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Protective effects of N-acetylcysteine against irinotecan – induced pulmonary toxicity in female albino rats

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Abstract

Background and objective: Idiopathic pulmonary fibrosis (IPF) is a deadly lung illness characterized by progressive lung fibrosis and deteriorating lung function, with the uncertain origin and few therapeutic options. Chemotherapy such as Irinotecan (IRI) is the major antineoplastic agent that produces IPF and induces lung damage. N-acetylcysteine (NAC) has a glutathionepre cursor that protects lung function. The present study was designed to evaluate the protective effects of N-acetylcysteine in Irinotecan induced lung toxicity.

Methods: In total, 18 female Albino rats were divided into three groups, aging from 8-12 weeks and weighing between 180-250 g. The control group G1 received 1 ml intraperitoneal (IP) normal saline for 14 days, G2 Irinotecan group was administered a single high dose of Irinotecan (300 mg/kg) intraperitoneal instillation. And G3 N-acetylcysteine group (500 mg/kg/day) orally one week before Irinotecan (300 mg/kg) injection continued to the end of 21 days of the experimental period. Histopathology, cell counts, cytokine responses in the serum (TGF-β, TNF-α, and IL-6), and weight measurements were studied to evaluate the protective effects.

Results: Histopathological study indicated the Irinotecan–induced pulmonary fibrosis with a significant increase in the levels of inflammatory cytokines, and the protective impacts of N-acetylcysteine administration attenuated the degree of inflammation and fibrosis with a reduced level of cytokines concentrations in serum.

Conclusion: N-acetylcysteine can protect the lungs from both histological alterations and activation of cytokines caused by pulmonary toxicity induced by Irinotecan.

Keywords: Lung; Idiopathic pulmonary fibrosis (IPF); Irinotecan (IRI); N-acetylcysteine; Collagen.

Introduction

Pulmonary toxicities are among the most common diseases, placing a heavy financial burden on healthcare systems everywhere.[1] Idiopathic pulmonary fibrosis (IPF) is the most common form of chronic lung pathology of interstitial pneumonia seen as a result of pulmonary toxicity in both human and animal models, with unknown causes and pathogenesis, characterized by persistent and unrepaired epithelial lung damage, with no effective therapeutic strategy to treat IPF.[2]

The most hallmarks of pulmonary toxicity are associated with inflammation, oxidative stress, aberrant proliferation and activation of fibroblasts to myofibroblasts accompanied by excessive production of extracellular matrix (ECM) like increasing in collagen deposition (fibrosis) in the pulmonary interstitium in the early stage of the disease. Further, the destruction of the alveolar structure causes a breakdown in gas exchange, which ultimately results in respiratory failure and death.[3,4]

Lung toxicity associated with anticancer medications is one of the most obvious side outcomes in systemic administration

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of antineoplastic therapies, occurring in approximately 5-10% of cancer patients who take chemotherapy.[5] Interstitial pneumonitis, fibrosis, acute bronchoconstriction and hypersensitivity pneumonitis are the most common manifestations of chemotherapy administration.[6]

Irinotecan, is a topoisomerase I inhibitor, was approved in Japan in 1994 to treat colorectal cancer.[7] Irinotecan associated pulmonary toxicity is a potentially fatal condition that is rarely seen in clinical practices and animal study. Few case reports of pulmonary disease have been previously reported in the earliest Japanese registration studies accounting for only 1.8% of patient's pneumonitis, as a reverse reaction of Irinotecan especially in individuals who had primary lung cancer. Cough, dyspnea and fever with a reticulonodular pulmonary infiltration are the main clinical features of Irinotecan induced pneumonitis,[8] and the extent to which Irinotecan affected pulmonary events is unknown.

Irinotecan-associated IPF may involve direct cytotoxic impact and an inflammatory response, but the exact mechanism is not well understood. As a cytotoxic agent, Irinotecan was hypothesized to block DNA synthesis and/or repair in pneumocytes and interstitial cells, hence killing those cells. However, more researches need to confirm this theory that Irinotecan causes IPF.[9]

Much research has been done in order to demonstrate the possible medications for treating IPF. N-acetylcysteine (NAC) is the N-acetyl derivative of the amino acid L-cysteine, it exhibits direct antioxidant effect through its free sulfhydryl (thiol) group which can reduce the free radicals, and enhancing glutathione precursors in lung cells under oxidative stress, which declines the disease progression and decreases the lymphocyte production as well as collagen deposition in the interstitium of the lungs.[10] Kahraman et al.[11] discovered that N-acetylcysteine ameliorated drug-induced pulmonary toxicity in rat model due to its anti-oxidant and anti-inflammatory properties.

Also Al- Hamdany and Al- Hubaity.[12] found the effect of N-acetylcysteine against IPF induced by 5-fluorouracil.

With this study, we aimed to collect and analyze the clinical and imaging data that could be used to understand Irinotecan-induced pulmonary toxicity, as well as to investigate the potential protective roles of N-acetylcysteine on fibrotic lung tissue in rat models. Additionally, if there were any significant findings in biochemical and histological parameters.

Methods

Experimental Animals

The study was carried out at Hawler Medical University/College of Pharmacy in the Experimental Animal House from the beginning of November to the end of December 2021. For this study, eighteen female Albino rats, aging from 8-12 weeks, weighing between 180-250g, were kept in Animal House at College of Pharmacy in an environmental controlled room at constant temperature 22±3℃ and humidity 50-60% under a 12-hour light/dark cycles. The animals were housed into three groups of six rats in each cage.

The experiment was conducted in the accordance of the ethical guidelines of animal house HMU/College of Pharmacy and it was an internationally accepted principle for laboratory use and care in animal research (Reference NO: 2021 2508-215 Hmu.PH.EC).

Experimental design

In Table 1, the experimental design of research proposal with experimental method has been described.

At the beginning of the process, the rats were divided randomly into 3 different groups of 6 rats per cage, the weight of each rat were recorded. The control group G1 received 1ml intraperitoneal (IP) normal saline for 14 days, G2 Irinotecan group was administered a single high dose of Irinotecan (300mg/kg) intraperitoneal

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instillation. And, G3 N-acetylcysteine 500mg dissolved in 10 ml of distilled water, NAC 500mg/kg/day via oral gavage daily given by stainless steel feeding needle to the rats 7 days before administration of Irinotecan and continued until 21 days to protect the lungs. 24 hours after the last dose, the rats were weighted and anesthetized by ketamine and Xylazine IP. After few minutes, the blood was withdrawn by cardiac puncture for hematological analysis and the rats were dissected. The lungs were weighted and then kept in diluted formalin solution for histopathological evaluation.

Drug sources

Irinotecan (100mg/5ml IV) injection, purchased from (Accord, UK), and N-Acetyl cysteine (600mg) Powder (Kochakpharma, Turkey), and other chemical agents (Ketamin Fresenius, SA) and (Xylazine, Holland) for anesthesia were used from standard commercial sources.

Antibodies and Reagents

The primary antibodies described in this paper include (Tumor Growth Factor (TGF-beta), Tumor Necrosis Factor (TNF-α) and Interlukine-6 (IL-6) (KPG)), the kits were used for determining biochemical analysis enzyme-linked assay. Complete Blood Count (CBC), to describe the hematological analysis.

Induction of lung toxicity

The group 2 was administered with single high dose of Irinotecan (300mg/kg) starting from 0 day. After 14 days of IRI administration the rats were anesthetized with ketamine 0.4 ml and xylazin 0.1ml injection. The rats were sacrificed and autopsies were performed. Group 3 is started with administration of N-acetylcysteine 500 mg/kg/day orally one week before Irinotecan administration until the end of 21 days then sacrificed.

Fixation of lung tissue injury and histopathology evaluations

Following dissection, specimens of lung tissue were first fixed in 10% formaldehyde solution for 48 hours, after which they were trimmed, dried and finally, embedded in paraffin in line with the normal operating protocols for histological evaluation. In order to evaluate the severity of the alveolitis and pulmonary fibrosis, the blocks of paraffin embedding were sectioned into slides with a thickness of 5μm, and then they were stained with Hematoxylin and Eosin (H&E) staining and Masson's trichrome staining to detect the degree of pathological analysis of inflammation and collagen deposition/ fibrosis. And the slides were examined through the use of a light microscope. All histopathological changes were identified.

Body weight

During the experimental period, the body weight of each rat in each group was measured on day 0, 7 and 14. Before dissection, all the rats were fasted for roughly 12 hours, with water consumption

Table 1 The experimental design of research proposal with experimental method

Subject	**Protective Effects of N-acetylcysteine against Irinotecan-Induced Pulmonary Toxicity in female Albino Rats**		
Model	Control group	Positive (inducer) group	Protective group
Group	G1	G2	G3
Drugs	0.9% N/S	Irinotecan	N-acetylcysteine+Irinotecan
Dose (mg/kg)	1ml 0.9%	300 mg/kg[13]	500mg/kg/day[14]
Posology	Single high dose, IP	Single high dose, IP	Once daily, Orally
Inducer inj.	None	At day 0	At day 7
Duration	14 days	14 days	21 days
Mortality rate	0	2	0

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allowed throughout these periods, and the rat body weights were recorded.

Statistical analysis

The data were all reported with the mean and standard deviation for each group. To analyze the differences between the groups, one-way analysis of variance (ANOVA) was carried out, and then multiple comparisons were carried out with a post hoc LSD test to discover whether groups had statistically significant differences. Statistical analysis was carried out with SPSS software. The statistically significant difference between the groups was determined by using *P*-values less than 0.05.

Results

Mortality rate

This study found that a single injection of 300 mg/kg of Irinotecan caused untimely death of two rats after four days. Rats in Irinotecan group died for a variety of reasons, including severe diarrhea few days after injection, lack of appetite on a regular basis that led to a fall in body weight, and poor immunity as a result of administration of Irinotecan that promote immunodeficiency.

Body weight

Individual rat weights were recorded on day 0 (pre-experiment), 7, and 14 (post-experiment) over the course of the experiment. Within 48 hours of receiving Irinotecan to G2, treated rats have seen a drop in body weight; after that time, the rats either died or began to recover. Rats in the G1 exhibited no significant variations in their steady weight gain over time. When compared to the G2. Rats in G3 treated with NAC+IRI showed a statistically significant increase in body weight from the first week of administration of IRI compared to G2, as shown in Figure 1.

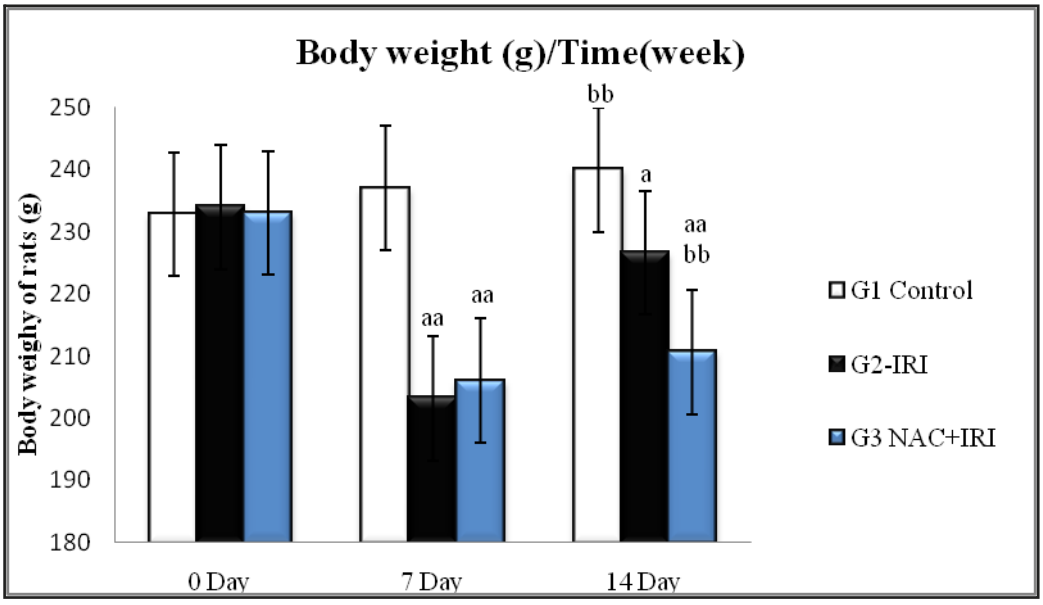

Figure 1 Effects of body weight/week in experimental groups
-(a, aa) Indicate a significant difference from the Control group *P* <0.05, 0.001
-(b.bb) indicate a significant difference from the Irinotecan group *P* <0.05, 0.001.

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Hematological analysis

Hematological analyses of cells count in blood samples with in different groups, control group, Irinotecan group and N-acetylcysteine +Irinotecan group.

In Table 2, analyses of the total amount of cells in hematological profile, revealed a significantly higher WBC and neutrophils count in G2 (Irinotecan group) compared to G1 (control group) (14.56±2.6,3.83±0.89) (7.76±0.93, 0.48±0.12), respectively, with *P* <0.05. In a G3 of N-acetylcysteine + Irinotecan, there were statistically significant decrease in WBC and neutrophils compared to the G2.

Concentration of cytokines in serum samples

Levels of TGF-beta, TNF-alpha and IL-6 in serum samples, there were significant differences observed among the groups.

Table 3, shows the mean serum levels of TGF-beta, TNF-alpha and IL-6 in control group G1, Irinotecan group G2, and treatment groups G3. The mean serum TGF-beta, TNF-alpha and IL-6 levels significantly increased compared to control group, *P* <0.05.

However, the levels of cytokines in the serum were lower in G3 of N-acetylcysteine+ Irinotecan than in G2 of Irinotecan (Table 3).

Table 2 The total number of WBCs, lymphocytes and neutrophils in hematological analysis

Results	**Cells count in serum**		
Groups	**WBC**	**Lymphocytes**	**Neutrophlis**
	10^3 cell/µl	**10^3 cell/µl**	**10^3 cell/µl**
G1 (Control group)	7.76±0.93 ^{bb}	5.90±0.67	0.48±0.12 ^{bb}
G2 (IRI)	14.56±2.6 ^{aa}	7.18±1.66	3.83±0.89 ^{aa}
G3 (NAC+IRI)	6.26±0.96 ^{bb}	4.35±0.59	1.20±0.287 ^{bb}
P- value	0.001	0.144	<0.001

-(a, aa)Indicatea significant difference from the Control group *P* <0.05, 0.001.
-(b,bb) Indicatea significant difference from the Irinotecan group *P* <0.05, 0.001.

Table 3 The expression of TGF-β, TNF-α and IL-6 in different groups, comparison of means using ANOVA test, detecting the significant points by using Post Hoc LSD test.

Cytokines biomarkers	**TGF-beta (ng/ml)**	**TNF-alpha (pg/ml)**	**IL-6 (pg/ml)**
Groups	**Mean ±SE**	**Mean±SE**	**Mean ±SE**
G1 (control)	86.92±4.10 ^{bb}	6.84±0.55 ^{bb}	22.68±2.28 ^{bb}
G2 (Irinotecan (IRI))	173.42±9.84 ^{aa}	16.67±2.32 ^{aa}	39.92±3.22 ^{aa}
G3 (N-acetylcysteine + IRI)	115.48±13.06 ^{a,bb}	12.09±1.21 ^{a,b}	31.55±4.67
P-value	<0.001	0.001	0.015

-(a, aa) Indicatea significant difference from the Control group *P* <0.05, 0.001
-(b,bb) Indicatea significant difference from the Irinotecan group *P* <0.05, 0.001.

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Histological evaluation of lungs

Examining the effects of NAC on the histological findings of Irinotecan-induced lung tissue damage in rats using H&E-staining and Masson's trichrome staining, (Figure 2).

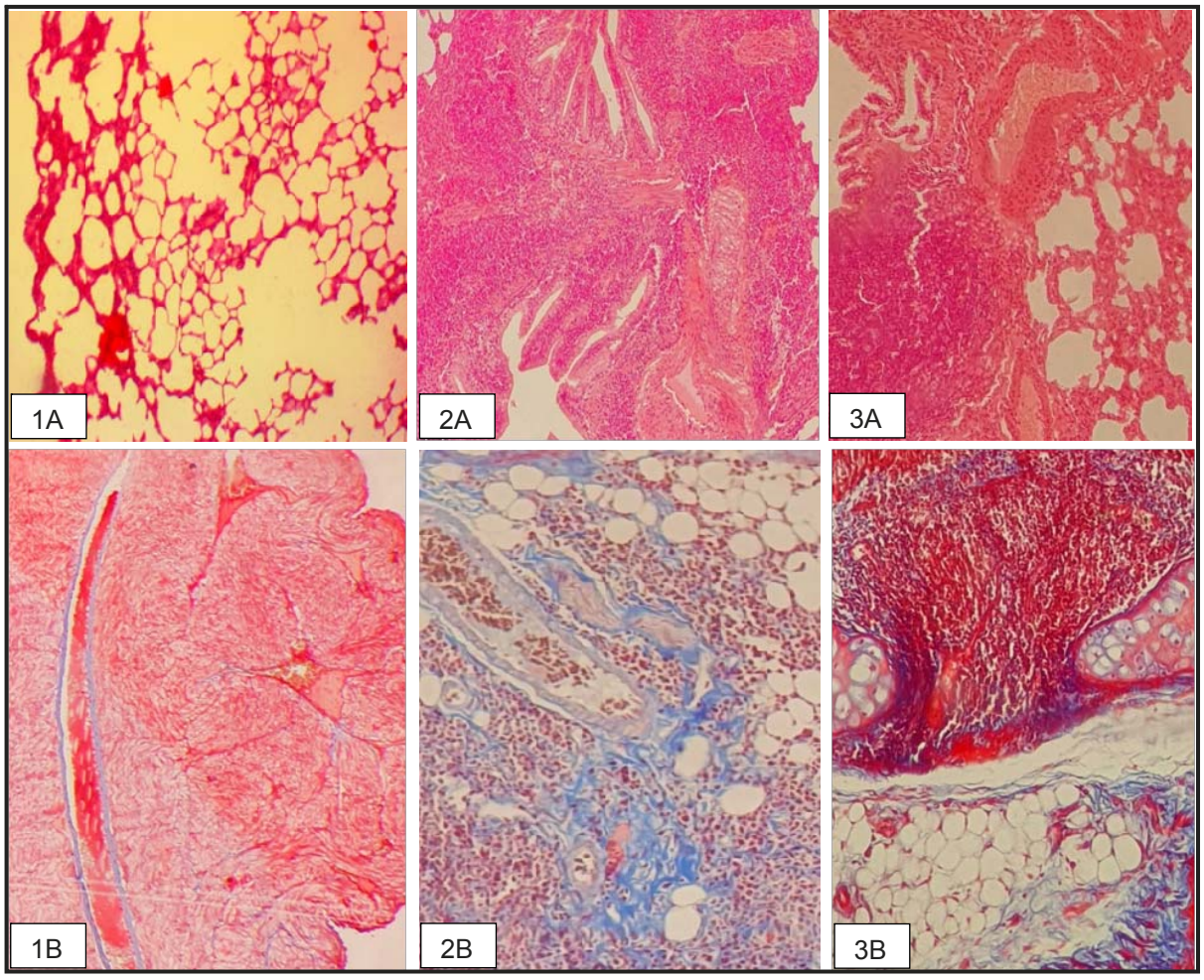

Figure 2 H&E, (1A) Section through the lungs of rats in the control group showing the normal histological structure of alveoli (100X). (1B) Section through the lungs of rats in the G2 (Irinotecan group) showing the loss of histological structure of alveoli with moderate inflammatory cell infiltration mainly lymphocyte, vascular congestion and fibroblast proliferation (100X). (1C) Section through the lungs of G3 (NAC+IRI) showing moderate inflammatory cell infiltration mainly lymphocyte, mild vascular congestion and fibroblast proliferation (100X). Masson's trichrome staining, (2A) Section through the lungs of rats in the control group showing the normal histological structure of alveoli, no fibrosis (100X). (2B) Section through the lungs of rats in the Irinotecan group, showing that blue color indicates fibrosis and collagen deposition (100X). (2C) Section through the lungs of rats in NAC+IRI, shows mild fibrosis and collage deposition, (100X).

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Discussion

Pulmonary toxicity is one of the most obvious adverse effects of systemic anti-cancer medications, such as Irinotecan, it can produce oxygen free radicals, which damages the lipid membrane in the lungs, and immune-mediated mechanism, including processes such as activation activities of the natural killer cells, this is the most important factors in the development of drug-induced lung toxicity.[15]

The main adverse effects of Irinotecan administration to patients were included suppression of bone marrow (leukopenia), persistent diarrhea, nausea/vomiting, anemia and alopecia. However, Irinotecan has also been linked to a small number of incidences of pulmonary injury. In addition, few case reports of Irinotecan-induced interstitial pneumonia have been published recently in literatures, showing IPF as one of the possible adverse effects of Irinotecan.[16,17] To know that, this animal study is used to demonstrate the possibility of pulmonary toxicity induced by Irinotecan in rat models.

In this study, hematological analysis and histological changes to the alveolar structure are support to the concept that Irinotecan induces IPF.

The current study showed that the G2 had a greater tendency for body weight loss of the rats during the first week after Irinotecan administration compared to G1, while G3 N-acetylcysteine + Irinotecan group had a lower propensity for weight loss induced by Irinotecan with P <0.05. Kim et al.[18] discovered that intratracheal administration of belomycin induced pulmonary fibrosis, results in a weight loss within the first week. Also, Hapitoglu et al.[19] observed a significant weight loss in a study involving belomycin-instillation in mice.

However, the severity of diarrhea in the group of Irinotecan is the main factor that causes rat dying during this period. Besides, other factors such as low immunity and loss of appetite can be attributed to the Irinotecan administration that induces immunodeficiency, another study done by Tang et al.[20] stated that Irinotecan can cause severe diarrhea.

In the present study, G2 Irinotecan causes increase in hematological cell count of WBC, lymphocyte and neutrophils (14.56±2.6, 7.18±1.66, 3.83±0.89) compared to G1 (7.76±0.93, 5.90±0.6, 0.48±0.120) respectively. As illustrated in table 2, these results in agreement with the results in belomycin induced pulmonary fibrosis in rat model with increasing in cell count in BALF.[18]

TGF-beta is the primary cytokine strongly associated with idiopathic pulmonary fibrosis and lung fibrosis, and its elevation is directly related to the degree of pulmonary fibrosis. In this study, the mean serum level of TGF-beta was significantly increased in the G2 Irinotecan group 173.42±9.84 ng/ml when compared to the G1 control group 86.92±4.10 ng/ml. Chitra et al.[21] established that TGF-β plays a critical role in lung fibrosis which confirms the findings of this study that administration of Irinotecan can sufficiently induce severe fibrotic activities in the lungs of rats.

The mean serum level of TNF-alpha was significantly increased in the Irinotecan group 16.67±0.55 pg/ml when compared to the control group 6.84±0.55 pg/ml. These findings corroborated the findings of a prior researcher who found that drug-induced lung toxicity increase in TNF-alpha cytokines were followed by increases in the expression of the profibrotic cytokine TGF-beta, which generated both high levels of oxidative stress and inflammation.[22]

Finally, the mean serum level of IL-6 was also significantly increased in the group of Irinotecan 39.92±3.22 pg/ml when compared to the control group 22.68±2.28 pg/ml and this is consistent with the research that proved the belomycin-induced pulmonary fibrosis.[18,22] IL-6 is a main cytokine which is strongly related to pulmonary fibrosis and its increase is directly proportion with the severity of pulmonary fibrosis.[23]

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Up to 2022, a substantial amount of research has been carried out to demonstrate the potential drugs for the treatment of IPF. On the other hand, N-acetylcysteine is an antioxidant medication that was developed recently with the intention of slowing down the progression of IPF.

Giving N-acetylcysteine + Irinotecan together, significantly reduced the mean serum TGF-beta, TNF-alpha and IL-6 levels comparing with Irinotecan group as administered 500 mg/kg/day via oral gavage for 21 days. Orally taking N-acetylcysteine, effected lymphocyte infiltration of the lung fibrosis, these results agree with Zhang et al.[24] who found that N-acetylcysteine improved the antioxidant protection of the lungs and is used to reduce lung damage produced by drug induced lung toxicity.

To confirm that Irinotecan induced pulmonary fibrosis and to decrease fibrosis by treatment with N-acetylcysteine, histopathological studies was conducted by Hematoxylin-Eosin (H&E) staining and Masson's trichrome staining to identify inflammatory cells and collagen deposition. The histological section of the control group revealed no pathology, normal alveolar structure, without cellular infiltration or fibrosis.

Histopathological examination of the rat's lungs in the Irinotecan group showed a loss of normal lung tissue architecture. The loss was characterized by; alveolitis, vascular congestion (VC), fibroblast proliferation (F), moderate to chronic diffuse inflammatory cells infiltration, primarily lymphocytes (L) and collagen deposition. However, according to Ozawa et al.[8] it has been implicated with severe pulmonary complications, the most common being pulmonary fibrosis and other disorders such as chronic interstitial pneumonitis after Irinotecan administration. Additionally, Kanaji et al.[25] attributed Irinotecan's inflammatory effects on lungs tissues as evidenced by inflammatory infiltration of lymphocyte.

Irinotecan-induced pulmonary toxicity has been demonstrated to progress to fibrosis. Masson's Trichome staining of a section of rat lungs in the Irinotecan group indicated that collagen deposition and fibrotic activities. The proliferation of fibroblasts to form predominant lining cells of the alveoli corroborates previous animal model experiments, which suggest that fibrogenesis is initiated at the reserve epithelial cells of the alveoli. Accordingly, Kim et al.[18] posited that both hyperplasia and fibrotic proliferation can be considered to be a manifestation of fibrotic repair often reflected in the underlying drug-induced lung injuries.

Conversely, histopathological sections of the G3 N-acetylcysteine + Irinotecan showed less diffuse inflammatory cells infiltration predominant lymphocyte, fibroblast proliferation, vascular congestions and collagen deposition compared to G2 Irinotecan group. Thus, concomitant administration of N-acetylcysteine with Irinotecan showed fewer symptoms of pulmonary toxicities in the lung parenchyma.

The observation made in the present study is consistent with those previously described by other researchers. Farag et al.[26] reported that administration of N-acetylcysteine could attenuate alveolar damage and reduce the rate of pulmonary collagen deposition due to assault by Irinotecan in the lungs. Moreover, Chen et al.[27] postulated that the positive effects of N-acetylcysteine against drug-induced pulmonary fibrosis in animal models could be attributed to its inhibitory effects on the secretion of cytokines released from macrophages. Examples of such cytokines include transforming growth factor-β_1 (TGF-β), which plays a central role in remodeling the extracellular matrix.

Conclusion

The results suggest that Irinotecan induce pulmonary fibrosis by increasing the inflammatory cytokines detected by biochemical and histological studies. Oral

treatment of N-acetylcysteine significant lyreduces the activity of alveolar proinflammatory cytokines in serum that attenuates lung fibrosis caused by Irinotecan in rat models.

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Competing interests

The authors declare that they have no competing interests.

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