Role of Tumor Necrosis Factor-alpha and Interleukin-6 in Nutmeg Induced Pulmonary Injury in Adult Albino Rats: A Light Microscopic and Molecular Study

Ghada Hasan El-saify; Nadia Said Badawy khair and Nermeen Mohamed Noor Eldien

Departments of Histology & Anatomy and Embryology, Faculty of Medicine, Menoufia University, Egypt

amarsmile2007@yahoo.com

Abstract: Background: Nutmeg is a popular spice & flavor that has a long list of associated health benefits. It is the shelled, dried seed of the plant Myristica fragrans. Nutmeg is used to treat diarrhea, nausea, stomach pain, and intestinal gas. It is also used for treating cancer, kidney disease, and trouble sleeping. Nutmeg is widely used for increasing menstrual flow, causing a miscarriage. Because it contains chemicals that affect central nervous system, many people used it as a hallucinogen and as a general tonic. Side effects such as thirst, dizziness, nausea, vomiting, feelings of pressure in the chest or stomach, dry mouth, stomach pain, and many other problems might occur in some people. Large dosage can be toxic, producing disorientation, double vision and convulsions, and even death.

Objectives: Evaluating the role of Tumor Necrosis Factor- alpha (TNF-alpha) and Interleukin-6 (IL-6) in Nutmeg Induced Pulmonary Injury in adult albino rats.

Material and Methods: Adult male albino rats (n=40) were used & classified into two groups: Group I (n= 20) served as control & group II (n= 20) treated with nutmeg in a dose of 500mg/kg orally daily for 12 weeks (1/10 LD50). At the time of sacrifice, the lungs were dissected and tissue samples were processed for light microscopic & molecular studies. Histological (haematoxylin and eosin, Toluidine blue & Masson trichrome stains) and immunohistochemical studies (CD68 to show alveolar macrophages) were done. Morphometric study was also done for septal alveolar wall thickness and number of alveolar macrophages. The mRNA expression of TNF-alpha and IL-6 in the lung tissue was quantified by competitive RT-PCR.

Results: Administration of nutmeg markedly disrupted the normal architecture in the form of thickening of inter-alveolar septa, over expansion of alveoli, congestion of blood vessels, cellular infiltration, proliferation and vacuolation of pneumocytes type II. Increase in TNF-alpha and IL-6 levels was also noticed. Conclusion: It could be concluded that prolonged administration of nutmeg in rats can induce lung damage with possible role of both TNF-alpha and IL-6 cytokines for further studies.

Key words: Nutmeg, TNF-alpha, IL-6, lung, albino rats, pneumocytes type II, CD68.

1-Introduction

The Nutmeg plant, Myristica fragrans Houtt, (family Myristicaceae) is a seed of an evergreen tree. It is one of the most commonly used spices in the world. In folk medicine, it is important in treatment of respiratory, skin, GIT diseases and CNS disorders [1]. Nutmeg is widely accepted as a flavouring agent and was used in higher doses (500 mg /Kg) as aphrodisiac and psychoactive agent in male rats [2,3] It consisted of phytochemicals that include alkaloids, saponins, antraquinones, cardiac glycosides, flavonoids and phlobatanins [4]. The active ingredient of nutmeg is Myristicin which has possible neurotoxic effects on dopaminergic neurons and monoamine oxidase [5].

Nutmeg has been shown to possess a spectrum of pharmacological activities, including anti-bacterial, anti-inflammatory, anti-cancer, anti-diabetes and hepato-protective activities [6] The flavonoids have been found to inhibit the development of chemically-induced cancers in animal models [7]. In-vitroanti-cancer effects in human as follows: Ovary cell line showed highest cytotoxic activity (97%); colon; prostate growth inhibition was 86%.; colon growth inhibition range from 73-86%, CNS 73% while Liver showed 72% but lung showed no significant activity (50%). However, epidemiological prospective cohort studies do not provide convincing evidence that high intakes of dietary flavonoids are associated with substantial reductions in human cancer risk [8].

Nutmeg is associated with toxicity and it is a cause of health concern when it comes to intoxication calls [9, 10]. It has been reported to reduce cell viability in a dose dependent manner. Cytotoxic and apoptotic effects of Myristicin have been reported such in a dose dependent manner [5]. Myristicin poisoning produces nausea, generalized body pain, palpitations, dehydration and even convulsions [10]. Investigations approved nutmeg toxicity on the brain [11] and parenchymatous excretory organs as kidney [12], and liver [13]. Evidence suggested the relation between inflammatory mediators released including...
cytokines and lung injury [14]. The term "cytokine" is derived from a combination of two Greek words - "cyto" meaning cell and "kino" meaning movement. Cytokines are cell signalling molecules that aid cell to cell communication in immune responses and stimulate the movement of cells towards sites of inflammation, infection and trauma [15]. Some cytokines act to make disease worse (proinflammatory cytokines) as they cause inflammation and marked tissue destruction, whereas others serve to reduce cytokines and lung injury [14]. The term "cytokine" is derived from a combination of two Greek words - "cyto" meaning cell and "kino" meaning movement. Cytokines are cell signalling molecules that aid cell to cell communication in immune responses and stimulate the movement of cells towards sites of inflammation, infection and trauma [15]. Some cytokines act to make disease worse (proinflammatory cytokines) as they cause inflammation and marked tissue destruction, whereas others serve to reduce inflammation and promote healing (anti-inflammatory cytokines) [16]. IL-1, IL-6, TNF-alpha and TGF-Beta are examples of proinflammatory cytokines, while IL-4, IL-10 and IL-13 are post inflammatory cytokines. TNF-alpha and IL-6 presenting cytokines that might play an important role in early and delayed development of lung injury [17]. Despite the wide use of nutmeg in many countries as an herbal remedy and its useful pharmacological effects, few studies have been published about its toxicological profiles specially on lungs. This work was conducted to clarify the toxic effects of nutmeg on lungs of adult male albino rats by histological, morphometrical and immunohistochemical assays and the role of TNF-alpha and IL-6 in these effects.

2-Material and Methods

Animals

The experiment was conducted on forty (40) adult male albino rats weighting 170-200 gm. The animals were kept in standard housing conditions and were freely supplied with food and water for 1 week before the experiment for acclimatization. They were randomly divided into two groups: A and B (n=20 in each group). Group A served as the control while group B served as treated group.

Nutmeg extraction & administration:

Dried seeds of nutmeg were collected from local market in Shebin El-Kom, Menoufia, Egypt. Alkaloid extraction was done in Faculty of Science, Menoufia University. According to the modified method of Harborne [18].

LD50 was 5.1g/kg [19]. Rats were given nutmeg in a dose of 500mg/kg orally daily for 12 weeks (1/10 LD50).

Group I (control):

The animals (n=20) were housed in stainless steel cages. Each cage contained five animals. The control group received feeds without nutmeg added for 12 weeks to investigate the basic parameters.

Group II (nutmeg treated):

The animals (n=20) were housed in stainless steel cages. We used 5 cages each one had partition that divide the cage into four parts. The dose was calculated according to each animal weight and mixed with small amount of food. We put four animals in the same cage and the partition separated each one from others, and then put food that contained required dose of nutmeg. After being sure that the rat ate the required dose, we removed the partition and gave them extra food and water. These steps were repeated every day for 12 weeks. The general condition and behavior of animals were noticed and recorded. Animals of each group were weighed at the beginning and at the time of sacrifice. The animals were killed by cervical dislocation then chest was opened and the lungs were dissected, weighted & prepared for histological, immunohistochemical & morphometrical studies. Small parts of the lungs were isolated & prepared for RT-PCR study. Semithin sections for Toluidine staining were done.

Histological Study:

The lungs were dissected and fixed in 10% formol saline, processed to paraffin wax and sections of 6um thickness were prepared for histological light microscopic study using hematoxylin and eosin and Masson trichrome stains [20].

Immunohistochemical study:

Immunostaining was performed using an avidin biotin–peroxidase technique for showing alveolar macrophages using CD68 mouse monoclonal antibody (purchased from Novocastra labs, UK, at a dilution of 1:20). This antibody has been shown to react selectively with a specific cyttoplasmic glycoprotein present in mononuclear phagocytes, microglia, and epidermal Langerhans cells [21]. Paraffin sections of the lung were incubated with biotinylated antimouse antibody (diluted 1:200) and the avidin biotin-conjugated peroxidase complex (VectorLab. Inc., USA). The reaction was developed with 0.05% diaminobenzidine (Dakopatts Glostrup, Denmark) as the substrate for peroxidase; finally the slides were counterstained with Meyer’s hematoxylin [22]. The cytoplasmic site of the reaction stained brown whereas the nuclei appeared blue. The specificity of the immunoreaction was tested by replacing the primary antiserum with phosphate-buffered saline as a negative control [23].

Semithin sections:

Lung specimens (1mm³ ) were cut and fixed in 3% glutaraldehyde for 24 h with 0.1 mol/l PBS at 4°C. The sections were dehydrated in a series of ethanol rinses, cleared with propylene oxide, and embedded in epon. Semithin sections were cut and stained with Toluidine blue [24].

Real-time RT-PCR study:

Expressions of mRNAs for the pro-inflammatory cytokines, TNF-α, and IL-6 were quantified by real-time RT-PCR. Total RNA was isolated from lung tissues (approximately 30 mg) using the RNA Easy kit (Qiagen, Germany), according to the manufacturer protocol. The extracted RNA was dissolved in 30 μL
nuclease-free distilled water. The concentration and purity of RNA were determined by Nanodrop Spectrophotometer (Thermo Scientific, USA). Real-time PCR was performed using 2 μL template in a 20-μL reaction containing 0.25 μM of each primer and 12.5 μL Sybr Green Real-time PCR Master Mix (Applied Biosystems, USA). Each run consisted of 50°C for 2 min and 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 60 s in a real-time qPCR machine (Stratagene, Agilent Bionsciences, USA). GAPDH was used as a housekeeping gene for normalizing the expression data [25].

Quantitative assessment and statistical analysis:

Data were obtained using the ‘Leica Qwin-C500’ image analyser computer system (Leica, London UK) for measuring the alveolar wall thickness & number of alveolar macrophages. The magnification used for all parameters was x 400. Digital images were captured from 10 randomly chosen non-overlapping fields from each of two separate slides per animal. Alveolar wall thickness was measured using perpendicular lines (5per field) drawn across the narrowest section of the alveolar walls to minimize the number of tangential sections included in the analysis.

The primer sequences are given in Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α-FW</td>
<td>ACT GAA CTT CCG GGT GAT TG</td>
</tr>
<tr>
<td>TNF-α-RW</td>
<td>GCT TGG TGG TTT GCT ACG AC</td>
</tr>
<tr>
<td>IL-6-FW</td>
<td>TGA TGG ATG CTT CCA AAC TG</td>
</tr>
<tr>
<td>IL-6-RW</td>
<td>GAG CAT TGG AAG TTG GGG TA</td>
</tr>
<tr>
<td>GAPDH-FW</td>
<td>GTA TTG GGC GCC TGG TCA CC</td>
</tr>
<tr>
<td>GAPDH-RW</td>
<td>CGC TCC TGG AAG ATG GTG ATG G</td>
</tr>
</tbody>
</table>

Data were recorded, tabulated and statistically analyzed using the statistical package SPSS, version14. One-way ANOVA and T-test were used to estimate the differences in quantitative variables. All data were expressed as mean ± S.D. p value ≤ 0.05 is significant [26].

3-Results:

I-General behavior and body weight, lung weight and relative lung weight (Table2)

At the start of the experiment all animals were in a good condition and showed normal behavior, activity, eating and growth. Then nutmeg treated rats showed loss of appetite and moderate irritability. Two rats of the treated group showed marked loss of appetite, severe diarrhea and salivation followed by in-coordinated movements, convulsion and death. They discarded from the result. The treated group showed significant decrease in body weight (after12 weeks) as the mean body weight was 158±21 while that of the control group was 193±17. There was significant increase in lung weight of treated group when compared with the control as mean lung weight of treated group and control was 1.98±0.15 and 1.42±0.36 respectively. There was significant change in the relative lung weight as it was 0.73±0.08 in control group and became 1.2±0.11 in treated group.

II- Histological, morphometrical and immunohistochemical assessments:

Light microscopic examination of haematoxylin and eosin-stained lung sections from control rats revealed spongy structure of the lung with thin interalveolar septa and clear alveolar sacs & alveoli, normal bronchi & bronchioles(Fig. 1A). Semithin sections showed the epithelial lining of the alveoli which composed of squamous alveolar cells with flattened nuclei (type I pneumocytes) and large cuboidal cells with large central nuclei (type II pneumocytes). The pulmonary alveolar macrophages were found bulging from the interalveolar walls as large oval cells with polymorphic nuclei (Fig. 2A). By Masson trichrome stain, the adventitia of the bronchioles as well as the pulmonary interstitium showed minimal amount of collagen fibers as tinge of blue color (Fig. 3A). Immunohistochemical stain with anti-CD68 showed normal distribution of a few alveolar macrophages in the lung tissue of the control group (Fig. 4A).

Table 2: Body weight, lung weight and relative lung weight of control and experimental rats after 12 weeks of Nutmegadministration.

<table>
<thead>
<tr>
<th>Weight in grams</th>
<th>Group (Number of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (N=20)</td>
</tr>
<tr>
<td>Body weight</td>
<td>193±17</td>
</tr>
<tr>
<td>Lung weight</td>
<td>1.42±0.36</td>
</tr>
<tr>
<td>Relative lung weight</td>
<td>0.73±0.08</td>
</tr>
</tbody>
</table>

T student test Each value represents the mean ± SE. * = Significant at p value ≤ 0.05

Light microscopic examination of haematoxylin and eosin-stained lung sections of nutmeg treated rats revealed thickening of the interalveolar septa with apparent narrowing of some alveolar spaces and compensatory dilatation of contiguous ones with interstitial haemorrhage (Fig. 1B1). Large emphysematous alveoli that showed destruction of a part of their lining epithelium with appearance of cell debris inside their lumina (Fig. 1B2). There was marked mononuclear cellular infiltration, sometimes forming nodules in the interalveolar septa & peribronchiolar causing obliteration of the bronchioles (Fig. 1B3). The blood

148
vessels were congested and dilated with interstitial hemorrhage (Fig. 1B2&B3).

Semithin sections showed extravasation of RBCs in the alveolar spaces. Type II pneumocytes were prominent in the alveolar wall and some of them appeared vacuolated. Multiple foamy macrophages were seen in the alveolar spaces; most of them were large with pale vacuolated cytoplasm and eccentric nuclei (Fig. 2B). Masson trichrome stain showed an increase in the collagen fibers content of the interalveolar septa as well as at the peribronchiolar and perivascular as compared to control group (Fig. 3B).

Immunohistochemical stain with anti-CD68 showed that the nutmeg-treated lung showed markedly increased brown positively stained cells within the interalveolar septa (Fig. 4B).

Fig. (1): A photomicrograph of Hx & E stained sections in rat lung of:
1A: control group with normal spongy appearance showing a bronchiole (b), alveolar sacs (s) and alveoli (a) separated by thin interalveolar septa (arrow) and lined by type I (arrow head) and type II pneumocytes (arrow). Note alveolar macrophages (m) and pulmonary vessel (p).
1B1-B2-B3: nutmeg treated group showing in B1: thickening of the interalveolar septa with apparent narrowing of some alveolar spaces and compensatory dilatation of contiguous ones, emphysematous alveoli (E) & interstitial haemorrhage (astriks). In B2: many emphysematous alveoli (E) and some showing interalveolar cell debris (de). congested thickened blood vessels (BV) could be seen. In B3: there are obliterated bronchi surrounded with extensive mononuclear cellular infiltration and marked thickening of the interalveolar septa with mononuclear cellular infiltration (Astriks) causing narrowing of some alveolar spaces and compensatory dilatation of contiguous ones (D). Thickened blood vessels (BV) are observed. (Hx& E x 400)
Fig. (2): A photomicrograph of Toluidine blue stained semithin sections in rat lung of:

2A: control group showing alveoli (a) lined by type I pneumocyte(I) and type II pneumocyte(II). Alveolar macrophages (arrow) are seen bulging from the interalveolar walls as large oval cells with polymorphic nuclei.

2B: nutmeg treated group showing multiple large foamy macrophages (m) with pale vacuolated cytoplasm and eccentric nuclei (n) in the alveolar spaces. Prominent type II pneumocytes(II) lining the alveolar spaces, which appear rounded with rounded nuclei and vacuolated cytoplasm (arrow). The extravasation of R.B.C.'s in the alveolar spaces(arrow head). (Toluidine blue x 1000)

Fig. (3): A photomicrograph of Masson trichrome stained sections in rat lung of:

3A: control group showing minimal amount of collagen fibers in the adventitia of the bronchiole (B) as well as in the pulmonary interstitium (arrow head).

3B: nutmeg treated group showing increase of collagen fibers in the pulmonary interstitium (arrows head) as well as peribronchiolararea (B) and perivascular area (BV). (Masson trichrome x 200)

Fig. (4): A photomicrograph of anti-CD68 stained sections in rat lung of:

4A: control group showing a few brown positively staining cells of alveolar macrophages (arrows head).

4B: nutmeg treated group showing many brown positively staining cells (arrows head) for alveolar macrophages in the interalveolar septa. (Anti-CD68 x400).
III- Morphometric Statistical Analysis: (Table3)

There was significant increase in the number of alveolar macrophages in the nutmeg-treated lung and there was also significant increase in the septal alveolar wall thickness.

Table 3: Septal alveolar wall thickness (pm) and mean number of alveolar macrophages/high-powerfield in the different studied groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Septal alveolar wall thickness</th>
<th>Mean number of alveolar macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.8 Um ±2.244</td>
<td>2.36 ±0.92</td>
</tr>
<tr>
<td>Nutmeg treated</td>
<td>25.07 ±5.157*</td>
<td>33.98 ±5.87*</td>
</tr>
</tbody>
</table>

T student test Each value represents the mean ± SE. * = Significant at p value ≤ 0.05

IV- TNF –α and IL-6 expression assessment:

The study revealed that TNF- α mRNA expression in lung was highly increased in treated group (3870±38) when compared to control group (207±13). This difference was statically significant. While IL-6 mRNA expression was increased to a lesser extent as it was 235±18 in control group and became 2009±27 in the treated group.

Table 4: TNF –α and IL-6 expression assessment in control and nutmeg treated group

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Group (Number of animals)</th>
<th>Control (N=20)</th>
<th>Nutmeg treated group (N=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/ ml)</td>
<td></td>
<td>207±13</td>
<td>3870±38*</td>
</tr>
<tr>
<td>IL-6 (pg/ ml)</td>
<td></td>
<td>235±18</td>
<td>2009±27*</td>
</tr>
</tbody>
</table>

T student test Each value represents the mean ± SE. * = Significant at p value ≤ 0.05

4-Discussion:

Nutmeg is widely used in a variety of ways and for various purposes. It has been known for its anticonvulsant, aphrodisiac and psychoactive properties which includes antidepressant, anxiogenic and hallucinogenic [1,4]. It is accepted as flavoring agent in food [5,7].

Despite the wide use of nutmeg in many countries as an herbal remedy and its usefulness pharmacological effects, few studies have been published about its toxicological profiles specially on lungs. Therefore, the aim of the present study was to assess effects of oral nutmeg on the rat lungs and the role of TNFα and IL-6 in these effects.

Change in the body weight and organs weight is considered a good indicator for intoxication effect [27]. The present study revealed dramatic decrease of the body weight which can be attributed to decreased appetite and absorption of nutrients from the gut [28]. In contrast, it was reported by Aisha [29] that there was increase in the rat weight that given aqueous extract of Myristica fragrans for 6 weeks. Other authors had reported that no significant difference in body weight of male mice treated with the aqueous extract of nutmeg seeds at the end of the 6weeks of injection with low dose range employed 20-80 mg/kg [30]. The study revealed marked increase in lung weight as compared to control. It can be attributed to increase thickening of interalveolar septa, overexpansion of alveoli congestion of blood vessels, and cellular infiltration. Focal areas of collapsed alveoli and thickened interalveolar septa side by side with emphysematous areas were found. The increased thickness of the interalveolar walls observed in experimental rats could be explained by the presence of excess inflammatory cells, congested capillaries, increased interstitial connective tissue and the associated alveolar collapse[31]. In addition to depletion of the pulmonary surfactant lead to partial collapse of the lung[32]. The intrabronchial cellular debris observed in most bronchioles was attributed to the direct toxic effect on the mucosa-lining bronchioles [33].

The reported vacuolated pneumocytes type II and foamy macrophages in this study might be due to inhibition of lysosomal phospholipases resulting in an abnormal degradation of phospholipids promoting its intracytoplasmic accumulation and permitting phagocytic cells to accumulate large quantities of lipids leading to the appearance of vacuolated pneumocytes type II and foamy macrophages [34].

The significant increase in macrophages was in concomitant with significant increase in TNFα level. This was enforced by the increasing evidence suggested correlation between macrophages and inflammatory mediators released, including cytokines as pathogenic process in lung injury. Alveolar macrophages become activated and release increased quantities of cytokines such as TNF-α, IL-1, IL-6, and IL-10, as well as chemokines and growth factors such as TGF-β and PDGF [14].

It was reported that in response to injury, inflammatory cells enter the lung and together with resident lung cells release mediators that stimulate fibroblast proliferation and collagen deposition within the lung interstitium. The cytotoxic proteins secreted by eosinophils may promote tissue injury and exacerbate alveolitis which drives the fibrotic response[35]. The role of macrophage-derived factor in the control of fibroblast proliferation and collagen synthesis [33].

The interstitial as well as intra-alveolar hemorrhage noticed in these rats could be explained by the increased vascular permeability. This vascular permeability was a result of release of polypeptide
mediators from the injured cells[36]. In addition to that, nutmeg resulted in impairment of endothelial function due to increased oxidative stress and enhanced formation of oxygen-derived free radicals as had been previously reported by Motoyama et al. [37].

The pulmonary vascular congestion noticed in nutmeg treated rats could be due to toxic effects of it on blood vessels through the release of vasodilator substances into blood stream. The stagnant blood in the dilated capillaries would cause hypoxia to lung tissue resulting in more pulmonary congestion[38].

**Conclusion: From the foregoing**, we concluded that administration of nutmeg in rats can induce lung damage in the form of disrupting the normal architecture with thickening of interalveolar septa, overexpansion of alveoli alternating with collapse of others, congestion of blood vessels, cellular infiltration, proliferation and vacuolation of pneumocytes type II. More collagen deposition associated with significant increase in macrophages number were present. Intra-alveolar cellular debris and vascular congestion were also observed. Also, the TNF-alpha and IL-6 levels showed elevation that was more in TNF-alpha.

**Recommendation:** It is important to do further studies on the effects of nutmeg on the lungs in different periods and doses, as there is lack of studies and researches in this field especially with the possible role of both TNF-alpha and IL-6 cytokines. The medicinal use of nutmeg and its use as a spice suggest that it contains some constituents which are responsible for the reported biological activities. Some of these active principles may at the same time possess adverse effects when dosage is abused. So it is also recommended that caution should therefore be advocated in the intake of this product & pulmonary functions should be periodically evaluated.

**References**


