Effect of Pistacia lentiscus oil on experimental pulmonary fibrosis

Effet de l'huile de Pistachier Lentisque sur la fibrose pulmonaire expérimentale

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R É S U M É

Introduction : La fibrose pulmonaire idiopathique (FPI) est une maladie chronique caractérisée par un aspect histopathologique de pneumopathie interstitielle commune. C'est la plus fréquente et la plus grave des pneumopathies interstitielles diffuses idiopathiques. Les traitements actuels sont fondés sur l'association de corticoïdes et d'immunosuppresseurs, mais leur efficacité demeure discutée.

But du travail : Tester l'effet préventif de l'huile de pistachier lentisque, connue pour ses effets anti-oxydants, antimutagènes et antiprolifératifs, sur un modèle de fibrose pulmonaire expérimentale.

Méthodes : Deux groupes de rats ont reçu une injection intra trachéale de bléomycine (4,5 mg/kg). Le premier groupe, témoin (n=20 rats) n'a reçu aucun traitement. Le deuxième groupe, traité par l'huile de pistachier lentisque (n=20 rats) a subi pendant 30 jours précédant l'induction de la fibrose, un gavage quotidien par l'huile de pistachier lentisque (3,33ml/kg). Ce traitement a été poursuivi pendant 10 jours. A la fin du protocole expérimental, tous les rats ont été sacrifiés et les poumons fibrosés ont subi une analyse anatomo-hitologique et immunohistochimique du Transforming Growth Factor (TGF).

Résultats : Le profil chromatographique de l'huile de pistachier lentisque montre la prédominance de deux acides gras qui sont l'acide linoléique et l'acide palmitique représentant respectivement 70,57 et 24,67%. Nos résultats montrent également une diminution de la distribution du TGFß aussi bien au niveau de l'infiltrat inflammatoire qu'au niveau des histiocytes du parenchyme pulmonaire des rats traités à l'huile de pistachier lentisque comparativement aux rats témoins. Cependant, ces modifications ne s'accompagnent pas de modifications statistiquement significatives du score de la fibrose et de l'indice inflammatoire.

Conclusion : Nos résultats sont intéressants à considérer. Des études complémentaires utilisant des doses plus importances de l'huile de pistachier lentisque s'avèrent nécessairses surtout que cette huile ne présente pas de toxicité.

M o t s - c l é s

S U M M A R Y

Background: Idiopathic pulmonary fibrosis (IPF) is a chronic disease characterized by histopathological lesions in lung tissue. This is the most common and most severe idiopathic interstitial pneumonias. Current treatments are based on the combination of corticosteroids and immunosuppressants, but their effectiveness is still debated.

Purpose of work: Testing the preventive effect of Pistacia Lentiscus oil, known for its antioxidant, anti-mutagenic and anti-proliferative effects, on a model of experimental lung fibrosis.

Methods: Two groups of rats received an intratracheal injection of bleomycin (4.5 mg / kg). The first group, control (n = 20 rats), has received no treatment. The second group was treated with Pistacia Lentiscus oil (n = 20 rats) for 30 days before fibrosis induction, by daily gavage oil Pistacia Lentiscus oil (3,33ml / kg). This treatment was continued for 10 days. At the end of the experimental period, all rats were sacrificed and the lung tissue was examined histopathologically and immunostained for TGFβ.

Results: The chromatographic profile oil Pistacia Lentiscus oil shows the dominance of two fatty acids that are linoleic acid and palmitic acid representing respectively 70.57 and 24.67%. Our results also show a decrease in the distribution of TGFβ both at the level of the inflammatory infiltrate and at the level of the pulmonary parenchyma histiocytes of rats treated with Pistacia Lentiscus oil compared with control rats. However, these changes are not accompanied by statistically significant changes of fibrosis score and inflammatory index.

Conclusion: Our results are interesting to consider. Further studies using higher doses of Pistacia Lentiscus oil are important to conduct.

K e y - w o r d s

Pulmonary fibrosis, inflammation, TGFβ, Pistacia Lentiscus oil, rat, Bleomycin.

Idiopathic diffuse interstitial fibrosis is a chronic disease of poor prognosis [1, 2, 3, 4]. It is characterized by an elementary lesion of lung tissue with increased fibrillar components of the extracellular matrix (ECM). In advanced disease, there is a profound disruption to lung architecture that causes impairment of mechanical function, gas exchange and alveolar-capillary diffusion [5]. The pathophysiology of pulmonary fibrosis is still poorly elucidated and the current treatment methods are anti-inflammatory drug therapy, immunosuppressive and immunomodulatory agents, anti-fibrosis drugs, antioxidants, anti-leukotriene drugs, and lung transplantation. However, these treatments are associated with controversy, and large sample epidemiological surveys have shown that the evidence for the treatment is not persuative [1, 6, 7, 8, 9]. Despite a number of advances in basic and clinical reseach, currently, pulmonary fibrosis remains a progressive and fatal disease.

Thus, there is an urgent need to identify or develop novel and effective therapeutic agents for pulmonary fibrosis. Bleomycin (BLM), a chemotherapeutic agent, is indicated in the management of some types of cancers. This drug produces a dose-dependent pulmonary fibrosis (PF) in most patients as well as experimental animals through oxidative injury. Using this experimental model, we showed, in previous studies, that treatment with All-Trans-Retinoic Acid significantly attenuated the increased pulmonary damage induced by BLM in rat [10]. Otherwise, we showed that fenugreek's polyphenol had a potent anti-inflammatory activity against bleomycin induced lung fibrosis but no major effect on structural disorganisation resulting from BLM, indicating that the inflammatory response and the fibrotic response can be dissociated [11]. On the other hand, Pistacia lentiscus L. (Anacardiaceae) is an evergreen shrub widely distributed throughout the Mediterranean region. North African people used Pistacia lentiscus oil in traditional medicine particurlaly to treat sore throats, burns and wounds, as well as respiratory allergies. *Pistacia lentiscus oil* is rich in essential fatty acids, vitamin E and polyphenols. It was found to have liver antioxidant [12], antimutagenic and antiproliferative effects [13, 14] but, and to our knowledge, it had never been tested in case of pulmonary fibrosis.

This study aimed to investigate the preventive effect of a fixed oil extracted from Pistacia lentiscus in experimental pulmonary fibrosis induced by bleomycin in rat.

METHODS

Animals

This study was carried out in fourty young wistar male rats (average weight 120 ± 30g), reared in the animal house of the Faculty of Medicine of Tunis. During the experiment, rats were housed according the recommendations of the International Council of Laboratory Animal Science (ICLAS). The room temperature was between 20 and 25 ° C with a normal cycle of day and night (12 hours light and 12 hours darkness). Food and water were provided *ad libitum*.

Experimental protocol

Rats were housed in individual cages and randomly divided into two groups: a treated group consisting of 20 rats received daily gavage with pistachio oil mastic (3.33 ml / kg) and a control group of 20 rats that were force-fed daily with water (3.33 ml / kg). The gavage of rats in both groups lasted 30 days before the induction of fibrosis by bleomycin and one week after induction of fibrosis.

Induction of fibrosis

Thirty days after start of the feeding, all rats underwent anesthesia by intraperitoneal injection of 75 mg / g of pentobarbital sodium solution (Sandoz laboratory, France). Each anesthetized rat was immediately suspended from a gallows. Induction of fibrosis was done by intra-tracheal injection of 4 mg/kg of bleomycin sulfate solution (Bleomycin ®, Laboratories Aventis, France) in 50 µl saline, as previously described [15].

Animals sacrifice

Anesthetized rats underwent laparotomy and were bled by incision of the abdominal aorta. Section of the diaphragm and of the anterior thorax allowed us to extract the heart-lung block.

Histological and immunohistochemical analysis

For histological studies, the lungs were perfused through their main bronchus with fixative solution (10% neutral-buffered formalin), immersed in the fixative for 24 h, and the blocks were taken thereafter. Tissue blocks were placed in formalin dehydrated in a graded series of ethanol, embedded in paraffin, cut into 4 mm thick serial sections, and stained with haematoxylin-eosin (H&E) to identify the inflammatory cells of Masson's trichrome for collagen deposition. Histological grading of lesions was performed using a blinded semi quantitative scoring system for extent and severity of inflammation and fibrosis in lung parenchyma. The severity of inflammation was estimated using the semi quantitative grading system which considers the following categories: Grade 0 = "absence of inflammation", Grade 1 = "minimal inflammation", Grade 2 = "minimal to moderate inflammation", Grade 3 = "moderate inflammation with thickening of alveolar walls", Grade 4 = "moderate to severe inflammation" and Grade 5 = "severe inflammation with presence of follicles which replace the parenchyma". The severity of interstitial fibrosis was also determined using the semi quantitative grading system, described by Ashcroft et al. [16]. The entire lung section was observed at a ×100 magnification and a score ranging from 0 (normal lung) to 8 (total fibrosis) was assigned. The

adopted categories of grading pulmonary fibrosis were as follows: Grade 0 = "normal lung", Grade 1 = "minimal fibrous thickening of alveolar or bronchial walls", Grades 2 to 3 = "moderate thickening of walls without obvious damage to lung architecture", Grades 4 to 5 = "increased fibrosis with definite damage to lung architecture and formation of fibrous bands or small fibrous mass", Grades 6 to 7 = "severe distortion of structure and large fibrous areas", "honeycomb lung" was placed in this category; Grade 8 = "total fibrotic obliteration of the field". The mean score of all fields was taken as the fibrosis score of that lung section. The immunohistochemical studies were performed on one representative block from each case. Sections of 3 to 4 µm were deparaffinezed with xylem and ethanol. Endogenous peroxides activity was blocked with 3% hydrogen peroxide for 10 min. Microwave epitope retrieval was used. Immunohistochemical analysis was performed using *TGFβ* antibody (R&D System Laboratories, France). The density of *TGFβ* in lung tissue was scored on a scale ranging from 0 to 3: 0 = "absent", 1 = "low", 2 = "medium", and 3 = "important". Micrographics were obtained by Nikon Cooplix 4500 camera.

Gas chromatographic (GC) analysis

Fatty acid composition of Pistacia lentiscus oil were determined using GC after derivatization to fatty acid methyl esters (FAME). The preparation of FAME was performed via saponification in 0.5M NaOH–MeOH solution and methylation with 14% BF3–MeOH (Sigma, USA), according to the 5509 ISO method FAME separation and identification were carried out on the gas chromatograph (6890 N, Agilent Technologies, USA) equipped with a flame ionization detector and capillary column HP-Innowax (30m×0.32mm×0.25 m).

The amount of each sample injected was 1.0 ml. Nitrogen, at a constant flow 1.0 ml/min, was used as the carrier gas and a spilt/spiltless injector was used with a split ratio of 50:1. The injector temperature was 230°C and the detector temperature was 280°C. The column temperature was programmed according to the following: initial temperature was 150 °C for 1 min and then increased 15°C/min to 210°C and maintained for 5 min before being readjusted upward again 5°C/ min to 250°C and then maintained until the end of the analysis that takes 25 minutes.

Fatty acid methyl esters were identified by comparison with the standard fatty acid methyl esters (Sigma, USA). Fatty acid methyl esters were quantified as percentages of the total methyl ester peak areas.

Statistical Analysis

SPSS 17.0 was used in data analysis. Data were analyzed with one-way analysis of variance (ANOVA) followed by a post hoc test (LSD alpha) for multiple comparisons. Alveolitis, fibrosis scores of lung tissue and TGFβ density were evaluated using the Mann-Whitney *U*-test and fatty acids comparison were evaluated using independent sample *t*-test. The data were expressed as mean± standard derivation (SD). P values < 0.05 were considered statistically significant.

RESULTS

Fatty acids compostion in *Pistacia lentiscus* oil

Figure 1 and table I show the results of chemical analysis of Pistacia lentiscus oil by Gas Chromatography (GC) performed at the National Institute of Nutrition of Tunis. Two major fatty acids were determined: linoleic and palmitic acids. Linoleic Fatty acid was the main fatty acid presenting more than 70% of the total fatty acid content. Whereas, palmitic acid presented almost 20%.

Table1: Chemical analysis of Pistacia lentiscus oil by gaz chromatography

Fatty acids	Retention time	Percentage (%)
Caprylic acid (octanoic)	17.68	0.854%
Capric acid (decanoic)	1.811	0.134 %
Myristic acid (tetradecanoic)	3.553	0.042 %
Palmitoleic acid (cis_9_hexadecenoique)	5.000	1.792 %
Palmitic acid (hexadecanoic: cetyl)	5.137	24.672 %
Heptadecanoic acid (margaric)	6.115	0.087 %
Heptadeconic acid	6.330	0.060 %
Linoleic acid (omega 6)	7.595	70.572 %
Stearic acid (octadecanoic)	7.919	1.414 %
Gadoleic Acid	12.110	0.208 %
Arachidonic acid (eicosanoic)	12.754	0.167 %

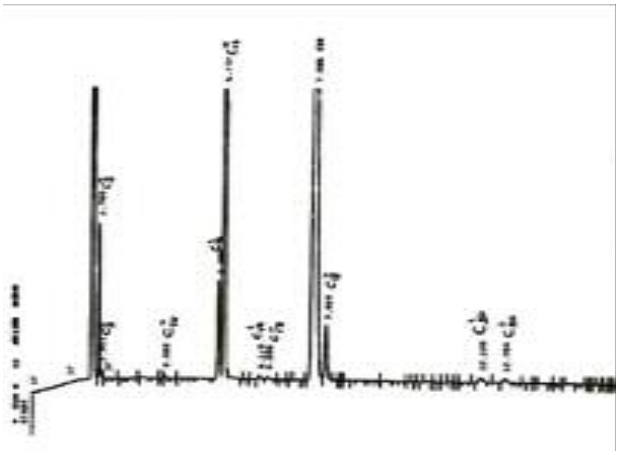

Figure 1 : Curve of chemical analysis of the pistacia lentiscus oil by GC

Histology and immunohistochemical analyses

The histological analysis showed that treatment with *Pistacia lentiscus oil* had no effect on inflammatory. In fact, inflammatory index was comparable in both groups (p= 0.82) (Table 2).

Otherwise, BLM caused high score fibrosis in control group, whereas treatment with *Pistacia lentiscus oil* induced a slight decrease of this score (4.1±1.852 vs 3.2±1.135, p= 0.22) (Table 2).

Table 2: . Score fibrosis, inflammatory index and TGFβ density in different lung areas

Groups	Control	Treated	P
Score Fibrosis	4.1±1.852	3.2±1.135	0.22
Inflammatory index	3.5±1.081	3.5±0.527	0.82
TGFβ density in alveoli	0.3±0.483	0.1±0.316	0.46
TGFβ density inflammatory infiltrate	1.8±1.033	0.5±0.707	0.030*
TGFβ density in fibrocytes	2.8±0.421	1.2±1.135	0.022*

Values are expressed as means ± SD; number of rats: n = 20.

The evaluation of TGFβ immunostaining (figure 2 and table 2) in different regions of the lung (alveol, fibrocytes and inflammatory infiltrate) in studied groups, revealed a net decrease of distribution of this cytokine in the lung fibrocytes (1.2±1.135 vs 2.8±0.421; p=0.022) and in inflammatory infiltrate (0.5±0.707 vs 1.8±1.033; *p*= 0.030) of rats in treated group compared to those of the control group.

DISCUSSION

To the best of our knowledge, this is the first report of the effect of Pistacia lentiscus oil on induced pulmonary fibrosis by BLM in rats. In this study, we aimed to evaluate the protective effects of Pistacia lentiscus oil on the bleomycin-induced early lung injury and fibrosis. The anesthesia and bleomycin instillation procedure were well tolerated by the rats. No adverse effects were observed in rats that received Pistacia lentiscus oil and all of the animals from both groups survived until the end of the experiment. In this work, we proposed to test the

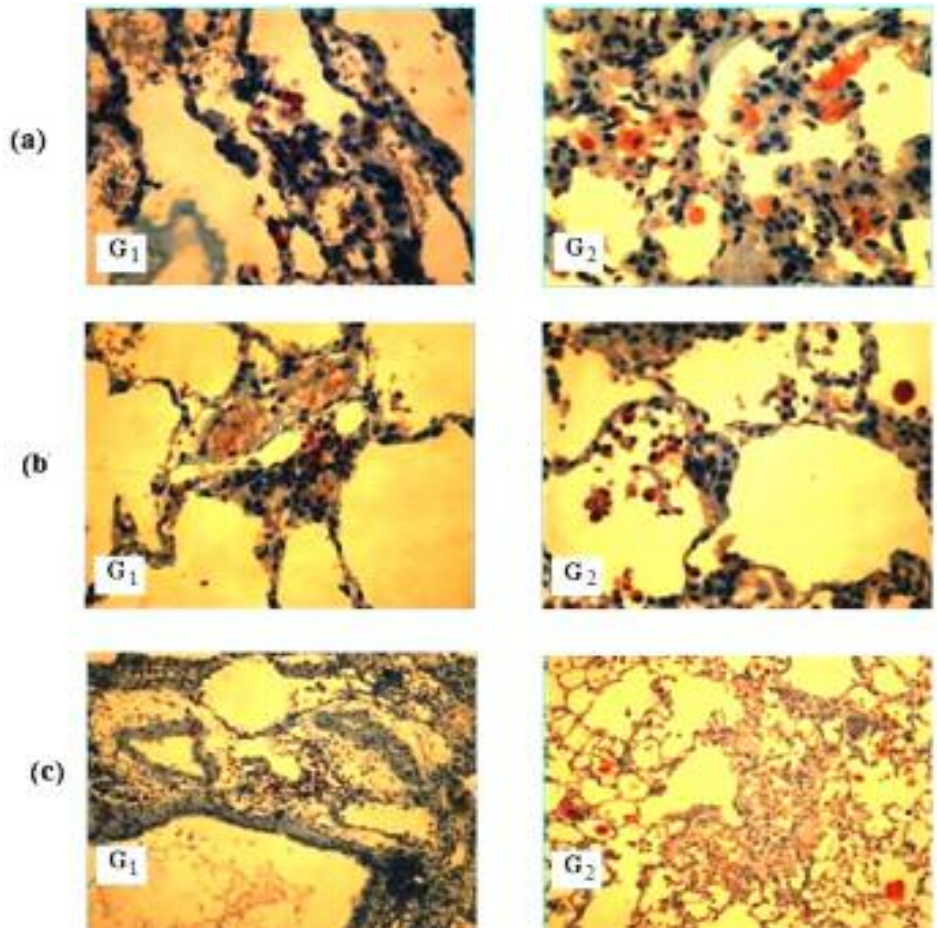

Figure 2 : Immmunohistochimical analysis: section of control rat received BLM (G1) and rat received BLM + Pistacia lentiscus oil (G2). One representative example is shown for each group. Original magnification × 400. (a):Histiocytes (b):Alveoli (c):Inflammatory infiltrate

effectiveness of Pistacia lentiscus oil on an experimental model of pulmonary fibrosis induced by bleomycin in Wistar rats. Evaluation of parenchymal fibrosis was based on anatomicopathological analysis, degree of inflammation and evaluation of the density of TGFβ by immunohistochemistry.

BLM is a drug used to treat different types of neoplasms. BLM's most severe adverse effect is lung toxicity, which induces remodeling of lung architecture and loss of pulmonary function, rapidly leading to death. While its clinical role as an anticancer agent is limited, its use in experimental settings is widespread since BLM is one of the most widely used drugs for inducing lung fibrosis in animals, due to its ability to provoke a histologic lung pattern similar to that described in patients undergoing chemotherapy. Up to now, the mechanisms involved in the development of pulmonary fibrosis have not been fully understood. Several studies have analyzed various potential biological molecular factors, such as transforming growth factor beta 1, tumor necrosis factor alpha, components of the extracellular matrix, chaperones, interleukins and chemokines [17].

In our study, we used BLM at the dose of 4mg/kg. This dose is fairly well tolerated by rats and sufficient to induce the development of a significant interstitial diffuse fibrosis as shown by fibrosis scores in control group. Treatment with Pistacia lentiscus oil induced a slight decrease of this score but the difference was not statiscally significative (p=0.22). Based on the microscopic results, the two groups did not differ in inflammatory index (Table 2). Our data suggest that Pistacia lentiscus oil has no apparent effects on inflammatory.

Pistacia lentiscus oil is commonly used in the north west of Tunisia for its therapeutic properties. It is known for its anti-oxidant [18] and antiproliferative effects [19]. This oil is rich in linoleic acid (Omega 6) (70.57%), which has anticancer activity [10, 20], antibacterial [21], antiproliferative and anti-inflammatory effect [22].

Omega 6 is one of the polyunsaturated fatty acids that have a beneficial effect on the nervous system, cardiovascular balance, immunity, healing.

The significant presence of palmitic acid «hexadecanoic: Cetyl» and vitamin A in this vegetable oil (24.67%) enhances its antioxidant and anti-proliferative activity.

The antioxidant and antiproliferative activity is manifested by a decrease in inflammation, immune cell activation and inhibition of xanthine oxidase and lipid peroxidation [10, 20, 23].

Since its discovery in the early 1980s, TGFβ emerged as a growth factor involved in essential physiological processes like embryonic development, tissue repair, differentiation and control of cell growth [24, 25]. This cytokine plays a central role in the pathogenesis of pulmonary fibrosis in association with some thirty other proinflammatory cytokines (TNF, TGFα, PDGF, FGF, EGF, IGF-1) [26]. It is a cytokine that plays a key role in the initiation and tissue repair. A prolonged production of TGF beta is involved in the development of fibrosis [27]. It can stimulate or inhibit the proliferation depending on cellular context, controlling the turnover of extracellular matrix: The rate of this cytokine increases in lung fibrosis. By stimulating the biosynthesis of fibronectin and collagen, TGFbeta exerts a powerful pro fibroblast. It also promotes the growth of fibroblasts and regulates their differentiation into myofibroblasts [28]. The increased density of TGF β has also been reported in experimental fibrosis induced by bleomycin. A positive correlation exists between the tissue levels of TGF β and the severity of fibrosis induced by bleomycin [25].

Also, the role of TGFβ in the excessive synthesis of collagen in lung fibrosis is demonstrated [29, 30]. So, intense production of this cytokine and its attachment to its specific receptor type II favors the induction of the inflammatory response and subsequent installation of fibrosis [31].

The results of this study agree well with those of other experimental work of the literature that show an increased density of TGFβ in different areas of fibrotic lung tissue (histiocytes, cells) and in the level of the inflammatory infiltrate in response to pulmonary fibrosis induced by bleomycin [32].

For treatment with Pistacia lentiscus oil, our study shows partial effectiveness of this oil especially in the inflammatory infiltrate in which it induced a significant difference in density of TGFβ of treated rats compared to the control group.

In light of this work, Pistacia lentiscus oil led to a decrease in the production of TGF β in the inflammatory infiltrate. These results could be due to the use of low doses. Higher doses could be tested to verify a possible dose-response.

CONCLUSION

Our study showed significant anti-inflammatory effects of natural oil extracted from Pistacia lentiscus. These results are encouraging and pave the way for other future therapeutic trials. Thus, we consider and test the dose-response effect of this oil could be more effective at higher doses, include the mode and site of action of this oil, also test the curative effect of this oil on fibrosis lung induced by bleomycin and try to test the effect of this oil in combination with other natural substances.

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