













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## Therapeutic potential of branched-chain amino acids against carbon tetrachloride-induced cardiopulmonary injuries in Wistar rats

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### ABSTRACT

**Background:** Carbon tetrachloride (CCl<sub>4</sub>) is a xenobiotic hepatotoxic agent that causes pathophysiological disorders in tissues and the liver. CCl<sub>4</sub>-induced cardiopulmonary toxicity is attributed to free radical-induced dyslipidemia and oxidative damage. Branched-chain amino acids (BCAAs) stimulate systemic defense against oxidative stress, which is probably caused by CCl<sub>4</sub>, through an improvement in the antioxidant system.

**Aim:** This study aimed to explore the therapeutic benefits of BCAAs against CCl<sub>4</sub>-induced cardiopulmonary pathologies.

**Methods:** A total of 28 inclusive Wistar rats stand under control was standard; group 2 comprised CCl<sub>4</sub>-received rats, whereas groups 3 and 4 comprised CCl<sub>4</sub>-rats supplemented with two varying levels of BCAAs.

**Results:** Biochemical findings indicated that CCl<sub>4</sub> provoked a rise in glucose and cholesterol levels compared with the control. The oxidative profile suggested increased malondialdehyde level and decreased superoxide dismutase activity in the CCl<sub>4</sub>-intoxicated heart. Supplemented BCAAs downregulated dyslipidemia and restored atrial changes in antioxidant components to be close to normal. Histopathological investigations using hematoxylin-eosin stain revealed damage to the inflamed heart-lung parenchyma of CCl<sub>4</sub>-intoxicated rats. An increase in fibrosis expression was detectable, simultaneously with a deprivation of protein content in cardiopulmonary sections, as shown by Masson trichrome and bromophenol blue techniques. Co-treated BCAAs intensified cardiopulmonary integrity, inducing scarce histological fibrosis with abundant protein. Accordingly, BCAA supplementation in CCl<sub>4</sub>-treated rats mitigated complications by reducing serum toxicity, enhancing antioxidant capacity, restoring protein expression, and improving organ health.

**Conclusion:** The findings support the protective role of BCAAs with antioxidant benefits against CCl<sub>4</sub>-induced damage, suggesting their potential as a therapeutic agent for cardiopulmonary pathology.

**Keywords:** CCl<sub>4</sub>, BCAAs, Dyslipidemia, Antioxidant profile, Cardiac injury.

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## Introduction

Cardiovascular ailments, including coronary diseases, stroke, and myocardial infarction, are the dominant cause of morbidity and mortality worldwide (Balanesu *et al.*, 2010). Some clinical studies have suggested a distinct relationship between dyslipidemia and the incidence of cardiovascular hazards (Eisen *et al.*, 2008). Certain dyslipidemia indices, involving a pronounced rise in blood total cholesterol (TC) and triglyceride (TG) levels, are identified as a pervasive factor for the development of cardiovascular risks in the world population (Jung *et al.*, 2022). Possibility of persistent oxidative stress, a mediator of allostatic burden, probably causes prolonged alterations in lipid signal with subsequent disturbance of the antioxidant machinery, accompanied by severe sequelae involving atherosclerosis (Goldstein and McEwen, 2002). Carbon tetrachloride (CCl<sub>4</sub>) is involved in multisystem dysregulation and oxidant/antioxidant imbalance. The hypercholesterolemia ascribed to CCl<sub>4</sub> is implicated in oxidative stress (Niemann *et al.*, 2017), owing to its possibility to impair the  $\beta$ -oxidation of fatty acids (Mahmoodzadeh *et al.*, 2017), stimulate the esterification lipid process (Mesalam *et al.*, 2021), and block the action of the lysosomal lipase enzyme (Marimuthu *et al.*, 2013). CCl<sub>4</sub> is a deleterious hepatotoxin (Xiao *et al.*, 2012), suggesting the progression of profound complications consequent to the heart by producing free radicals (Islam *et al.* 2017). At a high dose, CCl<sub>4</sub> profusely induces oxidative stress, cell necrosis, and inflammation, accompanied by acute tissue injury and apoptosis. Toxicity by CCl<sub>4</sub>, which offers a critical role in degenerative tissue conditions mediated through over generation of free radicals (CCl<sub>3</sub> and CCl<sub>3</sub>OO) via the metabolism of the NADPH-cytochrome P450 system, ultimately promoting lipid peroxidation (Shi *et al.*, 2013). Generated free radicals are involved in many pathological events, including dyslipidemia, atheroma development, endothelial damage, low-density lipoprotein oxidation, plaque injury, myocardial ischemic dysfunction, and recurrent thrombosis (Gupta *et al.*, 2015). Oxidative stress is also responsible for diabetic heart conditions and congestive cardiomyopathy (Bugger and Abel, 2010).

Various chemically induced methods *in vitro* and *in vivo* are available for provoking cardiac diseases and heart failure (Ahsan *et al.*, 2020). The mechanism of CCl<sub>4</sub>-induced toxicity in laboratory animals has often been demonstrated via inhalation, oral, and intraperitoneal administration (Taslidere *et al.* 2014). Earlier studies have suggested that the toxicity of CCl<sub>4</sub> can accelerate the generation of free radicals in various tissues (Khan and Ahmed, 2009). Free radicals and several associated species prompt oxidative damage through profound alterations in cell metabolism, thereby raising the calcium ions within cells, which then destroys the membrane permeability and cells via lipid peroxidation. Earlier supported findings through

CCl<sub>4</sub> model is not only associated with the rising level of malondialdehyde (MDA) but also with alleviating the antioxidant catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) enzymes levels in the heart cells, elucidating that the heart is certainly targeted by CCl<sub>4</sub> (Eshaghi *et al.*, 2012). The release of reactive oxygen species (ROS) into the myocardium leads to subsequent mitochondrial dysfunction and cell injury. In this context, elevated ROS levels have been clearly identified in several models of heart dysfunction. Formerly, a growing scale in oxidative injury owing to increased ROS production and a close shortage in the antioxidant system has been proposed as a possible biomarker of cardiotoxicity/cardiac injury (Abdel-Wahab and Metwally, 2015).

Many reports have suggested that CCl<sub>4</sub> is often involved in the occurrence and progression of significant tissue damage by aggravated induction of free radicals rather than experimental cardiotoxicity induction (Ahsan *et al.*, 2020). CCl<sub>4</sub> is a powerful toxicant to the lungs and constitutes a potent peroxidizing model that usually creates systemic toxicity (Unsal *et al.*, 2021). Pulmonary diseases are also devastating complications in patients with liver disease (Ma *et al.*, 2013). The mortality experienced by patients, in addition to pulmonary diseases, is clinically relevant (Krowka *et al.* 2012). Generally, CCl<sub>4</sub> causes lung injury and pneumonia during inhaler exposure (Garcia De Acilu *et al.*, 2015). However, CCl<sub>4</sub>, when applied intraperitoneally at low doses, prompts liver cirrhosis, lung injury, and pulmonary circulation disruption (Das *et al.* 2014). Similarly, the intraperitoneal application of 1 ml/kg CCl<sub>4</sub> for 10 consecutive days induced an appreciable alveolar deficit (Ahmad *et al.*, 2015). To achieve equilibrium between the extremely produced radical species and antioxidant machinery, a variety of potentials have been detected to lower the toxicity of CCl<sub>4</sub> (Wang *et al.* 2021). Growing attention has confirmed that dietary supplementation with specific nutrients ameliorates oxidative injury in mammals (Hao *et al.*, 2021). This evidence indicates that antioxidant nutrients in diets are an essential approach to minimize the possible damage emitted by ROS (Liu *et al.*, 2018). Supplementation with branched-chain amino acids (BCAAs), mainly comprising isoleucine, leucine, and valine, confers beneficial roles in the regulation of ROS (Hu *et al.* 2017). Furthermore, dietary BCAAs prevent cardiovascular issues associated with ameliorating hypercholesterolemia (Li *et al.*, 2022). The anti-atherogenic potential of valine and leucine has been shown to conquer the cardiovascular risks by Cojocar *et al.* (2014), as well as to help modulate serum hyperlipidemia. Valine and leucine exhibited a protective impact on antioxidant activities with a restoring effect in rats with hypercholesterolemia. Dietary BCAAs could improve the role of pulmonary rehabilitation in chronic lung diseases associated with low BCAAs (De Bisschop *et al.*, 2021). To date, dearth

concerns describe the consequences of CCl<sub>4</sub> toxicity in the incidence of cardiopulmonary injury. However, this protocol was prepared to decode the prophylactic measures of BCAAs by evaluating their consequences on different signaling pathways, such as oxidative stress and dyslipidemia profile, in cardiopulmonary-induced CCl<sub>4</sub> experimental toxicity. In this study, toxicity of the heart and lung was induced through the administration of CCl<sub>4</sub> by adult Wistar rats, and the scavenging free radical properties of the BCAAs were measured by assessing the SOD activity level as a known antioxidant and the extent of MDA content as a lipid peroxidation index.

## Materials and Methods

### Ethical approval

All research assays were conducted in accordance with the Ethics Guidelines for the Laboratory Animals Use of the Faculty of Science. Ethical approval and protocol were obtained on 3rd September 2024 from South Valley University, Qena, Egypt, with a protocol No. 003/09/24.

### Experimental rats

Animals of this experiment were twenty-eight male Wistar rats (200 ± 20 g), aged 3–4 months, supplied by Animal House from the University of Sohag, Egypt. All Wistar rats were secured in safeguarded cages after arrival into the laboratory room. Animals were kept in housing conditions and randomly assigned in 2–3 rats per cage to avoid any overcrowding between group-housed albino rats. Cages were changed biweekly and established using a constant light/dark system of 12 hours for each, with an air conditioning system of 22°C temperature and 50% relative air humidity. Animals were fed standard pellets with free-to-access tap water *ad libitum* for 2 weeks before the experimentation for acclimatization.

### Chemicals

Carbon tetrachloride is a chemical compound with the chemical structure of CCl<sub>4</sub>. Al-Nasr Chemicals, Cairo, Egypt, provided it. Using corn oil, CCl<sub>4</sub> was liquefied in a 1:1 ratio and injected intraperitoneally into rats three times a week for 3 weeks (Khedr and Khedr, 2017). BCAAs were produced by Sigma-Aldrich, Louis (MO, USA). High-quality reagents and analytical standard chemicals are used in laboratory research.

### Experimental design and dosage

Twenty-eight Wistar rats were obtained from the Laboratory Animal House of Sohag University, Egypt. Rats were acclimatized for two successive weeks in controlled environmental conditions with access to water and a suitable pelleted diet. Rats were randomly bifurcated under the random selection based on the body weights, into four categories as shown in Figure 1; seven rats were categorized for each group as follows: Group 1 (Control) consisted of seven un-treated animals, which employed a negative control, where the rats supplied standard pellets with free access to

freshwater *ad-libitum* through the experiment period. Group 2 (CCl<sub>4</sub>-treated group) consisted of seven animals injected with 1.0 ml CCl<sub>4</sub> dissolved in corn oil i.p. for 3 weeks, where the rats supplied standard pellets with free access to freshwater *ad-libitum* along the experiment. Group 3 (CCl<sub>4</sub>+10 g BCAAs-treated group) consisted of seven prior animals injected with 1.0 ml CCl<sub>4</sub> dissolved in corn oil i.p. for 3 weeks and then supplied 10 g BCAAs g/kg bwt for an additional seven weeks. Group 4 (CCl<sub>4</sub>+20 g BCAAs-treated group) consisted of seven animals prior injected with 1.0 ml CCl<sub>4</sub> dissolved in corn oil i.p. for 3 weeks and then supplied 20 g BCAAs g/kg bwt for additional 7 weeks. The injection of CCl<sub>4</sub> was trice i.p. per a week for 3 successive weeks in groups 2, 3 & 4 Khedr and Khedr (2017) till cardiopulmonary injury fulfilled. After the induction of CCl<sub>4</sub> toxicity through cardiopulmonary injury using diagnostic biopsies, groups 3 and 4 were orally supplemented with 10 g and 20 BCAAs g/kg but, respectively, according to the protocol of Marrone *et al.* (2023). Iqbal *et al.* (2021) recommended a dose of 10 g/kg bwt BCAAs containing 5.026 g leucine, 2.835 g isoleucine, 2.734 g valine, and 189.405 g casein once a day. A dose of 20 g/kg but BCAAs containing 9.446 g leucine, 6.002 g isoleucine, 5.224 g valine, and 179.328 g casein was also administered once a day for an additional 7 weeks. Rats in groups 1 and 2 were fed standard pellets deprived of BCAAs. However, the same diet was provided by groups 3 and 4 and full in BCAAs, with free access to freshwater *ad libitum* throughout the experiment period.

### Clinical signs and body weight (BW)

Changes in behavioral patterns or clinical aberrations were observed in experimental animals, and the mean body weight was noted at 0 days, 3 weeks, and 10 weeks.

### Sample collection

After the feeding period, the rats of all groups were immediately euthanized through cervical dislocation. Blood and tissue samples were immediately extracted from each group for further biochemical and histopathological analysis after 12 hours of fattening. To segregate serum, blood samples were obtained through the tail vein in sterilized plain vacutainer tubes. Serum was centrifuged at 4,000 rpm for 10 minutes and collected for biochemical monitoring. Collected tissues from the heart and lungs were carefully immersed in a proper fixative for 24 hours until histological analysis (Bancroft *et al.*, 2013). Other aseptic cardiac autopsies were further homogenized by washing in buffered saline, grinding, and centrifugation at 4°C. The heart tissues were assessed for oxidative and antioxidant activity profiles.

### Biochemical analysis of serum

Using standard diagnostic kits, spectrophotometric analyses of serum cholesterol and triglyceride levels (CAT. No. CH 12 20 & TR 20 30, Dokki, Egypt), respectively and were performed according to the

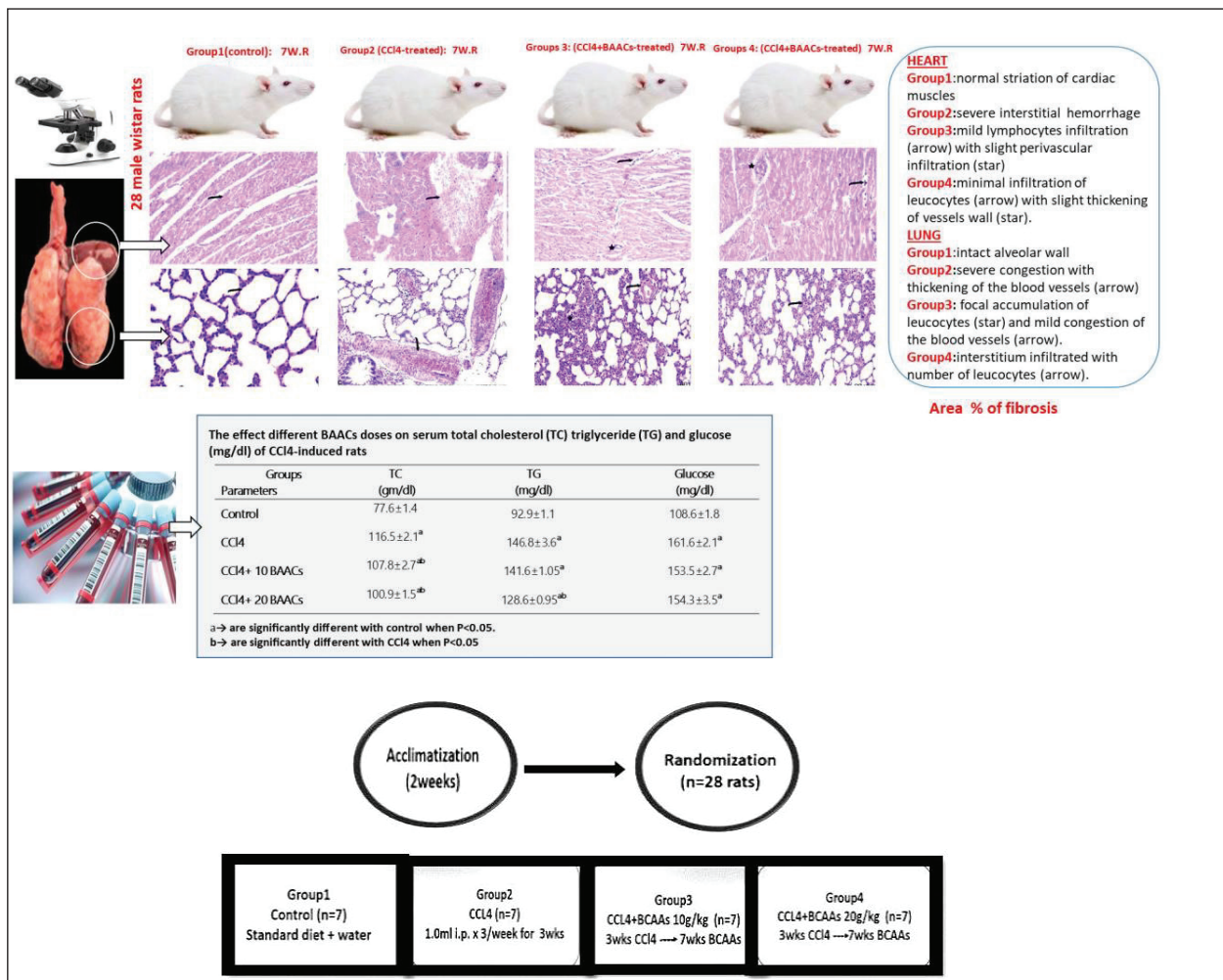


Fig. 1. Experimental design and dosage.

methods of Richmond (1973) and Fossati and Prencipe (1982), respectively. Moreover, the enzymatic colorimetric method (Trinder, 1969) was described for assessing serum glucose levels (CAT. NO. GL 13 20, Dokki, Egypt).

#### Oxidative stress analysis

The MDA level, a lipid peroxidation biomarker, was measured in the heart tissue following a spectrophotometric procedure (CAT. No. MD 25 28, Dokki, Egypt) illustrated by Ohkawa *et al.* (1979). Nishikimi *et al.* (1972) assessed SOD activity (CAT. No. SD 25 20, Dokki, Egypt). Kits for oxidative stress markers were procured by a bio-diagnostic company, Giza, Egypt.

#### Histological evaluation

Fresh small-sized specimens of the heart and lungs with an average diameter of 2 cm<sup>2</sup> were immediately dissected from each rat of the existing groups and rinsed in 10% neutral formalin for a day. Then, the specimens were processed in an automated tissue processor through the completion of dehydration in

ascending scales of methanol (70%, 80%, 90%, and 95%), clearing in xylene to discard the methanol, and softening through embedding into a paraffin block built from paraffin waxes (melting point 55°C–60°C). Then, the sectioning process included mounting the tissue samples on a microtome and harvesting into desiccated sections of 4 μm thicknesses, blemished by hematoxylin and eosin (H&E), Masson's trichrome, and bromophenol blue (BPB) stains according to standard protocols (Drury *et al.*, 1980). The stained slides were inspected, photographed, and scored using a light microscope equipped with a digital camera. A total of 9 sections/3 slides were selected from each group for histopathological scoring. The ordinary semi-quantitative method was run using a scale sorted into none (0), minimal (+), moderate (++), and severe damage (+++) according to Gibson-Corley *et al.* (2013).

#### Fibrosis scoring

Analysis of the photomicrographs of the heart and lung of the control, CCl4, CCl4 + 10 g BCAAs, and CCl4 +

20 g BCAAs was performed using Masson trichrome-stained tissues by the ImageJ software (Collins, 2007). Collagen fibers were separated greatly from the background, and their intensity was calculated using the “Threshold” index tool to evaluate the density and area of collagen fibers in Masson trichrome-stained sections. The threshold was manually adjusted until the entire green area of collagen fibers was highlighted in red. Then, the threshold area was measured.

#### Statistical analysis of data

Statistical data analysis was performed using *one-way analysis of variance* using Windows Statistics, version 21.0 (SPSS Inc., Chicago, USA). The Scheffe post hoc test was used to evaluate all experimental data of the biochemical findings to evaluate significant variations between groups. Triplicates ( $n = 3$ ) were used for each treatment. Data are defined as means  $\pm$  SD. The significance of the variations between means is based on  $p < 0.05$  (Mustafa and Rahman, 2024).

### Results

#### Clinical signs and body weight

The CCl<sub>4</sub> rats exhibited lowered appetite, decreased feeding intake, dullness, and inactivity. On day 0 of the experiment, the mean body weights of the rats in the CCl<sub>4</sub>, CCl<sub>4</sub> + 10 BCAAs, and CCl<sub>4</sub> + 20 BCAAs groups were  $197.3 \pm 3.0$ ,  $199.6 \pm 4.7$  and  $198.3 \pm 2.1$  g, respectively, exhibiting no significant variations compared with the control weight of  $203.0 \pm 4.0$  g. In contrast, in the second week after the injection, the mean body weights of the CCl<sub>4</sub> group ( $217.0 \pm 3.0$  g), CCl<sub>4</sub> + 10 BCAAs ( $220.3 \pm 2.5$  g), and CCl<sub>4</sub> + 20 BCAAs ( $219.0 \pm 3.0$  g) were significantly decreased ( $p < 0.05$ ) compared with the mean body weight of the control group ( $239.6 \pm 4.0$  g). In a comparison with CCl<sub>4</sub> values ( $230.6 \pm 2.1$  g), the mean body weights at 10 weeks from experimentation were significantly increased ( $p < 0.05$ ) to  $256.3 \pm 2.1$  and  $260.6 \pm 2.5$  g of the CCl<sub>4</sub> + 10 BCAAs and CCl<sub>4</sub> + 20 BCAAs groups, respectively, exhibiting significant improvement compared with the CCl<sub>4</sub> group (Table 1).

#### Biochemical finding

Table 2 shows the obtained concentrations of some serum enzymatic lipograms (cholesterol and triglyceride) in the rats. Substantial increases ( $p < 0.05$ ) were observed in the total cholesterol and triglyceride

levels after CCl<sub>4</sub> administration ( $116.5 \pm 2.1$  and  $146.8 \pm 3.6$  mg/dl, respectively) compared with the control non-administrated rats ( $77.6 \pm 1.4$  and  $92.9 \pm 1.1$  mg/dl, respectively). However, the concomitant administration of low and high doses of BCAAs with CCl<sub>4</sub> significantly declined ( $p < 0.05$ ) the serum cholesterol ( $107.8$  and  $100.9 \pm 1.5$  mg/dl, respectively) and triglyceride levels ( $128.6 \pm 0.95$  mg/dl) compared with CCl<sub>4</sub>, indicating that the BCAA-induced diet supports the improvement in lipid levels in the blood. Notably, the mean glucose level displayed an observable increase ( $p < 0.05$ ) in the CCl<sub>4</sub> group ( $161.6 \pm 2.1$  mg/dl). However, they increased significantly ( $p < 0.05$ ) in both combined groups of CCl<sub>4</sub> plus BCAAs ( $153.5 \pm 2.7$  and  $154.3 \pm 3.5$  mg/dl, respectively) compared with the control ( $108.6 \pm 1.8$  mg/dl), as shown in Table 2.

#### Results of oxidative stress

CCl<sub>4</sub> toxicity tends to induce consequential changes in the biomarker of lipid peroxidative stress (LPO), characterized by a significantly higher ( $p < 0.05$ ) in the MDA ( $43.5 \pm 1.7$   $\mu$ mol/l) compared with the control values ( $12.5 \pm 1.2$   $\mu$ mol/l), opposing SOD activity, which manifested a significant reduction ( $p < 0.05$ ) among CCl<sub>4</sub>-treated hearts ( $52.5 \pm 2.2$   $\mu$ mol/l) compared with the activity of control ( $194.46 \pm 3.2$   $\mu$ mol/l). In comparison with the CCl<sub>4</sub> values, the combined treatment of CCl<sub>4</sub> with both 10 and 20 BCAAs resulted in a considerable decrease ( $p < 0.05$ ) in MDA level was  $24.26 \pm 1.7$  and  $17.76 \pm 2.1$   $\mu$ mol/l, respectively. A significant rise ( $p < 0.05$ ) in the SOD activity, however, was  $83.3 \pm 2.6$  and  $124.36 \pm 2.5$   $\mu$ mol/l, respectively. Accordingly, the administration of BCAAs decreased the unusual increase in LPO level and greatly enhanced the enzymatic antioxidant activity relative to the control findings (Table 3).

#### Histological and histochemical changes

The histological and histochemical alterations (Table 4) illustrated that the CCl<sub>4</sub>-received group had more extensive and intense alterations than the CCl<sub>4</sub> + BCAA-treated groups.

#### H&E stain

Histopathological examination of the heart in control group showed normalization of cardiac tissue, indicating good striation of the cardiac muscles and an intact vein (Fig. 2A). In contrast, CCl<sub>4</sub>-intoxicated rats showed severe hemorrhage with massive infiltration

**Table 1.** Changes in body weight (mean  $\pm$  standard deviation g) of rats treated with carbon tetrachloride and bovine serum albumin (BCAAs).

Groups Parameters	0 day (g)	Second week (g)	Tenth week (g)
Control	$203.0 \pm 4.0$	$239.6 \pm 4.0$	$293.0 \pm 3.0$
CCl <sub>4</sub>	$197.3 \pm 3.0$	$217.0 \pm 3.0^a$	$230.6 \pm 2.1^a$
CCl <sub>4</sub> + 10 BCAAs	$199.6 \pm 4.7$	$220.3 \pm 2.5^a$	$256.3 \pm 2.1^{a,b}$
CCl <sub>4</sub> + 20 BCAAs	$198.3 \pm 2.1$	$219.0 \pm 3.0^a$	$260.6 \pm 2.5^{a,b}$

$p$ -value\*, comparing differences between groups using one-way ANOVA; <sup>(a)</sup> significant versus control, <sup>(b)</sup> significant versus CCl<sub>4</sub> group.

of red blood cells, perivascular inflammatory edema, hyaline deposition, fibrotic collagen precipitates on the cardiac surface, ballooning degenerative changes of the myocardium, myolysis, and intense muscle necrosis replaced by focal mononuclear aggregation (Fig. 2B and C). BCAA treatment at varying doses resulted in an improvement in the heart architecture characterized by improved cardiac muscle cells, minimal mononuclear infiltrates, weak hyaline deposition, and minimally congested blood vessels (Fig. 2D–F). The histopathological examination of the lung alveoli and bronchi of the control rats was histologically normal (Fig. 3A). Conversely, in the lung parenchyma of CCl<sub>4</sub>-treated rats, there were distinct areas of emphysema and interstitial pneumonia associated with abundant leukocyte infiltration. Many alveoli had thickened walls containing inflammatory cells, mostly mononuclear cells, eosinophils, and neutrophils. Some alveoli also revealed necrotic alveolar walls, indicating diffuse damage. Numerous erythrocytes were observed in and around the alveoli. The interstitial cell population infiltrated and thickened with mononuclear cells. The inflamed areas exhibited focal collagen and connective tissue fiber accumulation. Several alveolar macrophages were also invaded. The blood vessels showed extensive congestion, dilatation, and thickening of their walls with numerous inflammatory cells and fibrous tissues (Fig. 3B and C). CCl<sub>4</sub>+BCAA-treated rats showed a restored normal histological structure of the lung. Moderate degree of alveolar thickening with moderate accumulation of leucocytes. Blood vessels were also slightly congested and dilated (Fig. 3D and E). Furthermore, the CCl<sub>4</sub>+BCAA-treated rats

showed mild alveolar thickening with a mild leukocyte accumulation (Fig. 3F).

#### Masson's trichrome staining method

The area of fibrosis (%) of the heart and lung sections depends on the average proportion and collagen distribution determined by morphometric analysis (Table 5). The area percentage of heart fibrosis was significantly increased ( $p < 0.05$ ) in all treated groups compared with the control, as evidenced by increased areas of the collagen depositions. The heart sections of the CCl<sub>4</sub> group recorded a higher percentage than those of the BCAA group. Co-treated CCl<sub>4</sub> with 20 BCAAs exhibited the fewest percentage of heart fibrosis, comparable with 10 BCAAs. Although the mean proportion (%) of lung fibrosis was significantly increased ( $p < 0.05$ ) CCl<sub>4</sub> and CCl<sub>4</sub> + 10 BCAA-treated groups compared with the control group, the CCl<sub>4</sub> + 20 BCAA-treated group recorded a smaller proportion (%) of lung fibrosis; however, BCAAs verified dose-dependent alterations. Accordingly, the Masson staining method exhibited almost hard collagen detection in the control heart (Fig. 4A). Significant fibrosis characterized by the deposition of large amounts of collagen was observed after CCl<sub>4</sub> treatment (Fig. 4B–D). Treatment with 10 and 20 mg/kg BCAAs significantly diminished collagen deposition (Fig. 4E and F). The same context of the control lung showed scarce collagen infiltrates in the pulmonary parenchyma (Fig. 5A). CCl<sub>4</sub> treatment was accompanied by the pronounced activation of fibrosis with high collagen and fibrous tissue infiltration (Fig. 5B–D). Lung fibrosis was alleviated after using varying doses of BCAAs, as evidenced by faintly stained fibrous tissues (Fig. 5E and F).

**Table 2.** Effect of different doses of branched-chain amino acids on serum total cholesterol (TC), triglyceride (TG), and glucose levels in carbon tetrachloride-induced rats (mean ± SD mg/dl each).

Groups Parameters	TC (mg/dl)	TG (mg/dl)	Glucose (mg/dl)
Control	77.6 ± 1.4	92.9 ± 1.1	108.6 ± 1.8
CCl <sub>4</sub>	116.5 ± 2.1 <sup>a</sup>	146.8 ± 3.6 <sup>a</sup>	161.6 ± 2.1 <sup>a</sup>
CCl <sub>4</sub> + 10 BCAAs	107.8 ± 2.7 <sup>a,b</sup>	141.6 ± 1.05 <sup>a</sup>	153.5 ± 2.7 <sup>a</sup>
CCl <sub>4</sub> + 20 BCAAs	100.9 ± 1.5 <sup>a,b</sup>	128.6 ± 0.95 <sup>a,b</sup>	154.3 ± 3.5 <sup>a</sup>

*p*-value\*, comparing differences between groups using one-way ANOVA; <sup>(a)</sup> significant versus control, <sup>(b)</sup> significant versus CCl<sub>4</sub> group.

**Table 3.** The effect of different branched-chain amino acids doses on carbon tetrachloride-induced rats on lipid peroxidative biomarker (MDA), and antioxidant activity (SOD) (mean ± SD μmol/l each).

Parameters Groups	MDA (μmol/l)	SOD (μmol/l)
Control	12.5 ± 1.2	194.46 ± 3.2
CCl <sub>4</sub>	43.5 ± 1.7 <sup>a</sup>	52.5 ± 2.2 <sup>a</sup>
CCl <sub>4</sub> + 10 BCAAs	24.26 ± 1.7 <sup>a,b</sup>	83.3 ± 2.6 <sup>a,b</sup>
CCl <sub>4</sub> + 20 BCAAs	17.76 ± 2.1 <sup>a,b</sup>	124.36 ± 2.5 <sup>b,c</sup>

*p*-value\*, comparing difference between groups using one-way ANOVA; <sup>(a)</sup> significant versus control, <sup>(b)</sup> significant versus CCl<sub>4</sub> group, <sup>(c)</sup> significant versus CCl<sub>4</sub>+10 BCAAs group.

**Table 4.** Nominal semi-quantitative histological scores of the heart and lung of the control, carbon tetrachloride, and branched-chain amino acid-treated groups.

Group lesions	Control	CCl4	CCl4 + 10 BCAAs	CCl4 + 20 BCAAs
<b>Heart</b>				
Necrosis of the cardiac tissues	–	+++	++	+
Degeneration of the cardiac muscles	+	+++	++	+
Hemorrhagic inflammation	–	+++	++	+
Cardiac fibrosis	–	+++	++	+
Protein granules	+++	+	++	++
Mononuclear aggregation	–	+++	+++	+
Hyaline degeneration	–	+++	++	+
Congestion and blood vessel dilation	–	+++	+	+
Thickening of the blood vessels	–	+++	+	+
Perivascular edema and inflammatory	–	+++	+	+
<b>Lung</b>				
Pulmonary emphysema	–	+++	++	+
Pulmonary fibrosis	–	+++	++	+
Interstitial inflammation	–	+++	++	+
Protein fragments	+++	+	++	++
Interstitial pneumonia	–	+++	++	+
Necrotic alveoli with a sloughed wall	–	+++	+	+
Thickened alveolar septa and wall	–	+++	++	+
Inflammatory accumulation of cells	–	+++	+++	+
Erythrocytes aggregation	–	+++	+	+
Collagen and fibers of connective tissue	–	+++	+	+
Congested and dilated blood vessels	–	+++	+	+

#### BPB technique

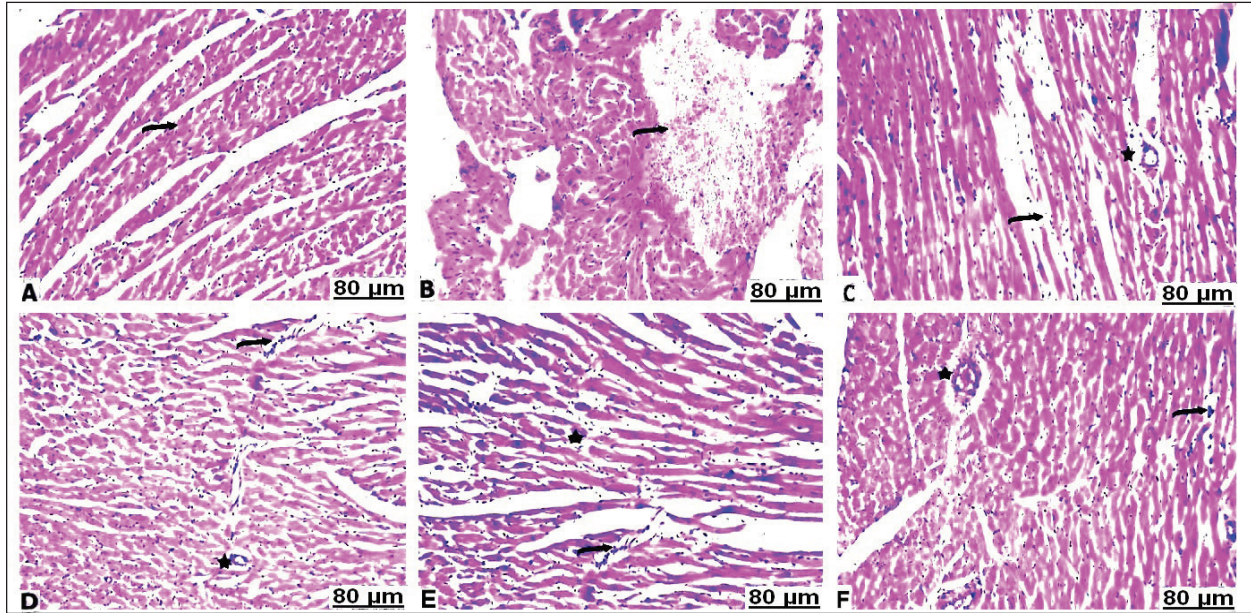
The control heart was specified by high total protein content (Fig. 6A). A highly positive bromophenol blue reaction was observed in the cytoplasm, characterized by equally distributed bluish granules of definite sizes. Total protein revealed a striking decrease in the cytoplasm of the heart cells of the CCl4 group, displaying high percentages of protein-devoid cells and cytoplasmic vacuolation, particularly around the blood vessels (Fig. 6B). However, oral BCAA administration concomitant with CCl4 could reduce alterations in protein content in rat hearts compared with the CCl4 group. The BPB reaction was increased, exhibiting dark-blue spotted granules of varying protein contents, peripheral in position or perinuclear, particularly adjacent to the vasculature (Fig. 6C and D).

The lungs of the control vehicle revealed marked protein content in the cytoplasm of the lung tissues, as indicated by the large number of bluish-colored fine materials of different sizes (Fig. 7A). Conversely, CCl4 injection into rats for three consecutive weeks resulted in a sustainable reduction in protein content, as evidenced by less BPB-stained cells. In these lungs,

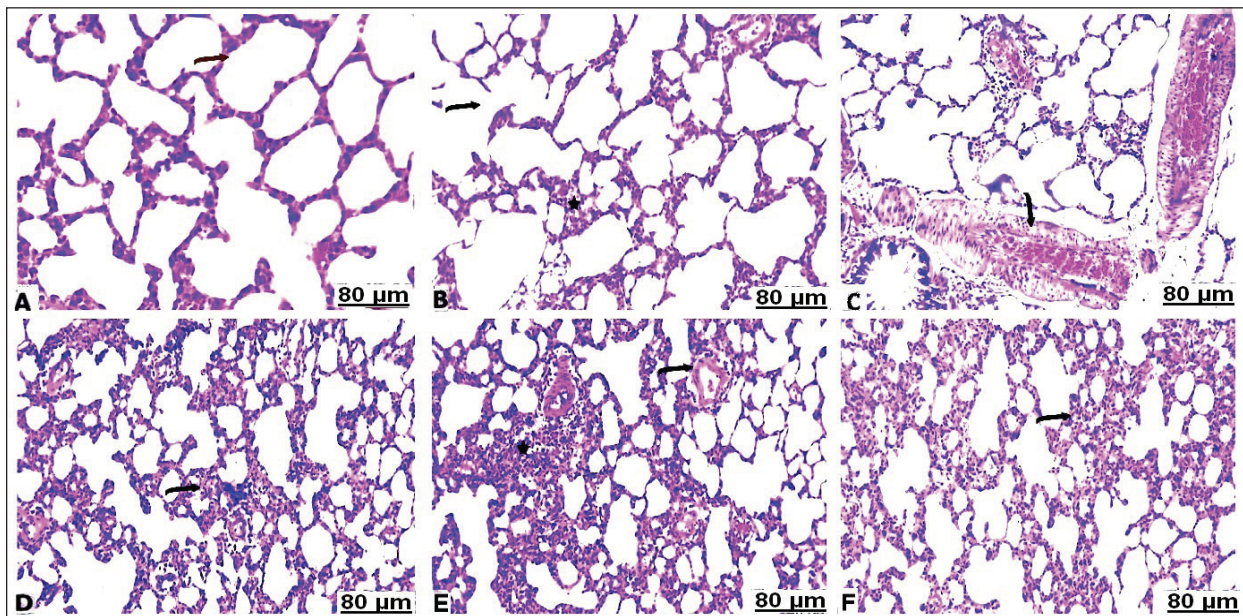
the protein content displayed minimal intensity of the reaction and became barely detectable (Fig. 7B). However, the combined administration of BCAAs and CCl4 strengthened the BPB reaction and resulted in a more detectable number of bluish-colored granules of varying sizes, indicating increased protein contents similar in intensity to those in the control lung (Fig. 7C and D).

#### Discussion

Oxidative stress is an imbalance between the radical species and the physiological antioxidant enzymatic machinery that neutralizes ROS or scavenges the resulting damage. The emitted ROS can destroy all cellular components, yielding fatal consequences (Shaban *et al.*, 2017), and excites a crucial part in the pathogenesis of multiple tissues, including the kidney, brain, spleen, liver, and lung (Ragab *et al.*, 2013). Reducing ROS expulsion is essential for cell survival and the prevention of further oxidative damage (Morgan and Liu, 2011). Several toxic chemicals, including carbon tetrachloride, methanol, bromobenzene, and aromatic hydrocarbons, can cause systemic injury by



**Fig. 2.** Heart sections of control (A), carbon tetrachloride (B, C), carbon tetrachloride + 10 g branched-chain amino acids (D, E), and carbon tetrachloride + 20 g branched-chain amino acids (F): A) normal striation of cardiac muscles. B) severe interstitial hemorrhage. C) necrosis of muscle fiber with a loss of striation (arrow), perivascular edema, and cellular infiltrates (star). D) Mild lymphocyte infiltration (arrow) with slight perivascular infiltration (star). E) Mild cardiac myolysis (star) and interstitial inflammation (arrow). F) Minimal leucocyte infiltration (arrow) with slight vessel wall thickening (star). [Hematoxylin and eosin staining].

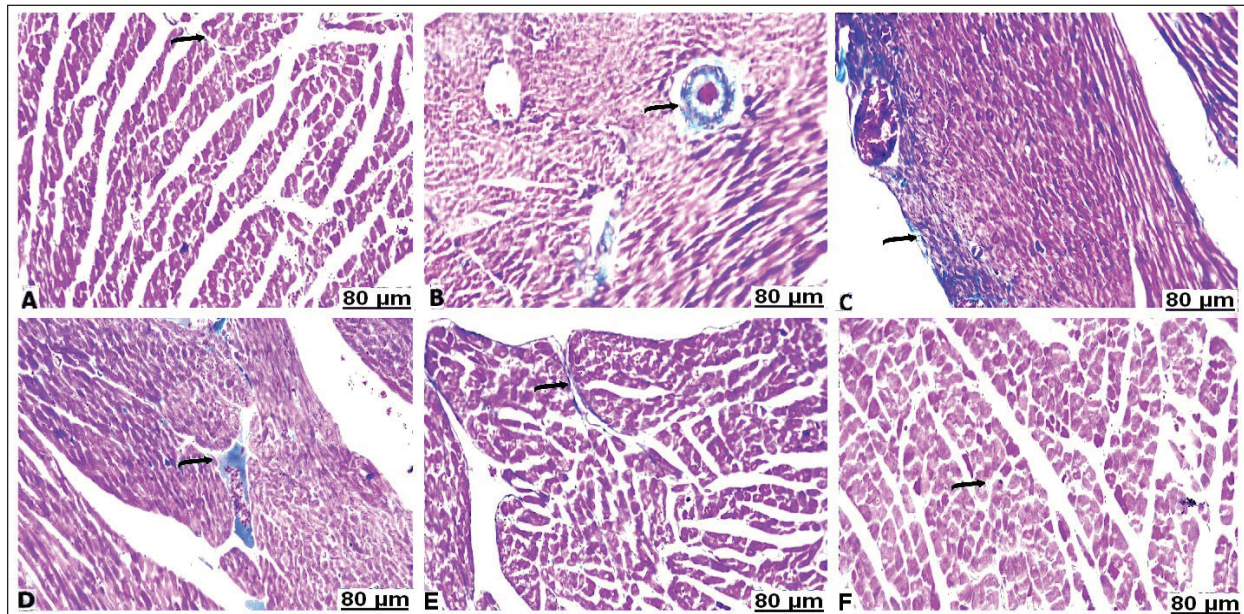


**Fig. 3.** Lung sections of control (A), carbon tetrachloride (B, C), carbon tetrachloride +10 g branched-chain amino acids (D, E), carbon tetrachloride +20 g branched-chain amino acids (F): A) showing intact alveolar wall. B) showing scattered areas of alveolar emphysema (arrow) and thickening of the interalveolar septa with mononuclear cells (star). C) showing severe congestion with thickening of the blood vessels (arrow). D) showing interstitial infiltration of alveolar septa with lymphocytes (arrow). E) showing focal accumulation of leucocytes (star) and mild congestion of the blood vessels (arrow). F) showing interstitium infiltrated with several leucocytes (arrow). [Hematoxylin and eosin staining].

**Table 5.** Fibrosis scoring area (%) of the heart and lung of the experimental groups compared with control tissues.

Area (%) of fibrosis Groups	Heart	Lung
Control	0.87 ± 0.42	2.43 ± 0.59
CCl <sub>4</sub>	5.72 ± 1.24 <sup>a</sup>	16.72 ± 2.59 <sup>a</sup>
CCl <sub>4</sub> + 10 BCAAs	1.83 ± 0.33 <sup>a,b</sup>	6.84 ± 1.17 <sup>a,b</sup>
CCl <sub>4</sub> + 20 BCAAs	1.18 ± 2.2 <sup>a,b,c</sup>	3.28 ± 1.10 <sup>b,c</sup>

*p*-value\*, comparing difference between groups using one-way ANOVA; <sup>(a)</sup> significant versus control, <sup>(b)</sup> significant versus CCl<sub>4</sub> group, <sup>(c)</sup> significant versus CCl<sub>4</sub>+10 BCAAs group.



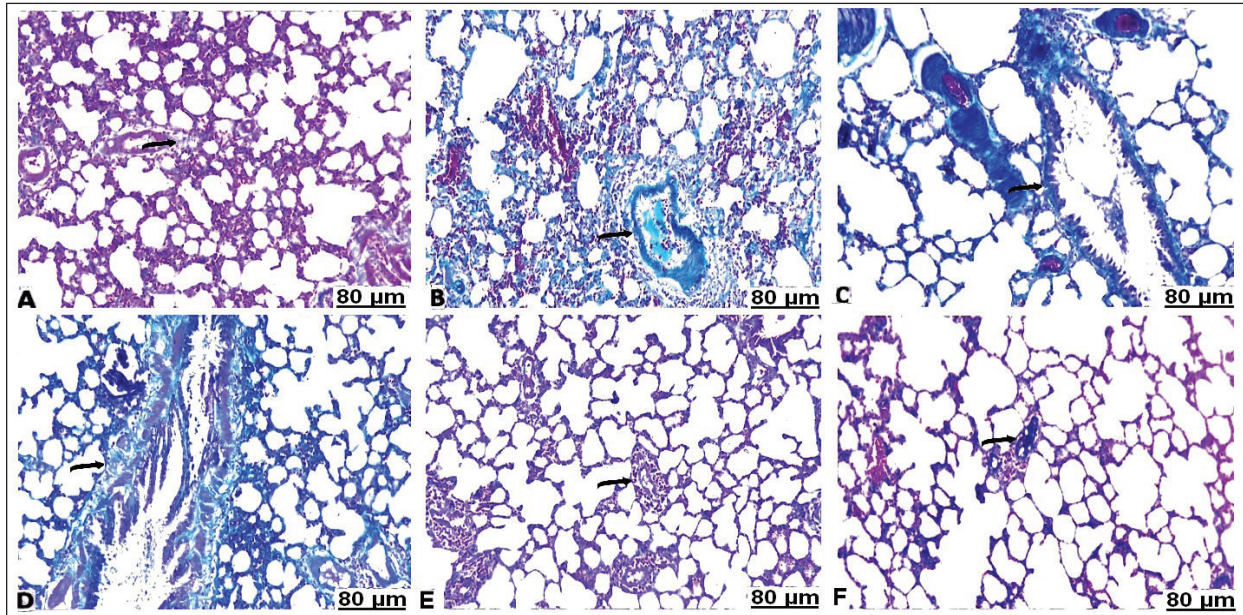
**Fig. 4.** Heart sections of control (A), carbon tetrachloride (B, C, D), carbon tetrachloride + 10 g branched-chain amino acids (E), and carbon tetrachloride + 20 g branched-chain amino acids (F): A) weak intensity of the collagen fibers staining (arrow). (B, C, D) increased fibrosis evident by a great rise in the stained collagen fibers (arrow). (E) Low percentages of the infiltrated collagen fibers (arrow). F) Scarcely distributed collagen fibers (arrow). [Masson's trichrome stain].

producing reactive oxygen species (Rahmouni *et al.*, 2017). Carbon tetrachloride is a copious environmental toxicant that can cause oxidative stress with over-created free radicals in various organs, notably the lungs and kidneys, motivating generalized toxicity (Habashy *et al.*, 2021).

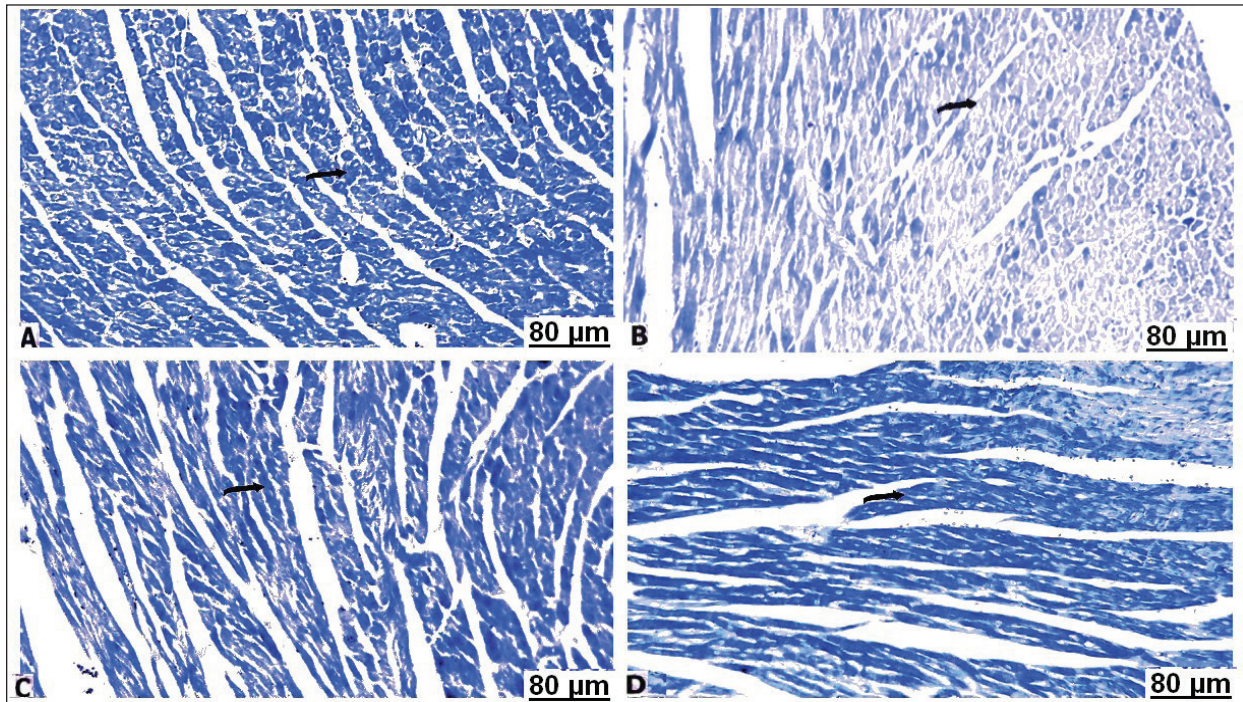
Carbon tetrachloride (CCl<sub>4</sub>), a vigorous carcinogenic agent, is an organic industrial solvent that may cause liver, kidney, nervous system, and lung dysfunction. CCl<sub>4</sub> is taken up by the respiratory tract, gastrointestinal system, and skin, and then, through cytochrome P-450 metabolism, it exerts toxic impacts via the free radicals of trichloromethyl and trichloromethyl peroxy metabolites (Khan, 2012). These radicals combine fatty acids in the lung cell membrane to enhance DNA fragmentation and lipid peroxidation. Besides this, they disturb the antioxidant enzyme system, encompassing superoxide dismutase, catalase, and glutathione enzymes. Because of aggregations of exaggerated fibroblasts, monocytes, macrophages, and

neutrophils in blood vessels, CCl<sub>4</sub> caused lung toxicity by interstitial cell degeneration, fibrosis, and ruptures of intra-alveolar septa. Increased ROS is involved in pulmonary fibrosis, lung carcinoma, emphysema, chronic bronchitis, and pleural diseases (Ahmad *et al.*, 2015). CCl<sub>4</sub> may cause excessive transforming growth factor beta1, leading to systemic fibrosis in multiple tissues, primarily the lung and liver (Fan *et al.*, 2013). CCl<sub>4</sub> promotes lung injury by accelerating lipid peroxidation and increasing MDA levels (Ganie *et al.*, 2010). CCl<sub>4</sub> induces cellular apoptosis by activating the caspase pathway, thereby promoting tissue injury (Xie *et al.*, 2015). Carbon tetrachloride induced heart dysfunction in rats. Intoxicated rats exhibited cardiac histopathology in the form of fibrotic strands in the heart wall, massive deposition of collagen on the cardiac layer, and ballooning degeneration of endocardial muscle fibers (El-Baz *et al.*, 2015).

In this study, the H&E-stained sections of CCl<sub>4</sub>-treated heart revealed necrosis and degenerative changes



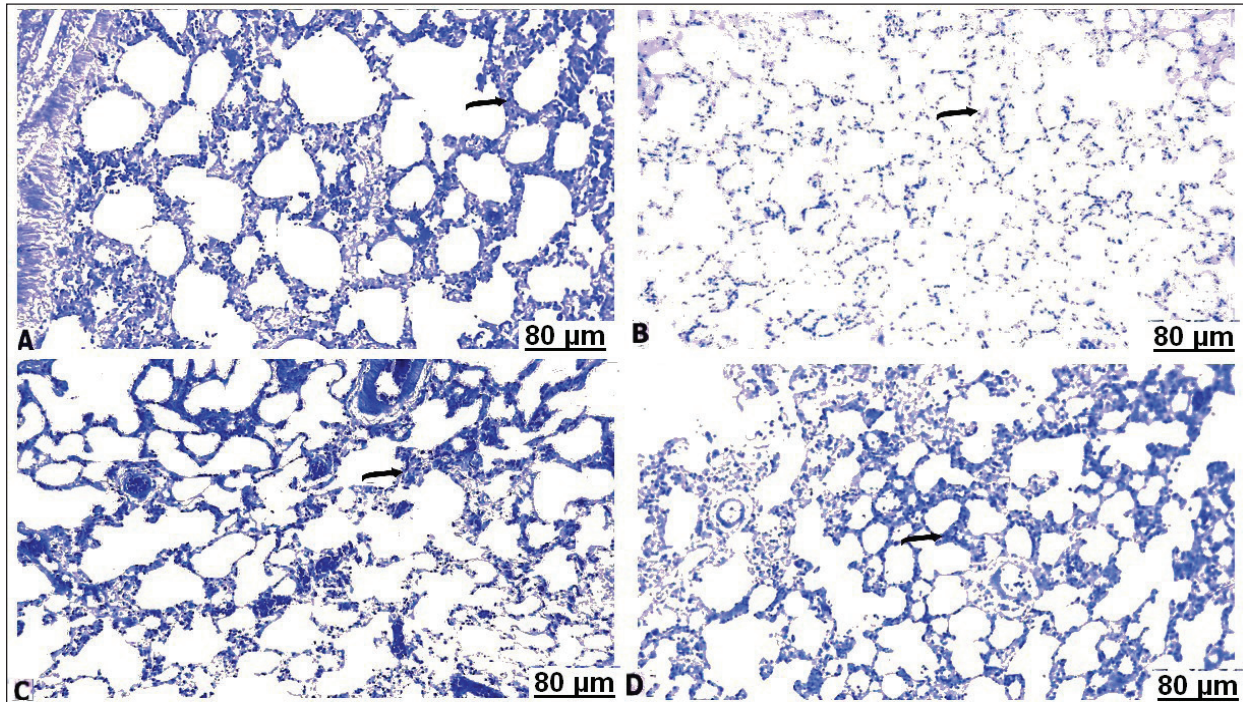
**Fig. 5.** Lung sections of control (A), carbon tetrachloride (B, C, D), carbon tetrachloride + 10 g branched-chain amino acids (E), and carbon tetrachloride + 20 g branched-chain amino acids (F): (A) faintly stained nuclei of collagen fibers (arrow). (B, C, D) marked incidence of perivascular and interstitial fibrosis (arrow). (E) significant depletion of collagen fibers (arrow). (F) weakly precipitated collagen fibers (arrow). [Masson's trichrome stain].



**Fig. 6.** Heart sections of control (A), carbon tetrachloride (B), carbon tetrachloride + 10 g branched-chain amino acids (C), and carbon tetrachloride + 20 g branched-chain amino acids (D): (A) a considerable distribution of protein elements within the cytoplasm (arrow). (B) a weak response of the cell's cytoplasm toward the BPB reaction (arrow). (C) a low decrease in protein elements (arrow). (D) a noticeable increase in protein content (arrow). [BPB stain].

of the cardiac muscles, inflammation, hemorrhage, and congestion of the blood vessels. Toxic response

of CCl<sub>4</sub>-enhanced heart infarction A single CCl<sub>4</sub> injection at 1.2 ml/kg induced myocardial damage



**Fig. 7.** Lung sections of control (A), carbon tetrachloride (B), carbon tetrachloride +10 g branched-chain amino acids (C), and carbon tetrachloride +20 g branched-chain amino acids (D): (A) Homogenous dense, blue-stained granules indicating positive affinity to BPB. (B) Very low protein particles (arrow). (C) A low decrease in protein elements (arrow). (D) A noticeable increase in protein content (arrow). [BPB stain].

identified by focal masses of huge degeneration with scattered, blue-stained fibrosis zones of fibrous tissues. CCl<sub>4</sub>-intoxicated rats had increased levels of serum pro-inflammatory biomarkers and C-reactive protein in heart cells (Al-Rasheed *et al.*, 2014). CCl<sub>4</sub>-mediated cardiotoxicity and cardiac injury interfere with antioxidant mechanisms and may involve oxidative stress. The elevated rate of lipid oxidation was more detectable in CCl<sub>4</sub>-induced cardiac injury. CCl<sub>4</sub> is linked to scattered necrosis, widespread inflammation, cytoplasmic vacuolization, interstitial edema, and disintegrated myofibrils (Alsaffar *et al.*, 2022). H&E-lung sections of the CCl<sub>4</sub> group exhibited interstitial pneumonia, abundant leukocyte infiltration, thickening of the interalveolar wall, and extensive congestion. Intense damage was detected in lung morphology after CCl<sub>4</sub> exposure, which was recognized by disorganized alveolar architecture, widespread thickening of the interalveolar space, and collapse of alveolar septa similar to those findings by Habashy *et al.* (2021). Acute and chronic lung injury in rats by i.p. CCl<sub>4</sub> injection for 2 and 5 weeks, respectively, exhibited features of discrete alveolar damage. Acute lung injury is distinguished by acute hemorrhagic interstitial pneumonia, whereas chronic interstitial pneumonia is caused by long-term injury. Connective tissue revealed intra- and interstitial alveolar fibrosis in patients with short-term injury. The

mechanism of CCl<sub>4</sub> lung injury is suggested by lipid peroxidation-correlated radicals (Pääkkö *et al.*, 1996). Light microscopy of the CCl<sub>4</sub>-exposed lung showed an abnormal histological appearance in the form of alveolar epithelial injury, edema, sloughed epithelial cells, and mononuclear inflammatory infiltrates in the lung's interstitial tissues. Intensive congestion of the large blood vessels, swelling of epithelial cells, shedding of cellular epithelium, and scattered visualizations, with intensive fibrosis of interstitial tissues with fibrous cells using Masson's trichrome. An examination of the lungs of cirrhotic mice treated with CCl<sub>4</sub> revealed the presence of strong fibrotic lesions in the lungs of CCl<sub>4</sub>-associated cirrhotic animals. H&E staining revealed that the most evident changes in lung sections were a lack of normal alveolar organization, significant pulmonary fibrosis, notable thickening of the alveolar interstitium, and prominent disorganization of the alveolar space (Das *et al.*, 2014).

The current design of Masson's trichrome-stained sections recorded an enormous increase in the collagen and fibrous tissue areas that induce fibrosis. Similar findings were consistent with those of Ding and Choi (2014), who detected the overexpression of collagen and fibrous tissues by mesangial cells of the kidney. Trichrome staining illustrated blue staining, intense extracellular matrix accumulation, and peribronchial and perivascular lung fibrosis in CCl<sub>4</sub>-cirrhotic mice.

Picrosirius Red staining showed substantial collagen deposition around the vasculature and in the lung parenchyma (Das *et al.*, 2014).

The incidence of protein elements by BPB-stained microscopic sections displayed a marked decrease in cardiac and lung cells following CCl<sub>4</sub> intoxication, while significantly reversed close to normal protein level in BCAA-treated groups. This alteration is attributed to oxidative damage to cell proteins and deterioration in cellular function and contents (Ohta *et al.*, 2000).

In this study, the total weight of rats dropped remarkably after intraperitoneal exposure to CCl<sub>4</sub> because of hyperlipidemia activity. CCl<sub>4</sub> supplementation tended to increase lipid and glucose levels in the serum, contrary to the BCAA-induced groups. Significant decreases in serum insulin and increases in blood glucose levels were observed after CCl<sub>4</sub> supplementation (Khedr and Khedr, 2017). The subsequent administration of CCl<sub>4</sub> is associated with hyperlipidemia activity characterized by a significant elevation in glucose and cholesterol levels. Changes in the lipid profile are caused by subsequent liver injury due to CCl<sub>4</sub> toxicity (Dutta *et al.*, 2018). Ultimately, Hussein and Khan (2022) showed that acute exposure to CCl<sub>4</sub> induced a marked increase in triglyceride, cholesterol, low-density lipoproteins, and glucose levels. CCl<sub>4</sub> intoxication triggered lipid metabolism parameters, leading to a significant increase in triglyceride and cholesterol levels. However, orally administered BCAAs lowered the serum levels of cholesterol and triglyceride in patients with liver cirrhosis (Takeshita *et al.*, 2009). In addition, Arakawa *et al.* (2011) reported that BCAA-rich meals improved and reversed triglyceride levels in a fat-rich mouse model. Generally, leucine supplementation improved body weight, attributed to marked improvements in glucose and lipid metabolism (Jiao *et al.*, 2016). Macotela *et al.* (2011) explored that leucine causes motivation in glucose tolerance and insulin index without changing the weight gain of mice with obesity. The improved influence of leucine on body weight and lipid markers is correlated with the regulation of lipid and carbohydrate metabolism (Chen *et al.*, 2012). Leucine supplementation induces the beneficial role of BCAAs in improving the lipid profile in the blood serum, provoking a marked decrease in microvesicular steatosis illustrated by decreased liver oxidation and improved lipid processing with concomitant inhibition of de novo lipid synthesis associated with obesity (Fu *et al.*, 2015). Improvement in liver lipid catabolism is associated with a prominent decline in insulin level, lipid peroxidation, liver diacylglycerol, and inflammation, further confirmed by profound repression of crucial inflammatory proteins and pathways. The valine and isoleucine constituents found in BCAAs serve as nutritional treatments to restore metabolic or inflammatory homeostasis in mice with obesity-associated steatotic livers (Gart *et al.*,

2022). Noguchi *et al.* (2006) detected the beneficial effect of BCAAs to regulate lipid metabolism and heart function, who confirmed that leucine and valine neutralized the deleterious effect of lipid levels immediately in atherosclerotic episodes. However, valine had a quicker response than isoleucine and leucine on the improvement of biochemical indices, according to the histopathological evaluation (Ifrim *et al.*, 2018).

In this experimental work, the oxidative component of MDA in CCl<sub>4</sub>-treated hearts was significantly higher than that in control animals, contrary to a significant decline in SOD antioxidant enzyme. The increased MDA level suggests the continual stimulation of the peroxidation process, inducing tissue injury and insufficiency of the antioxidant procedures to neutralize the excessively generated radical species (Ganie *et al.*, 2011). CCl<sub>4</sub>-induced lipid peroxidation was significantly attenuated by branched-chain amino acids, which was reversed by an improvement in antioxidant enzyme activity. The potential influence of BCAAs for the treatment of various disorders is shown through the enhancement of metabolic processes and the strengthening of mitochondrial bioenergetics for maintaining mitochondrial health (Ruocco *et al.*, 2021). BCAAs can reduce oxidative stress and modulate the antioxidant defense system in medium-aged mice (D'Antona *et al.*, 2010). Valine plays a favorable role in lipid metabolism (Bishop *et al.*, 2020). While studies on BCAAs, particularly leucine and isoleucine, have shown an advantageous impact on mitochondrial function, cellular metabolism, and oxidative stress (Tamanna and Mahmood, 2014), valine confers vital function in protecting mitochondrial viability by neutralizing cellular/mitochondrial harms activated by oxidative stress (Sharma *et al.*, 2024).

Dietary BCAAs had a protective effect on heart and lung architectures. The beneficial contribution of leucine, isoleucine, and valine supplementation results in significant mitigation of histopathological lesions due to a rich cholesterol diet. Similar histopathological examinations of leucine, isoleucine, and valine revealed mild lesions in the vascular endothelium (Ifrim *et al.*, 2018). The reduced microscopic lesions are a consequence of the addition of BCAAs to the diet of mice, which ultimately strengthens the ROS defense mechanism in the tissues and improves mitochondrial health status (Valerio *et al.*, 2011).

### Conclusion

Taken together, the current experimental results explicated the systemic toxicity of CCl<sub>4</sub> in the heart and lungs of rats and the significant therapeutic potential of BCAAs. The biochemical findings and histopathological results elicited the potency of BCAAs in alleviating CCl<sub>4</sub>-induced oxidative toxicity induced by CCl<sub>4</sub> through suppressing biochemical disturbance and fibrosis. Accordingly, this investigation suggests

that BCAAs, notably valine, leucine, and isoleucine, have an influential protector with strong radical scavenger function against CCl<sub>4</sub>-induced heart and lung damage. Thus, in dose-dependent amelioration, BCAAs are being proposed as a promising and efficient anti-toxic agent that targets different pathways in various organs, particularly the heart and lung tissues. However, specific mechanisms through BCAAs remain unclear and require further investigation.

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#### Conflict of interest

The authors declare no conflict of interest.

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#### Authors' contributions

All authors contributed to the study conception and design. Conceptualization: ZA-A, FAM, and HA-A; methodology: ZA-A, FAM, HA-A, AWB, and AMM; software: ZA-A, FAM, HA-A, AWB, AMM, and MMJ; validation: ZA-A, FAM, HA-A, AWB, and AMM; formal analysis: ZA-A, FAM, HA-A, AWB, AMM, MMJ, AE, IFR, FZ, and MZ; resources: ZA-A, FAM, HA-A, AWB, AMM, and MMJ.; data curation: ZA-A, FAM, HA-A, AWB, AMM, MMJ, AE, IFR, FZ, and MZ; writing—original draft preparation: ZA-A, FAM, HA-A, AWB, AMM, MMJ, AE, IFR, FZ, and MZ; writing—review and editing: ZA-A, FAM, HA-A, AWB, AMM, MMJ, AE, and IFR; visualization: HA-A, AWB, AMM, MMJ, AE, IFR, FZ, and MZ; supervision: ZA-A, FAM, and MMJ; funding acquisition: MZ and FZ. All authors have read and approved the published version of the manuscript.

#### Statement of the institutional review board

All research assays were conducted in accordance with the Ethics Guidelines for the Laboratory Animals Use of the Faculty of Science. Ethical approval and protocol were obtained on 3<sup>rd</sup> September 2024 from South Valley University, Qena, Egypt (Protocol No. 003/09/24).

#### Data availability statement

The datasets generated for this study are available upon request from the corresponding author.

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