in (Figs. 4, 5 and 6).

In the present study, we found that cells from the control mice had tightly compressed DNA and maintained the circular form of the normal nucleus, with little or no evidence of comet formation as shown in (Fig. 3). In contrast, cells from treated mice revealed an altered appearance as shown in (Figs. 4, 5 and 6).

The intensity of the comet tail relative to the head reflected the number of DNA breaks. Cells containing greater levels of DNA strand breakage generated comets with more intense 'tails' (Fig. 6). Single cell gel electrophoresis (comet test) results showed that the destruction degree of DNA was ranged within 4 grades (Table 2 and Fig. 7).

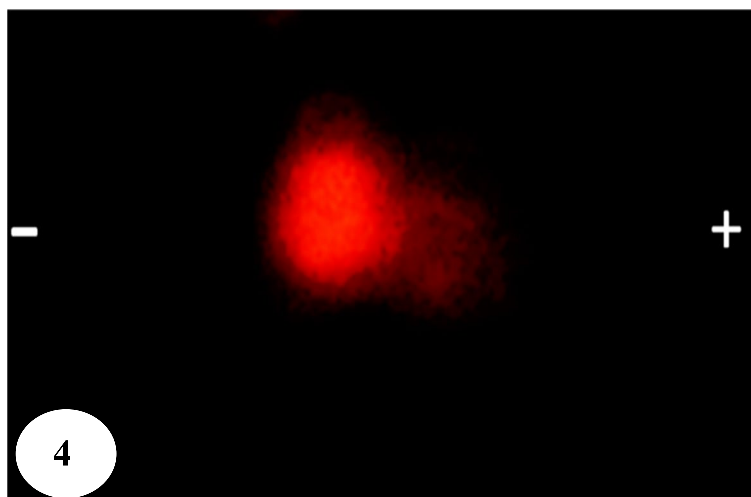

Fig. 4 Photomicrograph of comet assay showing the DNA migration patterns hepatocytes of mice which treated with (16 mg/kg b.wt.) of Tamiflu showing extensive DNA migration (slightly long tails). The symbols '-' and '+' represent cathode and anode, respectively, during the electrophoresis. (X: 1500)

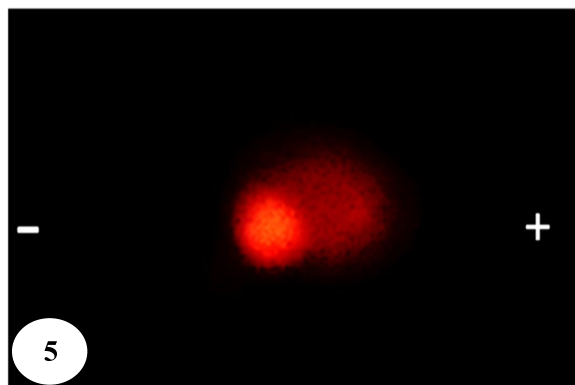

Fig. 5 Photomicrograph of comet assay showing the DNA migration patterns hepatocytes of mice which treated with (20 mg/kg b.wt.) of Adamine showing extensive DNA migration (slightly long tails). The symbols '-' and '+' represent cathode and anode, respectively, during the electrophoresis. (X: 1500)

Fig. 6 Photomicrograph of comet assay showing the DNA migration patterns hepatocytes of mice which treated with (16 mg/kg b.wt.) of Tamiflu concurrently with (20 mg/kg b.wt.) of Adamine extensive DNA migration (slightly long tails). The symbols '-' and '+' represent cathode and anode, respectively, during the electrophoresis. (X: 1500)

Table 2 The percentage of destruction degree of DNA as revealed by comet assay of hepatocytes of the control and treated male albino mice (*Mus musculus*)

Animal group	Destruction degree / %			
	0	1	2	3
C-group	66.6	28	5.3	0
T5	22.6	73.3	4	0
T10	14	34	52	0
T15	18	83.3	10.6	0
A5	40	18	30	12
A10	28.6	42.6	24.6	4
A15	26	32.6	32	2
TA5	8.6	32	55.3	4
TA10	15.3	36.6	42.6	5.3
TA15	12.6	53.3	34	0

qRT-PCR results

The expression levels of apoptosis regulator genes were evaluated after 5, 10 and 15 days of treatment. The mRNA expression of BAX (pro-apoptotic regulator gene) and BCL-2 (anti-apoptotic regulator gene) by qRT-PCR relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene in hepatocytes of control and all treated groups was detected in the present study.

The level of BAX and BCL-2 gene expression

The fold change and mean of Δ ct of BAX and BCL-2 mRNA expression of the control and all treated groups was given in (Table 3) and (Figs. 8 and 9). As shown in the table, the fold change of BAX was down regulated compared to the control except at T15, A10, A15 and TA5, while BCL-2 was up regulated except in A5 was down regulated compared to the control group.

The fold change of BAX was 0.44 ± 1.85, 0.35 ± 0.52 and 2.19 ± 0.86 after treatment with (16 mg/kg b.wt.) of Tamiflu for five, ten and fifteen days, respectively. The fold change was 0.74 ± 1.68, 2.85 ± 0.35 and 2.12 ± 0.52 after treatment with (20 mg/kg b.wt.) of Adamine for five, ten and fifteen days, respectively. The fold change was 1.07 ± 0.87, 0.68 ± 0.89 and 0.82 ± 0.6 after treatment with (16 mg/kg b.wt.) of Tamiflu simultaneously with (20 mg/kg b.wt.) of Adamine for five, ten and fifteen days, respectively compared to control group (fold change 1 ± 0.75).

While after treatment with (16 mg/kg b.wt.) of Tamiflu the fold change of BCL-2 was 9.98 ± 2.92, 2.67 ± 0.67 and 3.47 ± 3.26 for five, ten and fifteen days, respectively. The fold change was 0.70 ± 0.01, 7.84 ± 0.71 and 3.04 ± 0.96 after treatment with (20 mg/kg b.wt.) of Adamine for five, ten and fifteen days, respectively. The fold change was 5.01 ± 2.31, 8.13 ± 2 and 10.32 ± 1.64 after treatment with (16 mg/kg b.wt.) of Tamiflu simultaneously with (20 mg/kg b.wt.) of Adamine for five, ten and fifteen days, respectively compared to control group (fold change 1 ± 0.96).

Discussion

The observed genotoxic effect of Amantadine could be explained due to the results obtained by Staničová et al. [11], who studied the interaction of the antiviral agent Amantadine with calf thymus DNA by classical and UV- resonance Raman spectroscopy. It was found that: (i) the drug interacts with purine bases adenine and guanine via hydrogen bonds formation between N7 positions of purines and amino group of Amantadine and (ii) the interaction leads to partial DNA structure change, which is demonstrated by a deformation of the hydrogen bonds of the A–T base pairs and by a partial deformation of the sugar-phosphate backbone of

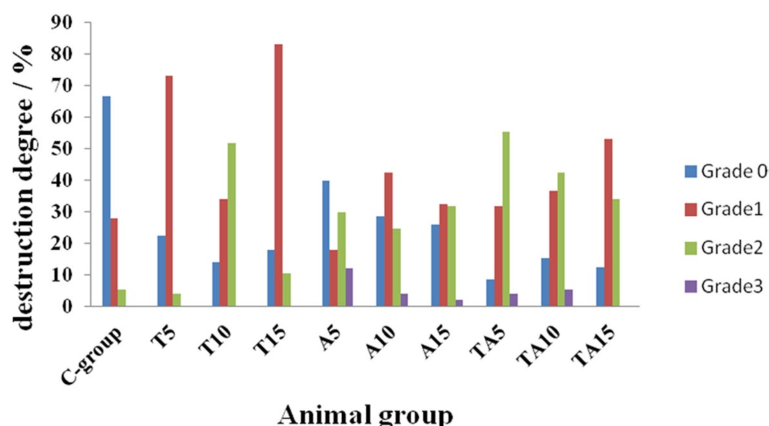

Fig. 7 Histogram represents the relationship between the percentage of destruction degree of DNA in hepatocytes of male albino mice in control and all treated groups as calculated in Table 2

Table 3 The Fold change of BAX and BCL-2 mRNA expression as revealed by qRT-PCR of hepatocytes of control and treated male albino mice (*Mus musculus*)

Animal group	Fold change		Mean of Δ ct ± SD	
	BAX	BCL-2	BAX	BCL-2
C-group	1	1	4.47 ± 0.75	9.58 ± 0.96
T5	0.44↓	9.98↑	5.67 ± 1.85	6.26 ± 2.92
T10	0.35↓	2.67↑	5.97 ± 0.52*	8.16 ± 0.67
T15	2.19↑	3.47↑	3.34 ± 0.86	7.79 ± 3.26
A5	0.74↓	0.70↓	3.94 ± 1.68	10.09 ± 12.36
A10	2.85↑	7.84↑	2.96 ± 0.35*	6.61 ± 0.71*
A15	2.12↑	3.04↑	3.39 ± 0.52	7.97 ± 0.96
TA5	1.07↑	5.01↑	4.38 ± 0.87	7.25 ± 2.31
TA10	0.68↓	8.13↑	5.03 ± 0.89	6.56 ± 2
TA15	0.82↓	10.32↑	4.76 ± 0.6	6.21 ± 1.64*

Level of significance:

Insignificant ($P > 0.05$)

*Significant ($P < 0.05$)

**Highly significant ($P < 0.001$)

DNA, which does not lead to the DNA conformation transition.

Also the results of the present work contradict the results obtained by Kaefer et al. [6], who examined deleterious effects on CNS induced by Amantadine, in adult male CF-1 mice that were treated with a systemic injection of Amantadine (15, 30 or 60 mg/kg b.wt.) 20 min before behavioral tasks on open field and inhibitory avoidance. The authors found that Amantadine was not able to induce chromosomal mutagenesis or toxicity on bone marrow, as evaluated by the micronucleus assay. At the lowest dose tested, Amantadine did not induce DNA damage and it was unable to impair memory, locomotion, exploration or motivation in mice. However, authors found that higher Amantadine doses increased DNA damage in brain tissue, produced locomotor disturbances severe enough to preclude testing for learning and memory effects, and induced stereotypy, suggesting neurotoxicity.

The results of the present study showed a decrease of BCL-2 expression after five days of treatment and an increase of BAX expression after ten and fifteen day of treatment; those results are in agreement with Lan et al. [7], who demonstrated that amantadine significantly induced apoptosis, reduced BCL-2 and increased the Bax protein and mRNA levels. Additionally, BCL-2/BAX ratios were lower in the two hepatocellular carcinoma cell lines following amantadine treatment.

In the present experiment, it showed that Tamiflu increases the expression levels of pro-apoptotic BAX gene in hepatocytes after fifteen days and after five days when it was given with Adamine and increases the expression levels of anti- apoptotic BCL-2 genes in mice hepatocytes after five, ten and fifteen days, While Adamine caused an increase in the expression levels of BAX and BCL-2 after ten and fifteen days. The results could be explained due to presence of BAX in the cytosol, under physiological conditions. An apoptotic trigger leads to its translocation into the mitochondrial membrane. Also, BAX can homodimerize or heterodimerize with pro-apoptotic members, thus forming mitochondrial pore and increasing membrane permeability, thereby releasing apoptogenic factors [3, 12]. The antiapoptotic protein, BCL-2, inhibits the ability of BAX to increase membrane potential [4] and antagonizing the apoptotic cascade by a direct interaction [14] and cell fate may be determined by balance of these proteins.

In this study we found that alteration in BAX/BCL-2 ratio is a key factor in the generation of apoptosis.

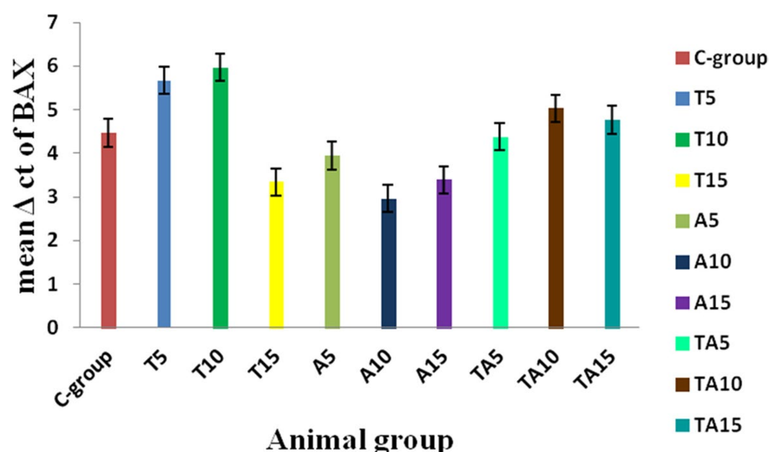

Fig. 8 Histogram represents the relationship between the means and standard deviation (± SD) of Δ ct of BAX in all nine treated groups and control group, as calculated in Table 3

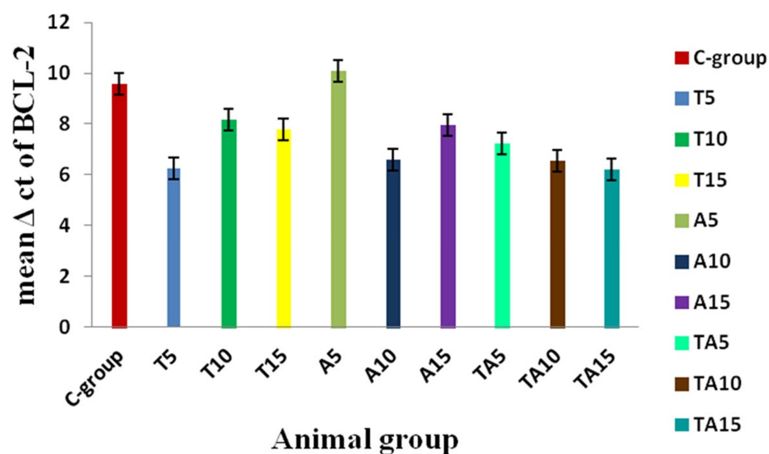

Fig. 9 Histogram represents the relationship between the means and standard deviation (± SD) of Δ ct of BCL-2 in all nine treated groups and control group, as calculated in Table 3

Increasing in this ratio may stimulate apoptosis, and a decrease in this ratio may reverse the injurious effect of cytotoxic stimuli and a nexus between liver injury and fibrosis [1, 2, 15].

Conclusion

In conclusion, the results of the present study illustrated that Tamiflu and Adamine separately or simultaneously caused deleterious impacts on DNA content of hepatocytes of treated mice. Whereas, the results of treatments with Adamine indicated pathogenic impacts more than that induced by Tamiflu.

Acknowledgements
Not applicable

Authors' contributions
HO performed the DNA examination in hepatocytes using comet assay and qRT-PCR, and was a major contributor in writing the manuscript. SS helped in the interpretation of the results and editing the manuscript. All authors read and approved the final manuscript

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Availability of data and materials
All the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate
Animal care and use protocols were carried out according to animal care guidelines approved by the authorities of Ain shams University. Committee's reference number isn't applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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