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Original Article

Potential Therapeutic Effect of Antioxidants on Toluene-induced Cardiotoxicity in Rats

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ABSTRACT

Workers are increasingly being exposed to the fumes of toluene, which is used extensively in many industrial processes. Toluene exposure has been connected to heart disorders such as ventricular tachycardia, coronary vasospasm, sinus bradycardia, and atrioventricular conduction difficulties. Alpha lipoic acid (ALA) and L-carnitine (LC) are antioxidants that may have cardioprotective benefits against chronic cardiotoxicity caused by toluene in male rats, therefore this study investigates these effects over a month in male rats. Toluene was administered for one month to male Sprague Dawley rats. ALA (50 and 100 mg/kg i.p.) and LC (150 mg/kg and 300 mg/kg i.p.) were administered during the last 15 days of treatment. Treatment with antioxidants had a strong therapeutic impact against myocardial damage caused by toluene, as evidenced by elevated levels of cardiotoxicity markers: The isoenzyme CK-MB (creatine kinase) and LDH (lactate dehydrogenase). Toluene significantly reduced glutathione levels, increased lipid peroxidation, and decreased the activities of glutathione reductase (GR) and glutathione peroxidase (GPx), antioxidant enzymes that are markers of oxidative stress. ALA and LC treatment significantly attenuated toluene-mediated-cardiac damage as well as oxidative damage, with LC being more effective than ALA. Furthermore, toluene induced apoptotic cardiac damage as evidenced by increased caspase-3 and caspase-12 activities. ALA and LC treatment attenuated these apoptotic actions of toluene. In addition, toluene increased calpain-2 activity, while ALA and LC ameliorated this effect. All of these results suggest that ALA and LC have a strong protective effect against the cardiotoxic effects of toluene by reducing oxidative stress, apoptotic tissue damage, and calpain-2 activity.

Keywords: *Toluene; cardiotoxicity; L-carnitine; alpha lipoic acid; calpain-2.*

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1. Introduction

One of the most often used industrial solvents is toluene, which can be found in a variety of paints, paint thinners, glues, and other commercial and residential goods. Toluene's quick effects after inhalation have made it the most overused solvent in the world change to **[1]**. Ninety-one percent of street children in Upper Egypt abuse inhalants, and thirty percent of those children misuse Kola glue, which contains toluene on a habitual basis with total ignorance knowing anything about its hazards to their health **[2]**. When it comes to substance misuse in Egypt, the research showed that the most popular kind was cigarettes, followed by sniffing glue, bango, pills, and a small percentage of users of combination substances **[3]**.

In Mexico, Colombia, and Japan, inhalant

use is among the most common kinds of substance misuse. Inhalant usage is widespread globally **[4]**.

In 2020, the National Institute on Drug Abuse **[5]** estimated that 3.8% of 12th graders, 7.4% of tenth graders, and 12.6% of eighth graders in the USA have ever huffed inhalants. In the eighth and tenth grades, the percentage of inhalers has dramatically grown (from 8.7 in 2018 to 12.6 in 2020) when compared to previous years. The majority of youngsters who reported using inhalants during the last year and within the last month were, once again, eighth graders (6.1% and 2.9%, respectively). This trend might continue when allegations of a rise in drug abuse linked to the COVID-19 epidemic surface **[6, 7]**.

In a study carried out in Pakistan, 416 kids in total, evenly distributed across the four cities of Karachi, Lahore, Peshawar, and Quetta, took part. Males made up the majority of respondents. The youngsters in Peshawar were marginally younger than the children in other cities, with a mean age of 14.3 years among those using solvents **[8]**. According to the study's statistical analysis, 88.46% of people engage in glue-sniffing. 40.38% of respondents in Nepal were between the ages of 9 and 12 **[9]**.

There are numerous detrimental consequences of toluene on various organs, including the heart. The action of toluene on several ion channels can account for the direct slowing of the sinoatrial node (SA) node and the susceptibility to certain cardiac arrhythmias. Toluene has been found to have an impact on calcium (Ca^{2+}), potassium (K^{+}), and sodium (Na^{+}) channels **[10]**. Heart rate left ventricular pressure, and perfusion pressure all rose after an acute toluene exposure. Lidocaine and nifedipine blocked these activities. According to our findings, acute toluene exposure alters the expression and function of voltage-gated sodium and calcium channels. This modification is most likely caused by a cardiac adrenergic mechanism, and these changes may contribute, if not entirely, to the development of ventricular arrhythmias **[11]**.

Toluene dose-dependently inhibited recombinant *N*-methyl-d-aspartic acid (NMDA) receptors at low concentrations in oocytes from *Xenopus laevis* **[12]**. Afterward, it was reported that toluene potentiates $GABA_A$ and glycine receptor-activated currents, and enhances 5-HT_3 receptor function **[13]** at similar concentrations to those found to be effective for the inhibition of NMDA receptors. Lately **[14]**, demonstrated that toluene also inhibited nicotinic acetylcholine receptors. Toluene's toxic effects on the kidney can lead to electrolyte imbalances and the development of severe arrhythmias **[15]**.

In biological systems, oxidative stress is the term used to describe abnormalities in redox biology. This phrase has been used to highlight the imbalance between antioxidants and oxidants, a topic of substantial investigation **[16]**. Antioxidants have received a lot of attention these days as a potential treatment for toluene-induced cardiotoxicity. To guard against the buildup of reactive oxygen species (ROS) and consequently, against oxidative stress, there are numerous antioxidant defense mechanisms in place. Non-enzymatic antioxidants such as α-lipoic acid (ALA), beta-carotene, ubiquinone, urate, reduced glutathione (GSH), and vitamins E and C are among them **[17]**. Plant sources naturally contain ALA, a naturally occurring antioxidant **[11]**. ALA is utilized as a nutritional supplement and can also be found in food **[18]**. One naturally occurring organosulfur component is called ALA **[19]**. Numerous diseases, including kidney and cardiovascular conditions, diabetes, neurological diseases, cancer, and aging, are largely caused by oxidative stress **[20]**. ALA is an antioxidant that is fat- and water-

soluble and has anti-inflammatory properties **[21]**. According to recent research, its therapeutic benefits are thought to be caused by changes in gene transcription and signal transduction, which raise the cell's antioxidant status **[22]**. Reduced vitamin C and vitamin E can be recycled from their oxidized states **[23]** later, which was proven to be an effective protective therapy against toluene-induced central nervous system damage **[24]**. The cardioprotective properties of ALA are explained by the following: decreased oxidative stress enhanced nitric oxide bioavailability and suppression of NADPH oxidase 4 (NOX4) activities, which results in nitric oxide synthase re-coupling and the preservation of mitochondrial function **[25]**.

L-carnitine (LC) also known as (β-hydroxy-γ-trimethylaminobutyric acid) is widely available in natural sources **[26]**. Skeletal muscles and heart muscles contain almost 90% of the body's stores **[27]**. LC can promote mitochondrial β-oxidation, which is necessary for the heart's energy metabolism to be balanced. By reducing oxidative stress, inflammation, and cardiac myocyte necrosis, long-chain fatty acids (LC) have a cardio-protective effect once they reach the mitochondrial matrix **[28]**.

This project is crucial for raising awareness about the deleterious effects of inhaling toluene on the heart among street children not only in Egypt, but also all over the world, and the potential ameliorative therapeutic effects of antioxidants in reversing toluene's deleterious effects. The current study aimed to investigate the potential therapeutic effects of the natural compounds, alpha lipoic acid and L-carnitine, on the cardiotoxicity associated with toluene abuse. To fulfill this aim, the following parameters were assessed:

- Cardiotoxicity markers: Lactate dehydrogenase and Creatine Kinase-MB (LDH and CK-MB)
- Histopathological examination: Hematoxylin and Eosin (H&E)
- Oxidative stress markers: Reduced glutathione (GSH) and the lipid peroxide marker: malondialdehyde (MDA)
- Antioxidant enzymes: Glutathione peroxidase (Gpx) and glutathione reductase (GR).
- Apoptotic marker: Caspase-12, caspase-3, and calpain-2.

2. Experimental Design

Six groups of ten rats each were randomly assigned, and the animals were handled as follows; Group 1: Paraffin oil injections were given to rats (1 mg, i.p, for one month daily) and served as negative control. Group 2: Toluene injections were given to rats (800 mg/kg, i.p, for one month daily) and acted as a positive control. This dose caused cardiac damage **[29]**. Group 3: Toluene injections were given to rats (800 mg/kg, i.p, for one month daily) + ALA (50 mg/kg, i.p for the final fifteen days of the daily treatment) **[30]**. Group 4: Toluene injections were given to rats (800 mg/kg, i.p) + ALA (100 mg/kg, i.p, for the final fifteen days of the daily treatment) **[31]**. Group 5: Toluene injections were given to rats (800 mg/kg, i.p, for one month daily) + LC (150 mg/kg, i.p, for the final fifteen days of the daily treatment) **[32]**. Group 6: Toluene injections were given to rats (800 mg/kg, i.p, for one month daily) + LC (300 mg/kg, i.p, for the final fifteen days of the daily treatment) **[32]**.

At the end of the trial, cardiovascular functions were examined. After that, the rats were anesthetized with ketamine (75 mg/kg, i.p.), blood was taken retro-orbitally, and tissues were removed and kept at -80 °C for biochemical research.

Ten hearts were extracted from each group, with half of the hearts removed for

histopathological analysis and the other half for biochemical investigations. Additionally, three sections were removed from each heart. Heart specimens were fixed in 10% buffered formalin for histopathological and immunohistochemical analyses.

All biochemical analyses were performed using tissue homogenate; however, serum was used for the assessment of CK-MB and LDH.

Since the toluene-treated group received injections for a month and had blood drawn after the treatment cycle, neither CK-MB nor LDH blood was collected at the day 15 timepoint.

Intraperitoneal injection of toluene produces the same effects as inhalation and this was discussed in several studies **[33, 34]**. At the same time, i.p injection is easier to handle than inhalation. Similar results to ours were reported in research by Arslan et al. on healthy individuals employed in the polishing industry who were exposed to toluene inhalation for three months. The study's HR and PR interval **[35]**. Another study on rats who breathed glue for ten minutes likewise found that the rodents developed bradycardia and had a longer PR interval **[36]**. Thus, toluene administered intraperitoneally was linked to cardiac depressive effects comparable to those seen with inhalation. Thus, toluene administered intraperitoneally was linked to cardiac depressive effects comparable to those seen with inhalation **[37]**. Our results showed that antioxidants were capable of protecting against the ECG changes and cardiac muscle injury caused by toluene, suggesting that administering toluene via the i.p. route did not interfere with the action of alpha lipoic acid and L-Carnitine.

3. Materials and Methods

3.1. Drugs and Chemicals

The supplier of toluene was Avantor Performance Materials in Poland. The supplier of ALA was Pharma ATOS in Egypt. LC was purchased from Mepacomedifood, Ellman's reagent [5,5'-dithio-bis (2-nitrobenzoic acid); DTNB], Ketamine (50 mg/mL), Orthophosphoric acid, Reduced glutathione (GSH), [1,1',3,3'-tetramethoxypropane] thiobarbituric acid (TBA) and From Sigma-Aldrich Co. (St. Louis, MO, USA), tris bases were acquired.

3.2. Animals

We utilized adult male Sprague-Dawley rats weighing 130–140 g. The animals were acquired from The National Research Center's breeding colony in Cairo, Egypt's animal home. Throughout the trial, every animal was kept in a typical laboratory setting with 25 ± 2 ℃ temperature, 60-70% humidity, and a 12 h light/dark cycle. They were also fed standard laboratory pellets (20% proteins, 5% lipids, and 1% multivitamins) and had unrestricted access to tap water. Before testing, the animals were given at least a week to acclimate. The hours that the experiments were conducted were 9:00 am and 3:00 pm. The National Research Center's Ethics Committee and the Ain Shams University Ethics Committee approved the use of animals in research operations.

3.3. Electrocardiography (ECG)

To guarantee that every animal under anesthesia had a normal ECG pattern, the experiment started with an ECG recording. Using a Bioscience recorder, the ECG of rats administered ketamine was recorded 24 h following the conclusion of the treatment cycle (Bioscience, Washington, USA). Rats that had been given anesthesia were put on a board and made to lie supine. At position II (right forelimb to left hind limb), needle electrodes were implanted under the skin. Each recording lasted for a minimum of five minutes. The voltage was 1 mV/cm and the ECG recording speed was 50 mm/s. To reduce noise, a digital filter was used. Analysis of ECG waves was conducted to

calculate the heart rate (beats/min), QRS duration (ms), QT interval (ms), which was corrected for heart rate using the Bazzer formula [QTc=QT/square root of RR interval)], and PR interval (ms). Every five minutes, three non-consecutive, randomly selected points were used to measure each parameter. The average of three segments chosen at random was used to report the results.

3.4. Assessment of Cardiotoxicity Indices

Using readily accessible commercial kits, the activities of lactate dehydrogenase (LDH) and creatine kinase isoenzyme-MB (CK-MB) were measured following conventional procedures (Spectrum Diagnostics, Cairo, Egypt).

3.5. Assessment of Oxidative Stress Markers

To quantify the reduced glutathione concentration (GSH), 0.5 mL of homogenate was added to 0.5 mL of 10% trichloroacetic acid in a tube. After being gently and irregularly shaken for fifteen minutes, the tubes were centrifuged for five minutes at 2000 rpm. An aliquot of the resulting supernatant (0.2 mL) was transferred to a tube containing 1.7 mL phosphate buffer and 0.1 mL Ellman's reagent, and the absorbance at 412 nm was recorded within 5 min. **[38]**. Millimoles per gram of moist tissue were used to express the results. Lipid peroxidation was determined by estimating the quantity of reactive compounds evaluated as malondialdehyde (MDA) by thiobarbituric acid **[39]**. In conclusion, the reaction mixture (0.5 mL homogenate + 3 mL 1% orthophosphoric acid + 1 mL 0.67% thiobarbituric acid) was heated for 30 min. in a boiling water bath. After cooling the liquid, the absorbance at 532 nm was determined. The information was displayed as moles of MDA for each gram of wet tissue.

3.6. Assessment of Antioxidant Enzyme Activities

Glutathione reductase (GR) and glutathione peroxidase (Gpx) activities were assessed in heart homogenate using kits provided by Biodiagnostics, Giza, Egypt. GR activity was determined according to the method of Goldberg **[40]**. Results were expressed as U/L. GPx was determined according to the method of Paglia **[41]**. The results were expressed as mU/mL.

3.7. Assessment of Apoptotic Markers

To evaluate apoptosis induced by the sarcoplasmic reticulum, the activity of neutral proteinase 2 (calpain 2), caspase-3, and caspase 12 were measured.

Using kits from Thermofisher Co., USA, and Sigma Chemical Co., USA, respectively, the activities of caspase-12 and calpain-2 were measured following the manufacturer's procedure. NBP275020 and AB81023 are the catalog numbers for caspase-12 and calpain-2, respectively. Calpain-2 and Caspase-12 results were given as ng/mL. Caspase-3 was analyzed immunohistochemically.

3.8. Histopathological Examination

Sections of the myocardium from the ventricles and atria of autopsied rats from various groups were preserved in 10% formol saline for a duration of twenty-four hours. After washing with tap water, the subjects were dehydrated using successive dilutions of alcohol (methyl, ethyl, and absolute ethyl). Specimens were cleaned with xylene and embedded in paraffin for 24 h at 56 degrees in a hot air oven. Using a sliding microtome, tissue blocks made of paraffin wax from bees were created for sectioning at a thickness of 4 microns. Following deparaffinization and staining with hematoxylin and eosin, the resulting tissue sections were placed on glass slides for routine examination using a light electric microscope **[42]**.

To illustrate toluene's Cardiotoxic effect on

the heart, the following were mentioned:

Focal inflammatory cell infiltration with edema and haemorrhages as well as congested blood vessels

Atrophied myocardium

3.9. Immunohistochemical Detection of Caspase-3

Heart tissue sections fixed in paraffin, measuring 3 µm in thickness, were rehydrated in xylene and then graded ethanol solutions. After that, the slides were blocked for two hours with 5% BSA in tris-buffered saline (TBS). Following overnight incubation at 4 ºC, the sections were immune-stained using a polyclonal rabbit anti-caspase-3 antibody at a concentration of 1 µg/mL in TBS containing 5% bovine serum albumin (BSA). The sections were treated with goat anti-rabbit secondary antibody after the slides were cleaned with TBS. Following a TBS wash, sections were incubated in a 0.02% diaminobenzidine containing 0.01% H_2O_2 solution for 5 to 10 min. After applying the H&E counterstain, the slides were examined under a light microscope **[43]**. Leica MDLSD image analysis software was used to quantify the immunostaining. For every rat segment, it was shown as optical density (O.D.) over ten distinct fields. However, it is not possible to test cleaved caspase-3. Moreover, in cell biology, caspase-3 antibodies that identify both cleaved and uncleaved forms of the enzyme are potent markers of the induction of cell death **[44]**.

3.10. Statistical Analysis

The findings were presented as mean ± SD. One-way Analysis of Variance (ANOVA) was used to compare means, and the Tukey-HSD post hoc test was used after. A significant threshold of $p < 0.05$ was established. Version 6 of Graph Pad Prism was utilized to conduct all statistical analyses.

4. Results

4.1. ECG

The control group's ECG trace revealed normal cardiac activity **(Fig. 1A)**. The ECG alterations in the toluene-treated group included bradyarrhythmia, QRS shortening, PR interval lengthening, and QTC interval shortening **(Fig. 1B)**. The intoxicated rats treated with ALA (50 mg/kg) and LC (150 mg/kg) **(Fig. 1C, E)** showed a moderate improvement in these ECG abnormalities; with ALA (100 mg/kg) **(Fig. 1D)** and LC (300 mg/kg) **(Fig. 1F),** the abnormalities showed a significant improvement. In contrast to the control group, the toluene group's heart rate dropped, while the ALA (100 mg/kg) group's heart rate returned to normal **(Table 1 and Fig. 1)**. LC (300 mg/kg) normalized the length of the QRS and QTc.

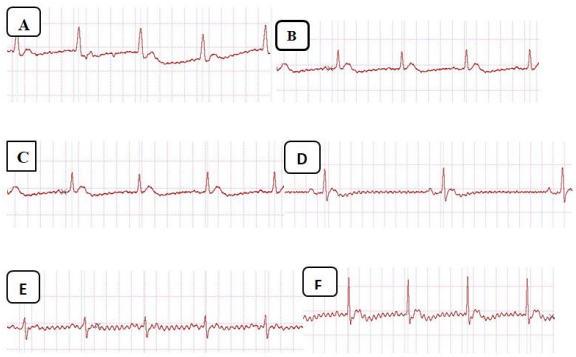

Fig. 1. A: normal ECG of negative control rats, B: ECG of positive control rats given Toluene (800 mg/kg) i.p. bradyarrhythmia, shortening of QRS, prolongation of PR interval and shortening of Qtc interval is shown, C: ECG of rats given Toluene (800 mg/kg) + ALA (50 mg/kg) i.p. showed improvement in ECG parameters, D: ECG of rats given Toluene (800 mg/kg) + ALA (100 mg/kg) i.p, normalized the four measured parameters, E: ECG of rats given Toluene (800 mg/kg) + LC (150 mg/kg) i.p, didn't normalize any parameter, F: ECG of rats given Toluene (800 mg/kg) + LC (300 mg/kg) i.p. normalized H.R, Qtc and QRS duration.

Table 1. Effect of treatment with ALA and LC on the ECG parameters (heart rate, Qtc duration, QRS duration, and PR interval) in toluene-induced cardiotoxicity in rats

Treated groups	Heart rate (beat/min)	QTc interval milliseconds (ms)	QRS duration (ms)	PR interval (ms)
Control –ve N= 10	256.8±4.215	0.1393±0.003327	0.04983±0.003061	0.04027±0.005636
Toluene (800 mg/kg) N= 10	53.72* ±2.923	0.05589*±0.003024	0.02333* ±0.003933	0.09398* ±0.004839
Toluene (800 mg/kg)+ALA (50 mg/kg) N= 10	179.2*$^{@bc}$±21.94	0.08999$^{*@bc}$±0.006676	0.03233$^{*@bc}$ ± 0.001366	0.07472$^{*@bd}$±0.001402
Toluene (800 mg/kg)+ALA (100 mg/kg) N= 10	250.7$^{@ac}$±1.633	0.1300$^{@ac}$±0.004116	0.0455$^{@ac}$ ± 0.001761	0.03413$^{@ad}$±0.001885
Toluene (800 mg/kg)+LC (150 mg/kg) N= 10	220.7*$^{@ab}$ ± 2.824	0.1146$^{*@abd}$±0.002623	0.0385$^{*@ab}$±0.001225	0.0534$^{*@a}$±0.001318
Toluene (800 mg/kg)+LC (300 mg/kg) N= 10	277.8$^{@a}$±8.909	0.1454$^{@ac}$±0.006648	0.05617$^{@a}$ ± 0.002787	0.02117$^{*@ab}$±0.001029

The mean ± SD of the data; N= rats number; -ve: negative

* $p<0.05$ indicates a significant difference from the control group; $^{@}$ $p<0.05$ indicates a significant difference from the toluene (800 mg/kg) group; ^{a} $p<0.05$ indicates a significant difference from the ALA (50 mg/kg) group; ^{b} $p<0.05$ indicates a significant difference from the ALA (100 mg/kg) group; ^{c} $p<0.05$ indicates a significant difference from the LC (150 mg/kg) group; ^{d} $p<0.05$ indicates a significant difference from the LC (300 mg/kg) group; One-way ANOVA was used for statistical analysis, and the Tukey-HSD test was used for multiple comparisons.

4.2. Cardiotoxicity Indices

Toluene administration caused a significant increase in the activities of cardiac markers CK-MB and LDH by 113.84% and 104.64% respectively as compared to the control group, indicating myocardial injury. Treatment of intoxicated animals with ALA (50 mg/kg) significantly reduced cardiac markers activities by 22.47% and 12.97% respectively compared to the toluene-treated group **(Table. 2)**. Noteworthy, ALA (100 mg/kg) normalized both CK-MB and LDH activities compared to the toluene-treated group. Moreover, LC (150 mg/kg) normalized CK-MB activity while it decreased LDH activity significantly by 27.34% compared to the toluene-treated group; while LC (300 mg/kg) normalized both of the cardiac markers activities **(Table. 2)**.

4.3. Oxidative Stress Markers and Antioxidant Enzymes

To identify the toluene-induced redox imbalance, the cardiac homogenate's GSH and MDA levels, as well as GPx and GR activities, were measured. As shown in **Table 3**, toluene

significantly decreased GSH level by 33.93% and increased lipid peroxide level by 73.10% as compared to the control group. In addition, compared to the control group, toluene caused a drop in antioxidant enzymes, GPx and GR, of 63.28% and 76.59%, respectively. When compared to the toluene group, treating intoxicated rats with lower doses of ALA (50 mg/kg) and LC (150 mg/kg) may have partially increased GSH levels, GPx and GR activity, and decreased MDA levels. In the meantime, the larger doses of LC (300 mg/kg) and ALA (100 mg/kg) administered to intoxicated animals resulted in a restoration of GSH, MDA, GPx, and GR activities to almost those of the control group.

Table 2. Effect of ALA (50 mg/kg and 100 mg/kg) and LC (150 mg/kg and 300 mg/kg) on cardiotoxicity markers creatine kinase-MB and Lactate dehydrogenase (CK-MB and LDH) in toluene-induced cardiotoxicity in rats

Treated groups	CK-MB (U/L)	LDH (U/L)
Control –ve N=10	125.7±7.537	131.5±5.023
Toluene (800 mg/kg) N=10	268.8^{*abcd}±13.52	269.1^{*abcd}±4.992
Toluene (800 mg/kg)+ALA (50 mg/kg) N=10	$208.4^{*@bc}$ ± 10.41	$226.4^{*@bd}$±8.235
Toluene (800 mg/kg)+ALA (100 mg/kg) N=10	$157.2^{@ ac}$ ± 4.403	$125.7^{@acd}$±5.681
Toluene (800 mg/kg)+LC (150 mg/kg) N=10	$124.7^{@ab}$ ± 3.779	$189^{*@bd}$±11.64
Toluene (800 mg/kg)+LC (300 mg/kg) N=10	$118.4^{@a}$ ± 10.25	$118.3^{@abc}$±4.663

The mean ± SD of the data; N= rats number; -ve: negative

* $p<0.05$ indicates a significant difference from the control group; $^{@}$ $p<0.05$ indicates a significant difference from the toluene (800 mg/kg) group; ^{a} $p<0.05$ indicates a significant difference from the ALA (50 mg/kg) group; ^{b} $p<0.05$ indicates a significant difference from the ALA (100 mg/kg) group; ^{c} $p<0.05$ indicates a significant difference from the LC (150 mg/kg) group; ^{d} $p<0.05$ indicates a significant difference from the LC (300 mg/kg) group; One-way ANOVA was used for statistical analysis, and the Tukey-HSD test was used for multiple comparisons.

Table 3. Effect of ALA (50 and 100 mg/kg) and LC (150 and 300 mg/kg) on oxidative stress markers (MDA and GSH) and antioxidant enzymes (GPx and GR) in toluene-induced cardiotoxicity in rats

Treated groups	MDA (nmole/g)	GSH (µmol/g)	GPx (mU/mL)	GR (U/L)
Control –ve N= 10	168.8±22.80	0.3560±0.004099	10.15 ±0.8591	12.16 ±0.1787
Toluene (800 mg/kg) N1=10	292.2^{*abcd}±7.252	0.2352^{*abcd}±0.003920	3.727^{*abcd}±0.7348	2.847^{*abcd} ±0.7564
Toluene (800 mg/kg)+ALA (50 mg/kg) N=10	$243.7^{*@bc}$±15.22	$0.2643^{*@bd}$±0.007038	$6.317^{*@bd}$±0.5166	$5.292^{*@bcd}$ ±0.1030
Toluene (800 mg/kg)+ALA (100 mg/kg) N=10	$146.7^{@a}$±8.076	$0.4605^{*@acd}$±0.01814	$13.45^{*@acd}$±0.8676	$14.11^{@acd}$ ±0.8838
Toluene (800 mg/kg)+LC (150 mg/kg) N=10	$206.5^{*@a}$±11.35	$0.3370^{@bd}$±0.01568	$10.63^{@bd}$±0.4453	$10.80^{@abd}$ ±0.7501
Toluene (800 mg/kg)+LC (300 mg/kg) N=10	$152.7^{@}$±6.428	$0.4010^{@abc}$±0.006573	$12.25^{@abc}$±0.8704	$12.9^{@abc}$ ±0.1038

The mean ± SD of the data; N= rats number; -ve: negative

* $p<0.05$ indicates a significant difference from the control group; $^{@}$ $p<0.05$ indicates a significant difference from the toluene (800 mg/kg) group; ^{a} $p<0.05$ indicates a significant difference from the ALA (50 mg/kg) group; ^{b} $p<0.05$ indicates a significant difference from the ALA (100 mg/kg) group; ^{c} $p<0.05$ indicates a significant difference from the LC (150 mg/kg) group; ^{d} $p<0.05$ indicates a significant difference from the LC (300 mg/kg) group; One-way ANOVA was used for statistical analysis, and the Tukey-HSD test was used for multiple comparisons.

4.4. Sarcoplasmic Reticulum-mediated Apoptosis

Additionally, the assessment of sarcoplasmic reticulum-mediated apoptosis was conducted by measuring the cardiac activities of caspase-12 **(Fig. 2B)** and calpain-2 **(Fig. 2A)**. Myocardial calpain-2 was significantly elevated by toluene (1331.28%) in comparison to the control group. When compared to the toluene group, treatment with ALA and LC at both doses resulted in a substantial decrease in calpain-2 activity.

Assessing caspase-12 activity allowed for additional confirmation of apoptosis. When compared to the control group, toluene greatly enhanced caspase-12 activity by 1268.05%. However, when compared to the toluene group, the administration of ALA and LC at both doses to intoxicated animals resulted in a significant decrease in caspase-12 activity, indicating anti-apoptotic actions.

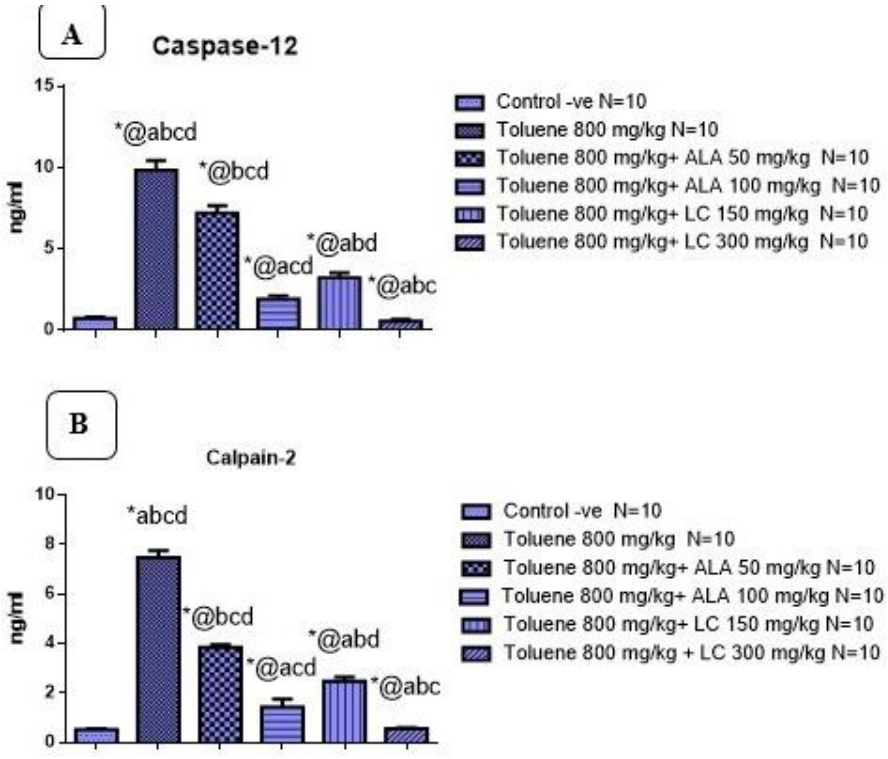

Fig. 2A. Effect of ALA (50 and 100 mg/kg) and LC (150 and 300 mg/kg)on calpain-2 level in toluene-induced cardiotoxicity in rats.

Fig. 2B. Effect of ALA (50 and 100 mg/kg) and LC (150 and 300 mg/kg) on caspase-12 level in toluene-induced cardiotoxicity in rats.

* $p<0.05$ indicates a significant difference from the control group; @ $p<0.05$ indicates a significant difference from the toluene (800 mg/kg) group; a $p<0.05$ indicates a significant difference from the ALA (50 mg/kg) group; b $p<0.05$ indicates a significant difference from the ALA (100 mg/kg) group; c $p<0.05$ indicates a significant difference from the LC (150 mg/kg) group; d $p<0.05$ indicates a significant difference from the LC (300 mg/kg) group; One-way ANOVA was used for statistical analysis, and the Tukey-HSD test was used for multiple comparisons.

4.5. Histopathological Examination

An investigation into the histology of the toluene-induced cardiotoxicity was carried out. The control group's heart sections **(Fig. 3A)** stained with H&E revealed regular cellular distribution and normal myocardial architecture. The group that was exposed to toluene contains multiple images because the various pathological damages are depicted in **(Fig. 3B, C, D, and E)**. The ventricular myocardium displayed fatty changes with cellular vacuolization of certain focal myocardial cells in the toluene group **(Fig. 3E)** and focal inflammatory cell infiltration **(Figs. 3B and C)** linked to blood vessel congestion **(Fig. 3D)**. The degenerated atrophied atrial myocardium in the group treated with ALA (50 mg/kg) showed focal inflammatory cell infiltration, edema, hemorrhages, and congested blood vessels **(Fig. 3F)**. It's interesting to note that the group receiving ALA treatment (100 mg/kg) did not exhibit any histological changes **(Fig. 3G)**. In the group treated with LC (150 mg/kg), the ventricular myocardium had focal inflammatory cell infiltration **(Figs. 3J and K)** and focal bleeding in between the bundles **(Figs. 3H and I)**. As seen in **(Fig. 3L)**, there was no histological aberration observed in the group treated with LC (300 mg/kg).

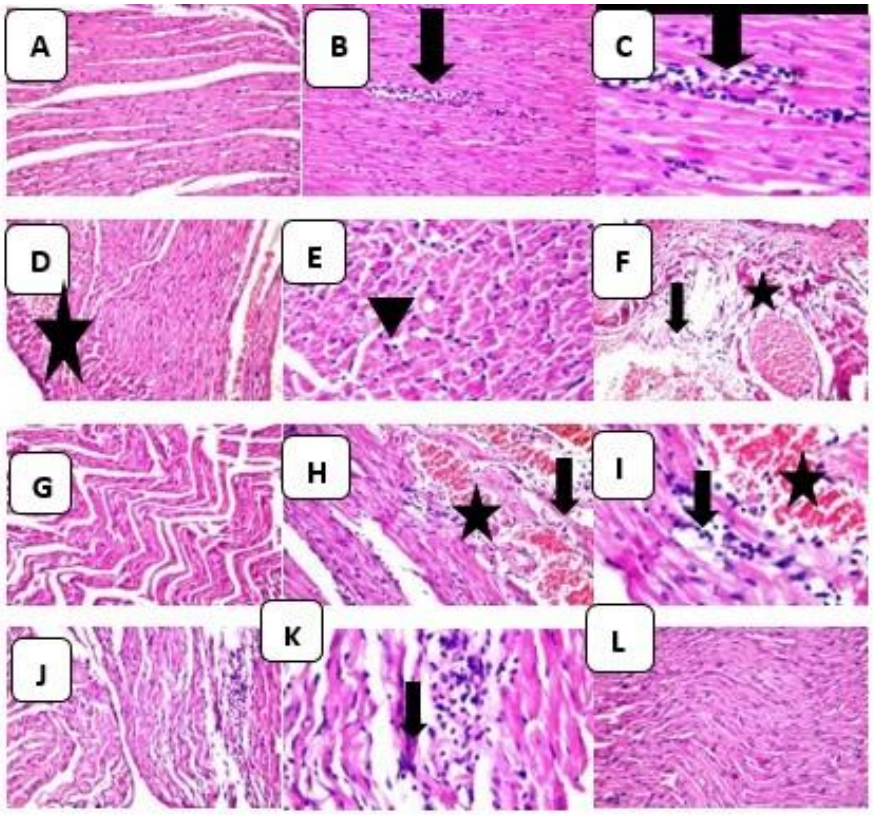

Fig. 3. Photomicrographs of hematoxylin and eosin-stained sections of the heart
(A): Photomicrograph of heart section of control group showing normal vesicular nucleas and normal structure of the myocardium (O.M.X 40); (B)&(C): Photomicrograph of heart section of toluene-treated (800 mg/kg) group that showing myocardium of the ventricles showed focal inflammatory cells infiltration (arrows) (O.M.X 40 and O.M.X 80) respectively; (D): Photomicrograph of heart section of toluene-treated (800 mg/kg) group showing congestion in the blood vessels (star) (O.M.X 40); (E): Photomicrograph of heart section of toluene-treated (800 mg/kg) group showing cellular vacuolization of some focal myocardial cells (triangle) (O.M.X 80); (F) Photomicrograph of heart section of toluene-treated (800 mg/kg) group + ALA (50 mg/kg) group showing Focal inflammatory cells infiltration (arrow) with edema and hemorrhages as well as congested blood vessels (Star) (O.M.X 40); (G): Photomicrograph of heart section of toluene-treated (800mg/kg) group + ALA (100 mg/kg) group showing normal structure of the myocardium (O.M.X 40); (H)&(I): Photomicrograph of heart section of toluene-treated (800 mg/kg) group + LC (150 mg/kg) group showing focal hemorrhages (star) (O.M.X 40 and O.M.X 80) respectively; (J)&(K): Photomicrograph of heart section of toluene-treated (800mg/kg) group + LC (150 mg/kg) group showing focal inflammatory cells infiltration (arrow) (O.M.X 40 and (O.M.X 80) respectively; (L): Photomicrograph of heart section of toluene-treated (800mg/kg) group + LC (300 mg/kg) showing normal structure of the myocardium (O.M.X 40).

4.6. Apoptotic Marker

The expression of caspase-3, an executive protein linked to apoptosis, was investigated by immunohistochemistry. The control group displayed negative immunostaining for the caspase-3 enzyme in terms of protein expression **(Fig. 4A)**. The myocardium displayed significant alterations following toluene injection, as demonstrated by the black arrow indicating tissue granulation and the positive expression of caspase-3 **(Fig. 4B and C)**. Caspases-3 was moderately expressed when ALA (50 mg/kg) was administered **(Fig. 4D)**. On the other hand, ALA (100 mg/kg) resulted in no caspase-3 expression **(Fig. 4E)**. Whereas LC (300 mg/kg) produced no expression in **(Fig. 4H)**, LC (150 mg/kg) produced considerable caspase-3 expression as shown in **(Figs. 4F and G)**.

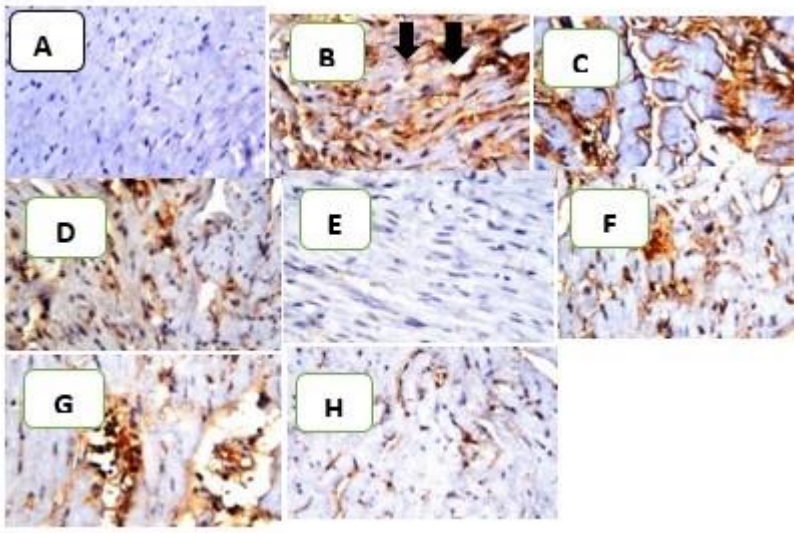

Fig. 4. Photomicrographs of caspase-3 stained sections of the heart

(A): Photomicrographs of caspase-3 stained sections of the heart showing control group nill caspase-3 expression (O.M.X 80); (B)&(C): Photomicrographs of caspase-3 stained sections of the heart showing toluene-treated (800 mg/kg) group showing tissue granulation (arrow) +++ caspase-3 expression (O.M.X 80); (D): Photomicrographs of caspase-3 stained sections of the heart showing toluene (800 mg/kg) + ALA (50 mg/kg) group showing (++) moderate caspase-3 expression (O.M.X 80); (E): Photomicrographs of caspase-3 stained sections of the heart showing toluene (800 mg/kg) + ALA (100 mg/kg) group showing nill caspase-3 expression (O.M.X 80); (F)&(G): Photomicrographs of caspase-3 stained sections of the heart showing toluene (800 mg/kg) + LC (150 mg/kg) group showing (++) moderate caspase expression (O.M.X 80); (H): Photomicrographs of caspase-3 stained sections of the heart showing toluene (800 mg/kg) + LC (300 mg/kg) group showing nill caspase-3 expression (O.M.X 80).

5. Discussion

The results of this investigation showed that a month-long exposure to toluene caused notable changes in the heart's biochemistry, histology, and immunohistochemistry. Toluene reduced the levels of GSH, GR, and GPx antioxidant enzymes, and raised the lipid peroxidation marker, MDA. Additionally, toluene raised the activity of caspase-12 in heart homogenate and calpain-2, which measures the amount of calcium in the heart. To evaluate toluene-induced cardiotoxicity, heart tissue was examined histopathologically, the cardiotoxicity markers LDH and CK-MB were biochemically analyzed, and an ECG was carried out. The toluene-treated group immunohistochemistry results for the apoptotic marker caspase-3 were positive. Histopathological examination revealed signs of cardiotoxicity as the ventricle's myocardium displayed cellular vacuolization of certain localized myocardial cells and focal inflammatory cell infiltration linked to blood vessel congestion.

It is believed that the lipophilic compound toluene alters the lipid composition of the cell wall and increases membrane fluidity by raising Na/K-ATPase activity **[45]**. When toluene is present in high concentrations, it is also believed to have an impact on the GABAergic, glutamatergic, serotonergic, and dopaminergic pathways **[46, 47]**. Studies have previously demonstrated that toluene causes apoptosis, with an increase in reactive oxygen radicals being identified as the trigger **[48, 49]**.

The primary mechanism of cellular damage resulting from exposure to toluene is the production of free oxygen radicals **[50]**. These O_2 radicals can result in the production of oxidative stress **[51]**.

Toluene toxicity may be largely caused by oxidative stress, disruption of the GSH: GSSG ratio, chronic inflammatory changes, and apoptosis **[52]**.

According to our findings, toluene significantly increased tissue MDA (lipid peroxidation product) as compared to the vehicle control, which is in line with our earlier findings from studies in which toluene was administered at a dose of 1132 mg/kg.p **[24]** and consistent with as previously described, **[53]** that administered toluene for 4 months 8h/day, 6 days/week at a high lethal inhalation dose of 3000 ppm. There were also decreased antioxidant levels where toluene-intoxicated rats showed significant depletion of reduced glutathione (GSH) as well significant inhibition of GPx and GR **[54]** already stated, decreased GPx activity in rats treated with toluene at a dose of 500 mg/kg for 14 days. The authors proposed that, depending on the dose that reduced the activity of antioxidant systems, free radicals are generated that are either directly or indirectly produced by toluene through the impairment of antioxidant defense mechanisms. Additionally, authors have previously noted a significant drop in GR activity and GSH levels following toluene treatment at a dose of 650 mg/kg for 45 days **[52]**.

Our results demonstrated that the administration of ALA and LC greatly reduced the oxidative stress caused by toluene. Furthermore, there was a noteworthy elevation in GSH levels and an improvement in GPx and GR activity. Both are recognized as strong antioxidants **[55]** and were shown to combat oxidative stress in experimental models **[56]**. ALA has been demonstrated to counteract the harmful effects of cadmium and mercury in some studies **[57]**.

Calpains are a type of cysteine proteases that are dependent on calcium and are widely expressed in mammals and various other animals. In damaged hearts, calpain activation is seen, and it is linked to inflammation, fibrosis, hypertrophy, and cardiac cell death **[58]**. When calpains are activated, they break down nuclear and membrane substrates, which causes the cellular structure to collapse and ultimately results in apoptosis **[59]**. In our investigation, the administration of toluene increased calpain-2 activity, while the two distinct dosages of LC and ALA both reduced calpain-2 activity in the cardiac homogenate. As far as the authors are aware, this is the first study to examine the impact of toluene on calpain, which is directly linked to the previously stated calpain-2 and myocardial injury **[60]**.

Calpain-2 cleaves caspase-12 and stimulates its activation during apoptosis produced by endoplasmic reticulum stress **[61]**. In a study using knockout mice, Caspase-12 was found to be the initiating caspase of endoplasmic reticulum stress-mediated apoptosis **[62]**. It was discovered that when caspase-12 is activated, caspase-9 is activated, which in turn activates caspase-3, ultimately resulting in apoptosis as previously mentioned **[63]**. Our current investigation also showed positive expression of caspase-3 assessed by immunohistochemistry and an increase in caspase-12 activity in cardiac homogenate as a result of toluene exposure **[64]**. High doses of toluene (6 ml/kg gavage) were administered, and histopathologic analysis of heart tissue sections verified positive immunoreactivity for caspase-3 protein in the toluene-treated group relative to the control group. The ventricles myocardium also revealed fatty changes with cellular vacuolization of some focal myocardial cells and infiltration of focal

inflammatory cells, which is consistent with the results of our study. In myocardial infarction rats, erythropoietin enhances cardiac function by suppressing caspase-12 expression, which may shield the myocardium by preventing endoplasmic reticulum stress-induced cardiomyocyte apoptosis and enhancing cardiac performance **[65]**. As opposed to the toluene group, ALA and LC improved the immunoreactivity of both caspase-12 and caspase-3 in this demonstration.

In our investigation, significant bradycardia, an elevated PR interval, and shorter QTc and QTS lengths all supported toluene's arrhythmogenic potential.

This supports a study that suggested long-term exposure to toluene impairs cardiac autonomy, specifically by inhibiting sympathetic activity and parasympathetic suppression. The study also showed bradycardia and an elevated PR interval, which are signs of a heart block **[66]**. Compared to the toluene group, ALA and LC restored the heart rate and ECG values to normal.

When we measured CK-MB and LDH-1 in serum, we found that the group treated with toluene had higher levels than the control group. According to a study, isoproterenol given S.C. at a dose of 5 mg/kg b.w. for two months boosted LDH activities, and CK-MB at a high dose of 54 mg/kg b.w. **[67]**.

Furthermore, a study revealed that lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) levels in the serum were significantly elevated by doxorubicin and were reduced after two weeks of LC 300 mg/kg administration **[68]**.

Limitations of the Study

The primary constraints of our research were the absence of cardiac echo measurements and the omission of Troponin T measurements. An additional constraint on our research was the inability to evaluate the impact of additional compounds (such as xylene and n-hexane) present in benzene at lower concentrations. Regretfully, we only looked into two antioxidants.

Conclusion

It was demonstrated that long-term exposure to toluene induced a state of oxidative stress in the heart as well as inflammation and apoptosis. It increased MDA level, reduced antioxidant enzymes; GR and GPx activities in tissue, and decreased GSH level, it caused conductivity and contractility defects and fiber damage as confirmed by increased cardiac enzymes CK-MB and LDH-1 levels in serum as well as by histopathological examination. It also resulted in increased caspase-3 expression and caspase-12 activity in tissue accompanied with increased calpain-2 activity in heart tissue. Histopathological changes indicative of apoptosis as well as focal inflammatory cell infiltration associated with congestion in the blood vessels and fatty change with cellular vacuolization of some focal myocardial cells were seen in the toluene group.

The harmful effects of LC and ALA were mitigated by toluene. They have anti-apoptotic and anti-inflammatory properties, significantly decreased oxidative stress, and strengthened antioxidant defense. Accordingly, our findings imply that due to their anti-apoptotic and antioxidant properties, LC and ALA may be significant cardio-therapeutic agents in the treatment of toluene addiction.

Recommendations

For future studies, we can investigate the toxic effect of toluene on diseased myocardium as well as in the presence of arrhythmia, in addition, we can measure more than two antioxidants. Moreover, we can measure more biomarkers like troponin T as well as do an ECHO of the heart, as well as more than two

antioxidants.

Declarations

Ethics Approval and Consent to Participate

Ain Shams University Ethics Committee permission number: 59

Consent to Publish

Not applicable.

Availability of Data and Materials

All data generated and analyzed in this study are included in the main published article and this manuscript.

Competing Interests

The authors declare that no competing interests exist.

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Authors' Contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Mariam Maged and Marwa El Shamarka. The first draft of the manuscript was written by Mariam Maged and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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