Defensive Effect of Garlic as revealed by Molecular, Biochemical and Ultra Structure Print after Toluene Stress on Mice

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Abstract: The present study represents a trial for using the natural garlic extract (Tomox) as a treatment for the asthma caused by the organic chemical (Toluene). Twenty four mice were divided into four groups; group I kept as control, untreated animals, group II included animals treated daily with 2.5 mg/kg b.w. garlic for one week via oral gavage, group III included animals treated daily with toluene as a spray all over the body for 10 min 3 times/day for one week and group IV included animals treated with toluene followed by garlic. IgE levels were measured as an indicator for the immune response. Toluene increased the level of IgE (4.2 µg/ml), while treatment with garlic decreased its level to 3.7 µg/ml compared with 3.2 µg/ml in control animals. Transmission electron microscopic examinations were performed to reveal the effect of toluene on lung tissues. A marked changes has been observed after the treatment with toluene.These changes were represented by vacuolations, ill-defined mitochondria, fragmented rough endoplasmic reticulum and pyknotic nuclei of type I and II. Macrophage with pyknotic nuclei and condensed heterochromatin on the inner surface of the nuclear envelope and rupture nuclear envelope in some spaces. Molecular genetic analysis has been performed for the F1 to assess the genetic changes occurred in the offspring due to the treatments. There was no an observable variation on the RAPD-PCR level using 5 random primers O6 (5'- CCC AGT CAC T-3'), O10 (5'- TCA GCG CCA C-3'), C5 (5'- CCG CAT CTA C-3'), C10 (5'- TGT CTG GGT G -3') and C 14 (5'- AAG CCT CGT C-3'). The results showed that, toluene induced damage in lung tissue and immunosuppressive effects in adult animals. In spite of that, toluene did not induce genetic variation in DNA of babies of treated females as revealed by RAPD-PCR.
[Sahar A. Mosallam and Hussein Sabit. Defensive Effect of Garlic as revealed by Molecular, Ultra Structure and Immunological Changes after Toluene Stress on Mice.Journal of American Science 2011:7(7):733-740]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Asthma, Garlic, Genetic fingerprint, IgE, Toluene.

1. Introduction:

Asthma is one of the most common chronic diseases worldwide. To treat this widespread disease there is a high prevalence of usage of herbal medicine. The use of plants is as old as humankind and it has been steadily increasing over the past 10 years. Plant-based remedies are now one of the most popular complementary treatments (**Szelenyi and Brune, 2002**). Chronic inflammatory pulmonary diseases such as Chronic Obstructive Pulmonary Disease (COPD) and asthma are highly prevalent and associated with a major health burden worldwide. Despite a wealth of biological and clinical information on normal and pathologic airway structure and function, the primary causes and mechanisms of disease remain to a large extent unknown as well as preventing the development of more efficient diagnosis and treatment **(Auffray *et al*., 2010)**.

The incidence of asthma has increased by more than 50% in the last 2 decades. The reasons for this increase are not entirely clear, but workplace exposures may cause asthma in some patients **(Linda *et al*., 2001)**.

Toluene (T), also known as methylbenzene, is a volatile organic compound with highly reactive industrial applications. It is an aromatic hydrocarbon that is widely used as industrial feedstock and as a solvent **(Streicher *et al.*, 1981)**. Toluene can exert neurotoxic and immunotoxic effects, although these effects have been studied extensively, the underlying mechanism remains obscure **(Win-Shwe *et al.*, 2011)**. Toluene is capable of inducing lymphocyte-dependent but IgE-independent tracheal hyper reactivity in the mouse, which is not associated with cellular infiltration in the airways **(Scheerens *et al*., 1996)**.

The potency of Garlic (G) (*Allium sativum*) has been acknowledged for 5000 years. In ancient times, the Babylonians, Egyptians, Phoenicians, Vikings, Chinese, Greeks, Romans and Hindus used garlic frequently as a remedy for many diseases **(Block, 1985)**. Garlic exhibits hypolipidemic, antiplatelet, and procirculatory effects. It prevents cold and flu symptoms through immune enhancement and exhibits anticancer and chemopreventive activities **(Amagase, 2006)**. The use of garlic to treat wounds surfaced repeatedly through diallyl disulfide

(DADS), a component of Garlic, inhibits the proliferation of human blood, colon, lung and skin cancer cells. Although DADS had been reported to induce apoptosis in human leukemia HL-60 cells, there are no reports regarding whether or not it affects leukemia cells in vivo **(Yang *et al*., 2006)**.

Hasbal *et al*. (2010) studied DNA damage as level of DNA strand breaks and formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites, which reflects oxidative DNA damage and glutathione (GSH) level in children with mild-to-moderate persistent asthma and to examine the effect of antiasthmatic therapy on these DNA damage parameters and GSH level.

The main aim of the present study is to evaluate the effect of garlic on toluene-induced asthma in mice and to determine the genetic background changes (RAPD- PCR) that might be happened in the F1 offspring of treated pregnant female mice.

2. Materials and Methods

Animals

In this study, twenty four adult female mice (*Mus musculus)* aged about 12 weeks (25 – 30 g) were purchased from the Animal House of the National Research Center, Cairo, Egypt. Mice were acclimatized to the laboratory conditions and given food and water *ad lib.*

Chemicals

Toluene which known as methylbenzene, $C_6H_5CH_3$ with molecular weight 92.14 and concentration 99.5 % was purchased from Al-Gomhoria Co., Cairo, Egypt. Garlic is available in local markets as tablets (TOMOX). Each tablet contains 200 mg of specially prepared garlic powder. Tablets purchased from Atos Pharma, Cairo, Egypt.

Experimental design

Animals were grouped into four experimental groups each consists of six animals; group I, served as a control; group II, treated daily with 2.5 mg/kg b.w. garlic for one week via oral gavage; group III, treated with toluene as a spray all over the body fur (3 times, 10 min. per day) for one week to induce asthma and group IV, were treated with toluene followed by garlic to evaluate the antagonistic effect of the two substances. The samples were taken to be tested for the occurrence of asthma after 3 days from the end of exposure.

Immunoglobulin assay

Blood samples were collected from each animal, the serum was separated and the indirect ELISA method was used. The level of Immunoglobulin E (IgE) was measured in all the samples.

Sample preparation for histopathology examinations

One animal from each group was dissected to obtain lung tissues which were then prepared for electron microscopy examination. The lungs were cut into small pieces and fixed in 2.5% glutaraldehyde and paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 4 h. Then samples were washed in cacodylate buffer and post-fixed in a buffered solution of 1% osmium tetroxide at 4 ºC for 90 min. This was followed by dehydration in ascending grades of alcohol, immersed in propylene oxide for two changes, 5 min. each, and embedded in Epon. Semithin sections were stained with 0.5% toluidine blue and examined under a bright field light microscope. Ultrathin sections were cut, mounted on formvar-section grids, stained with uranyl acetate and lead citrate **(Weakley, 1981)**. The prepared sections were examined and microphotographed using a transmission electron microscope (JEOL JEM-1200 EX II, Japan) operated at 60-70 KV, Faculty of Science, Ain Shams University, Cairo, Egypt.

Molecular genetic fingerprinting

DNA was isolated from lung tissue samples according to **(Ausubel *et al.*, 1989).**

Random Amplified Polymorphic DNA (RAPD-PCR) Analysis

The purified DNA samples were subjected to amplification using random primers via PCR. Five random primers were used in this study (C10, C5, C 14, O6, and O10, Operon Technology Inc., UK) (Table 1) to generate a distinguishable pattern. The PCR profile was as follows: 95ºC for 5 min. as pre-PCR step and the cycles were 94 ºC for 1 min., 40ºC for 1 min., and 72ºC for 1 min. and a final extension step at 72ºC for 10 min. were performed. The numbers of cycles were 35. The PCR product was then separated on 1.2 % agarose gel and illuminated on UV-transilluminator after being stained with ethidium bromide. The gels were photographed and subjected to analysis via gel documentation system (Gel Pro-Analyzer, version. 3.1).

3. Results

As shown in tabulation (1) toluene-exposure group revealed reduction in food intake (1.5 – 2 g media / day) accompanied with decrease in weight gain (15 - 20 g) compared with control and garlic group (4 – 5 g media / day). In addition, the mean offspring numbers of toluene-exposure group were 4 animals compared to control (12 animals) and garlic

groups (10 animals). Also toluene-exposure animals showed general weakness and fatigue compared with control animals.

Immunoglobulin assay

Toluene significantly increased the level of IgE (4.2 µg/ml) in comparison to control (3.2 µg/ml). Meanwhile, garlic treatment revealed decrease in the level of IgE (3.4 µg/ml). Moreover the level of IgE in the group IV which treated with toluene followed by garlic was 3.7 µg/ml. (Fig.1).

Scanning electron microscopic examination

Histological examination of the respiratory epithelium from lung in mice exposed to toluene 3 times / 7 days was performed under a light microscope. The alveolar epithelium didn't undergo any changes in toluene-exposed mice compared with control mice. Electron microscopic examination of the alveoli of the control lung revealed two types of cells: the pneumatocytes I (the major type) and pneumatocyte II (the minor one). The pneumatocyte I are thin squamous alveolar cells with attenuated cytoplasm containing pinocytic vesicles and ovoid nuclei. The cytoplasm contains rough endoplasmic reticulum, Golgi complex and mitochondria (Fig. 2). The second type pneumatocyte II (septal cells), are interpenetrated among the type I cells. These cells rest on the basement membrane and their cytoplasm have a well developed Golgi complex, mitochondria, rough endoplasmic reticulum, lamellar bodies and microvilli on their free apical surfaces (Figs. 3 & 4). The blood air barrier consisted of cytoplasmic extensions of pneumatocyte I resting on basal lamina of the endothelial cells lining blood capillaries (Fig. 2). Macrophages (dust cells) are observed in the intra-alveolar spaces and in the interstitial septum (Fig. 5).

Treatment of mice with toluene had produced marked changes in the ultrastructure of the pneumatocytes I and II. Many mitochondrial cristae were broken down and appeared more electron dense (Fig. 6). The cisternae of the rough endoplasmic reticulum were fragmented into small stacks. The nucleus appeared shrunken and pyknotic with irregular contour (Fig. 7). The micrograph of pneumatocytes II revealed marked cytopathological alterations. There were abundant RER with cisternae were fragmented into smaller stacks. The nucleus was also pyknotic with irregular nuclear envelope (Figs 7 & 8). The heterochromatin was condensed on the inner surface of the nuclear envelope (Fig. 8). The examination of lung sections of mice treated with toluene exhibited advanced degenerative changes. These changes were represented by vacuolations, ill-defined mitochondria, fragmented rough endoplasmic reticulum and pyknotic nuclei of type I and II. Macrophage with pyknotic nuclei and condensed heterochromatin on the inner surface of the nuclear envelope and rupture nuclear envelope in some spaces (Fig. 9).

Identification of DNA changes

Fig (10) revealed no variation between the samples under study using different primers, so the time of exposure or the toluene itself didn't cause any DNA change.

Table (1): The sequence, operon codes and GC content of random primers used in the experiment.

N0	Primer	Sequence (5'- 3')
1	O6	CCC AGT CAC T
2	O10	TCA GCG CCA C
3	C5	CCG CAT CTA C
4	C10	TGT CTG GGT G
5	C14	AAG CCT CGT C

Tabulation (1): Gram feeding / day, weight and mean offspring number for the mice under study after toluene treatment.

	C group	G group	T group	T & G group
Feeding / day	4 – 5 g of media	4 – 5 g of media	1.5 – 2 g of media	2 – 3 g of media
Weight	30 – 35 g	30 – 35 g	15 – 20 g	20 – 25 g
Mean offspring no.	12	10	4	7

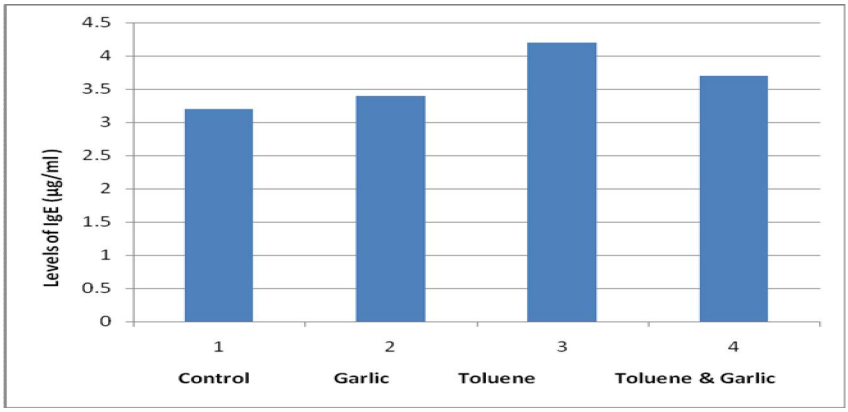
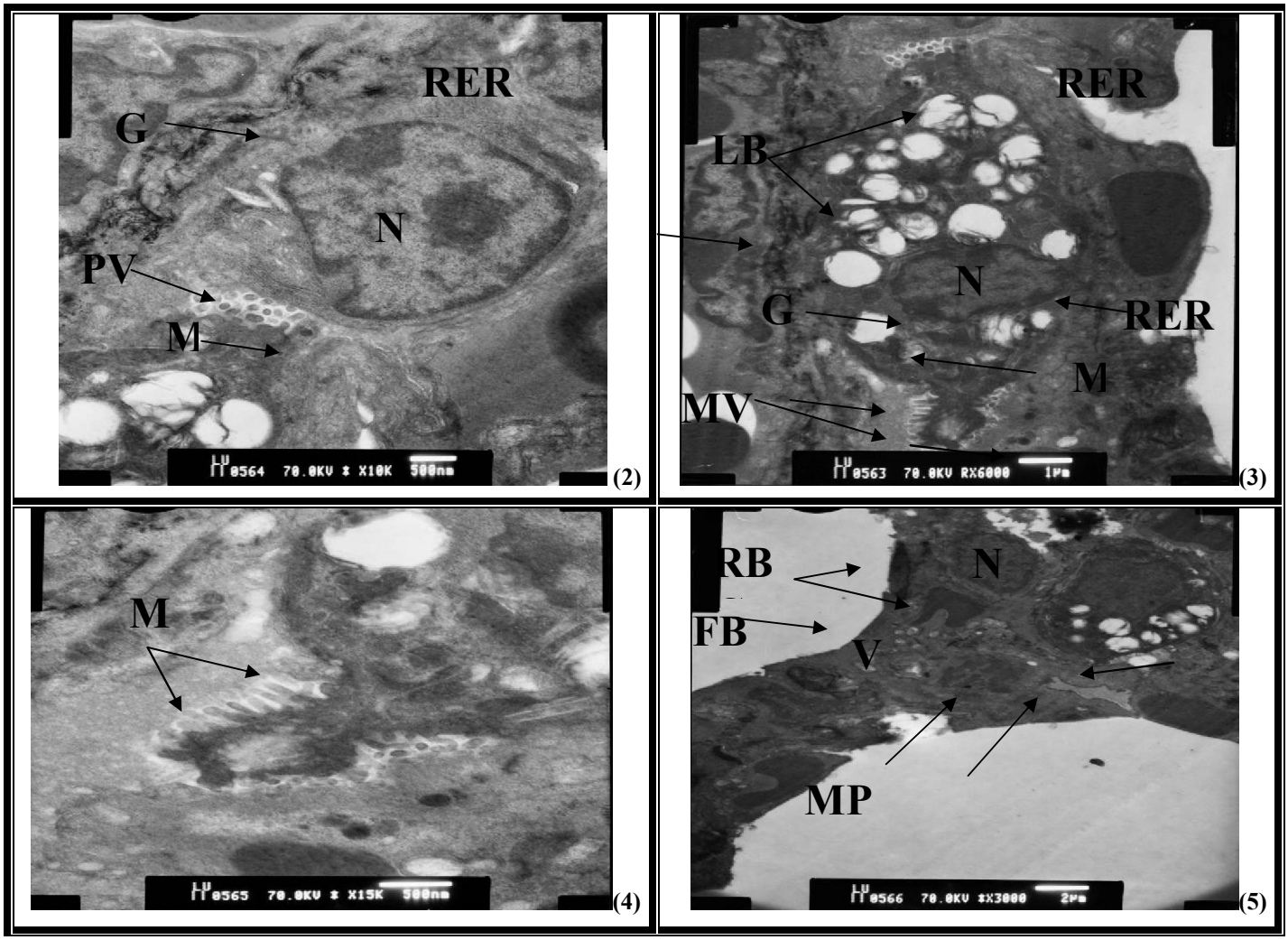
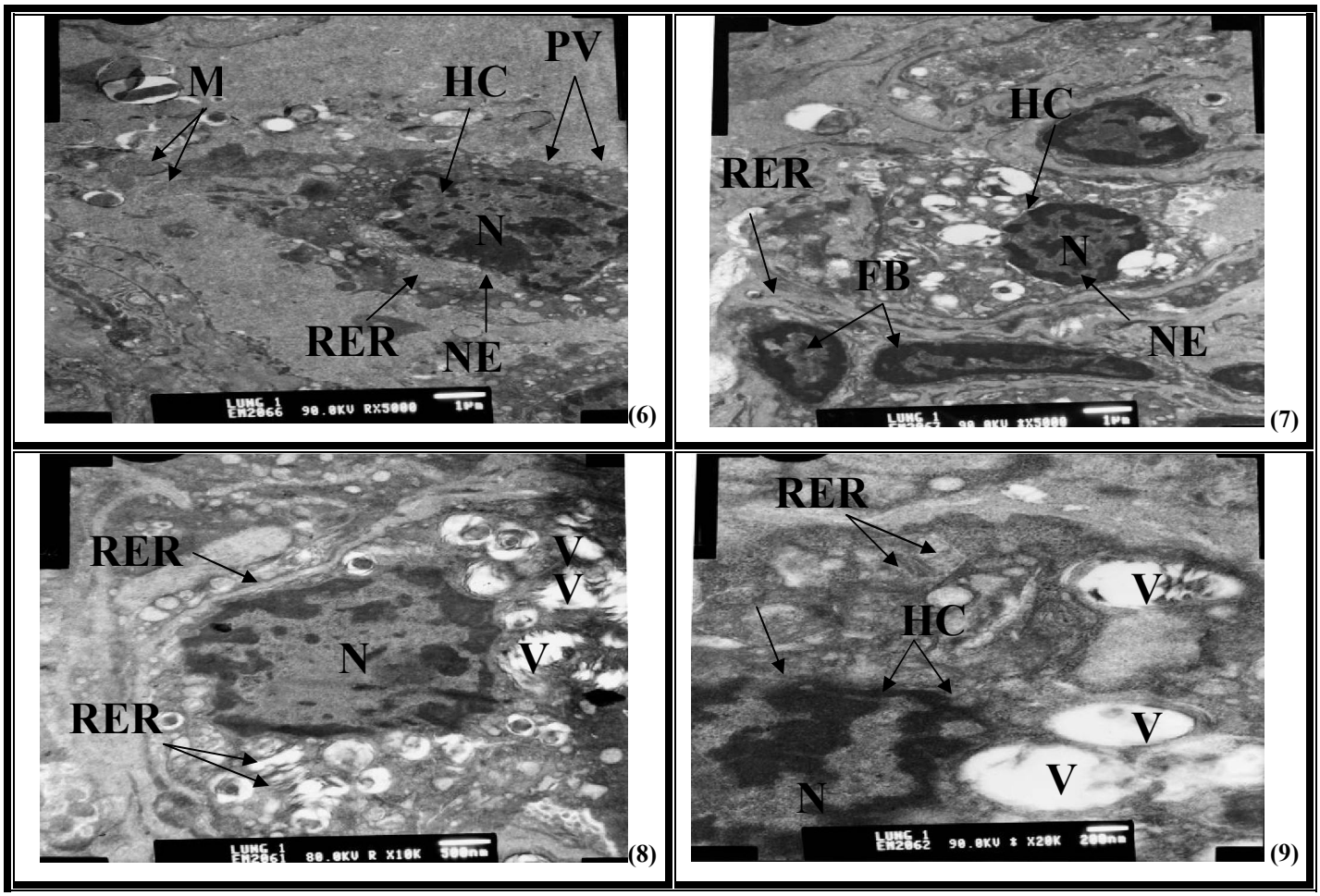


Figure (1): IgE levels in the control and the treated mice blood sample.





Figures (2, 3, 4 and 5): Are electron micrographs of lung of control mice. Figure (2): Showing squamous alveolar cell (pneumatocyte I) with attenuated cytoplasm. Notice, rough endoplasmic reticulum (RER), well developed Golgi (G) and pinocytotic vesicles (PV). (X.10.000). Figure (3): Illustrating cuboidal pneumatocyte II which rest on the basement membrane (arrow) and has microvilli (MV). Notice, a large number of lamellar bodies (LB), mitochondria (M), rough endoplasmic reticulum (RER), a well developed Golgi complex (G) and nucleus (N). (X.6000). Figure (4): Is an enlarged portion of pneumatocyte II illustrating apical microvilli (MV). (X.15000). Figure (5): Showing macrophage (MP) with pseudopodia (arrow), many vacuoles (V) and nucleus (N). (X.3000).

Figures (6, 7, 8 and 9): Showing electron micrographs of lung of mice treated with toluene for 7 days. Figure (6): Illustrating pneumatocyte I showing mitochondria (M), fragmented rough endoplasmic reticulum (RER), pinocytotic vesicles (PV), irregular nuclear envelope (NE) and nucleus (N) (X.5000). Figure (7): Showing pneumatocyte II with fragmented rough endoplasmic reticulum (RER), condensed heterochromatin (HC), irregular nuclear envelope (NE) and pyknotic nucleus (N). Interstitial fibroblast (FB) with irregular nucleus (N) (X. 5000). Figure (8): Is high magnification of pneumatocyte II illustrating highly vacuolated cytoplasm (V) and fragmented rough endoplasmic reticulum (RER). Notice the nucleus (N) with irregular nuclear envelope (X. 10000). Figure (9): Showing macrophage with vaculated cytoplasm (V), condensed heterochromatin (HC), fragmented rough endoplasmic reticulum (RER) and rupture in nuclear envelope (arrow) (X. 20000).

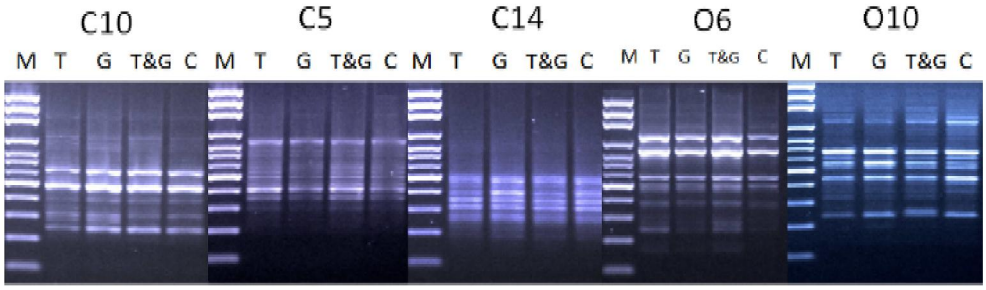


Figure (10): Random Polymorphic DNA –PCR analysis of the treated and non-treated samples. M: marker, T: Toluene, G: Garlic and C: Control.

4. Discussion

Asthma is an inflammatory disease and the attention has focused on the mechanisms of this inflammation. Over the past decade, animal models, including guinea pigs, monkeys, and mice have been employed to explain the inflammation associated with asthma **(Gleich and Kita, 1997)**. The incidence of asthma has increased by more than 50 % in the last 2 decades. The reasons for this increase are not entirely clear **(Linda *et al.,* 2001)**. In the present study, the effect of toluene as an inducer of asthma in mice was assessed and the effect of garlic as an IgE reducer agent was also assessed. Our data revealed the decrease effect of toluene on body weight, offspring No and gram feeding per day which in agreement with **Bowen and McDonald (2009).** Results also referred to the negative effect of toluene on central nervous system (CNS), it was seen as a loss of animal appetite and affect too on the sensory organs so it couldn't reach or define the food and this is in turn reflected on animals weight (**Bowen and McDonald, 2009**). That is in accordance with **(Ghaly *et al.,* 2007)** who stated that toluene primarily causes central nervous system disorders. In the short term, it can cause fatigue, nausea, weakness and confusion. Long term exposure to toluene can result in spasms, tremors, memory and/or coordination impairment, as well as liver and kidney damage.

Toluene reacts as a normal aromatic hydrocarbon towards electrophilic aromatic substitution **(March, 2001 and Wade, 3003)**. The methyl group makes it around 25 times more reactive than benzene in such reactions. The toxicity of toluene can be explained mostly by its very low water solubility, it cannot exit the body via the normal routes (urine, feces, or sweat). It must be metabolized in order to be excreted. The methyl group of toluene is more easily oxidized by cytochrome P450 than the benzene ring. Therefore, in the metabolism of toluene, 95% is oxidized to become benzyl alcohol **(Nakajima *et al.*, 1997)**. The toxic metabolites are created by the remaining 5% that are oxidized to benzaldehyde and cresols **(Chapman *et al.,* 1990 and Hanioka *et al.,* 1995)**. Most of the reactive products are detoxified by conjugation to glutathione but the remainder may severely damage cells **(van Doorn *et al.,* 1981)**.

The data obtained revealed that toluene when sprayed on the mice increased IgE (4.2 µg/ml) which may be indication of asthma induction. While treatment with garlic (*Allium sativum*) after toluene exposure decreased the level of IgE (3.7 µg/ml) compared to control (3.2 µg/ml). Recent studies in that concern indicated that peritoneal injections of the garlic extract in mice caused a significant decrease in the hallmark criteria of allergic airway inflammation levels **(Zare *et al.*, 2008)**. Furthermore, **Shields *et al.*, (1995)** indicated that numerous clinical studies showed that direct interference with the IgE response leads to a decrease or elimination of allergic symptoms and this was in agreement with our data. While adding garlic to animal feed showed remodulation of the immune response and decreasing the level of IgE. In the present investigation we measured the IgE level especially because of its central role in the pathogenesis of the eosinophilic inflammation as well as in the obstructive air way physiology of the bronchial hyperreactivity **(Mehlhop *et al.*, 1997)**. IgE antibodies are thought to play an important role in the induction of the allergic inflammation of the bronchi **(Saban *et al.*, 1994)**. The treatment with toluene induces asthma in mice and subsequently the level of IgE increased (4.2 µg/ml) in comparison to the control (3.2 µg/ml). The antagonistic effect was revealed when the mice were treated with garlic after toluene exposure (the IgE level was 3.7 µg/ml). This indicated that garlic could attenuate the allergic inflammatory response associated with asthma due to treatment with chemicals **(Owen, 2006)**.

The present investigation illustrated marked deleterious consequences of changes on the alveolar cells following treatment with toluene. These changes were in agreement with **(Mollenhauer *et al.,* 1990)** who found exposure of animal cells to toluene caused

cellular changes which included condensation of heterochromatin in nuclei and ultimately degeneration of organelles.These results coincides with the report of **(Lange *et al*., 1999)** who found that toluene diisocyanate vapor *in vitro* on differentiated human bronchial epithelial cells caused cytotoxicity as pyknosis, numerous mitochondria and DNA fragmentation, while *in vivo* implications of these finding include decreased ciliary movement and longer retention of toluene and hence increased exposure. **Kanter (2009) and Hussain *et al.* (2011)** found that toluene caused severe inflammatory cell infiltration and many alveoli were obstructed.

The destruction of pneumatocyte I and II referred to the action of toxic substances which is followed by a strong increase in the mitotic activity of the remaining type II cells, most of type II cells are transformed into type I cells, and the alveolar lining regains its normal appearance **(Junqueira *et al*., 1995)**.

The short time of the exposure revealed no change in DNA pattern of offspring obtained through (RAPD-PCR). This result don't judge that toluene is mutagenic or no.

It can be conclude that garlic causes improvement for the immune system and that may reflect its potentiality of attenuation of inflammatory features of allergic airway inflammations. We recommend that the garlic can be used as a potential treatment for the pulmonary diseases.

Acknowledgement

The authors represent their deep gratitude to Dr. Samia Sakr, assistant professor, Biology and Geology Department, Faculty of education, Ain Shams University for her great support and valuable guidance in commenting on the EM graphs. Also great thanks would be given to Mrs. Shimaa Eid Abdel-Ghany for providing a tool to analyze the RAPD-PCR profiles.

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6/2/2011