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Formaldehyde vapor-induced chronic tracheitis in relation to the expression of S-100 proteins

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Abstract

Background and objective: Formaldehyde is the most widely used chemical in daily life; thus, chronic exposure to formaldehyde has been shown to have adverse effects on different organs in humans and animals. Different of industries use formaldehyde including the medical, detergent, cosmetic, food, rubber, metal, wood, leather, petroleum, and agricultural industries and as a hydrogen sulfide scavenger in oil operations. This research was designed to investigate variable tracheal changes arising from chronic formaldehyde exposure by measuring different histomorphometric parameters in accordance with S-100 protein expression.

Methods: In this experiment, twenty Wister rats were used, divided into the control group (n=10) and the experimental group (n=10). Animals in the experimental group had approximately 21 days of formaldehyde vapor 40ppm. At the end of the study tracheal samples were collected and used for histopathologic assessments and IHC staining.

Results: In comparison to the control group, the experimental group revealed various histological lesions in tracheal structures from lining epithelium till adventitia including highest degrees and scores of; Epithelial necrosis, goblet cell hyperplasia, fibrosis in the submucosa, squamous metaplasia, chronic tracheitis and glandular changes. Results of IHC showed that the S-100 expression in the experimental group displayed higher percentages in different cells (epithelial cells, chondrocytes, and inflammatory cells) which were reported as strong intensity (3+), then moderate intensity (2+), and accompanied by weak intensity (1+), which revealed minimum percentages compared to the control-negative group in which the weak intensity (1+) recorded the highest percentages.

Conclusion: Chronic exposure to formaldehyde causes significant histological changes that may lead to cancer induction. S-100 may appear as pro-inflammatory and proliferative marker, also S-100 expression associated with chondrocyte hypertrophy.

Keywords: Chronic tracheitis; Formaldehyde vapor; Histomorphometric parameters; H-score and S-100 proteins.

Introduction

Formaldehyde is present in the natural system of the organism and is widely used in everyday life due to its chemical properties for many applications.[1]

Workplace exposure to formaldehyde occurs in the processing of acetal, melamine, urea, and phenol Formaldehyde resins. Such resins have many industrial applications and are used in the manufacture of wood, plastics, textiles and leather, adhesives, and binders. Moreover, formaldehyde plays a pivotal role in medicine as a tissue fixative and bactericide.[2,3] Different industries use formaldehyde including the medical, detergent, cosmetic, food, rubber, metal, wood, leather, petroleum, and agricultural industries and as a hydrogen sulfide scavenger in oil operations.[4] A relatively large number of workers were exposed to formaldehyde due to its widespread use.[5]

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It has the negative impacts on the human and animal bodies, especially on the eye and respiratory system, but also affects the nervous and reproductive systems. Recent formaldehyde studies demonstrated that it triggers the production of reactive oxygen species (ROS) which induces apoptosis and necrosis through lipid peroxidation and metabolic alterations.[6] According to the International Agency for Research on Cancer (IARC), formaldehyde is listed in Group I as known carcinogens including asbestos, vinyl chloride, formaldehyde or ionizing radiations and benzene.[7] Several studies found the link between formaldehyde exposure in humans and animals and the development of nasal cancer.[3,8]

The S-100 proteins, referring to a calcium-binding cytosolic protein family, are consisted of 25 known members. They have a wide range of intracellular and extracellular roles, including energy metabolism, calcium balance, protein phosphorylation, apoptosis, proliferation, differentiation, migration, inflammatory reaction, preserving both extracellular and intracellular conditions after infection, development of tumor and metastasis.[9,10] Depending on their functional activities, S-100 proteins are classified into three major subsets: S-100 proteins that act only intracellularly, S100 proteins are involved in aspects of regulation of proliferation, differentiation, apoptosis, Ca2+ homeostasis, energy metabolism, inflammation and migration/invasion through interactions with a variety of target proteins including enzymes, cytoskeletal subunits, receptors, transcription factors and nucleic acids[11] largely exert extracellular functions, and S-100 proteins with both intracellular and extracellular functions.[9,11] It has been reported that levels of S-100 proteins have changed with respect to various diseases, such as inflammatory and neurodegenerative disorders, cardiomyopathies, and cancer.[10] This study was aimed to demonstrate all histopathological alterations in the trachea formed after chronic formaldehyde exposure and joined to S-100 protein expression.

Methods

Animal model

Twenty adult Wister albino rats (10 males and 10 females, weighting 200-240gm) were purchased and housed in the Animal House in the College of Medicine, Hawler Medical University. A week before the experimental study, animals were acclimatized to conform to the laboratory environment; the rats were placed in four plastic cages (5 rats per cage) and housed under proper laboratory conditions with 12hrs light: 12hrs dark cycle at 22±2°C. Standard rat pellets were provided to the animals, formulated by using a computer program based on Pico Lab. Rodent diet as follows: 66.6% wheat, 25.6% soybean, 4.4% sunflower oil, 1.5% limestone 0.63% salt, 0.158% methionine, 0.062% choline chloride and 0.05% trace elements. The research procedure was accepted by the local Ethic Committee (No. 5 on 24/9/2021) for Animal Research in the College of Medicine/Hawler Medical University and all animals received humane treatment in accordance with the European Animal Care Protocol.

Experimental design

Animals were classified into two groups; group A (Control group, n=10) which were not exposed to Formaldehyde vapor and group B (experimental group, n=10) which were exposed to Formaldehyde vapor at 40ppm (animals were subjected to 40% formaldehyde for 3 hours/day, for 21 consecutive days,). In order to achieve these measurements, two special boxes were manufactured from PVC and glass with (60x60x60) cm dimension with one slide opening used for keeping the rats during exposure period (one box for males and the other for female, each prisoned separately). A piece of cotton was soaked with 20ml of 40% formaldehyde, put in a petri-dish and hanging 30 cm from the cabinet ground.[12]

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Sample collections

On the day 21 the experiment, animals were sacrificed by ketamine hydrochloride and xylazine. Tracheal samples were taken and fixed at 10% neutral buffered formalin, then processed and embedded in melted wax to obtain paraffin blocks. From each sample, two sections were cut at 4µm; one for hematoxylin and eosin (H&E) staining and the other for immunohistochemical staining.

Histomorphometry

Tracheal sections for histopathological evaluation were examined in each 5 distinct and randomly selected fields for epithelial alterations, changes in the tracheal gland, Goblet cells, evidence of fibrosis and infiltration of inflammatory cells. Microscopic changes were examined and the maximum score was 10 as presented in the table 1. Every rat sample was scored and values for each category were calculated. Additionally, Top View 3.7 image analysis system was used to measure the tracheal epithelial thickness (TET) with an x100 magnification fold. TET was estimated from the basement membrane to the highest point of the epithelial surface of the trachea. Sections were analyzed by utilizing a NOVEL research microscope.

Table 1 Histological scoring for chronic tracheitis.[13]

Lesions	Severity	Scores*
Epithelial necrosis	Normal	0
Goblet cell hyperplasia	Mild	1
Fibrosis in the submucosa	Moderate	2
	Severe	3
Epithelial metaplasia	Absent	0
	Present	1
Inflammatory cell infiltrations	Normal cellularity	0
	Mucosa	1
	Submucosa	2
	Muscularis or adventitia	3
Glandular changes	Normal	0
	Hydropic degeneration	1
	Dilation	2
	Gland abscess	3

*Sum of the scores ranged from 0-10.

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Evaluation of immunoreactivity and H-score

Immunohistochemical (IHC) staining was conducted according to the manufacturer's protocol (Polyclonal Rabbit Anti S-100 [Code 4010] Dako company, Denmark) to evaluate the expression of S-100 proteins.

For the assessment of the immunoreactivity of S-100 proteins, H-score[39] examination was utilized. During the H-score analysis, nuclear and cytoplasmic staining intensities of S-100 were assessed in four categories. The assessment found (0) no stain, (1+) weak but noticeable stain, (2+) moderate stain, and (3+) strong stain. Based on each category of staining intensity cells were counted and percentage values were calculated by rating the number of cells in the category to the total number of cells at ×400 magnification folds. For each tracheal specimen, the score assessment was performed at five different fields for epithelial cells, chondrocytes and inflammatory cells, and a mean score was determined. After that the total score was achieved by multiplying these percentage values with their own staining score, according to the following formula:[14,15]

H-score= 1 × (Percentage of score 1+) + 2 × (Percentage of score 2+) + 3 × (Percentage of score 3+).

Statistical analysis

The collected data from histomorphometric parameters were analyzed to detect any differences or correlations between two groups through utilizing SPSS program, version 19 (Both student T-test and Pearson's Correlation Coefficient) with regarding a *P*-value of <0.05 as statistically significant.

Results

Histopathological results

Tracheal section in control negative group showed intact lining epithelium (ciliated-pseudo stratified columnar epithelium, Figure 1a), while in control positive group various degree of epithelial necrosis (detachment or breaking of lining epithelium) were seen and scored as follows; Three cases showed mild epithelial necrosis (Figure 1b) with score (1), five cases displayed moderate epithelial necrosis (Score 2) as in figure 1c, whereas only two cases showed severe epithelial necrosis (Figure 1d and e) with Score 3, in comparison to the control negative group in which all cases scored by (0).

In control negative group the goblet cells which were found among the epithelial cells in tracheal section showed normal cellularity (Score 0) as in figure 1a, in comparison to the control negative group, five cases showed normal goblet cells features (Score 0), while 2 cases showed mild hyperplasia (increasing in number) of the goblet cells (Score 1), and 3 cases had moderate goblet cells hyperplasia (Score 2), as shown in figure 1e and f.

The lining epithelium of trachea in control negative group showed normal epithelium type (ciliated-pseudostratified columnar epithelium, Figure 1a) with score 0, also 8 cases in control positive group had score 0 with normal epithelial type, while two cases showed metaplasia and transformation from normal epithelial type to non-keratinized stratified squamous epithelium score 1 (Figure 1g).

The submucosa that contain tracheal gland showed normal mucous secretory acini in control negative group (Score 0) as in figure 1h, in comparison to the control positive group that showed various pathological lesions in the tracheal gland such as; Three cases exhibited hydropic degeneration in the lining epithelium of secretory portion (Score 1), 5 cases had oval-irregular dilation or distention of tracheal gland that contain a large amount of mucin (Score 2), and only 2 cases showed gland abscess (present of pus exudate inside the distended tracheal gland, Score 3), as seen in figure 1i-k.

In control negative group the sub-mucosa composed of loose connective tissue as in figure 2a with score 0, while in control positive group sub-mucosa showed fibrosis in mild degree in 6 cases (Score 1), and

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the remainder cases had moderate degree of fibrosis (Score 2) in the sub-mucosa of the trachea as in figure 2b-e.

The tracheal section in control negative group from mucosa till adventitia showed normal cellular infiltration (Score 0, Figure 2 f), in control positive group different degree of inflammation was found according to the extent of inflammatory reaction, infiltration of mononuclear inflammatory cells include macrophages, lymphocytes, fibroblasts, and plasma cells, the degree of inflammation ranged from; Mild degree of inflammation that extent only to the mucosal region (Score 1) found in five cases, in four cases moderate inflammatory reaction was seen that extent to the sub-mucosa (Score 2), while severe inflammatory degree (Score 3) which extent to the muscularis layer found only in one cases (Figure 2 g-i).

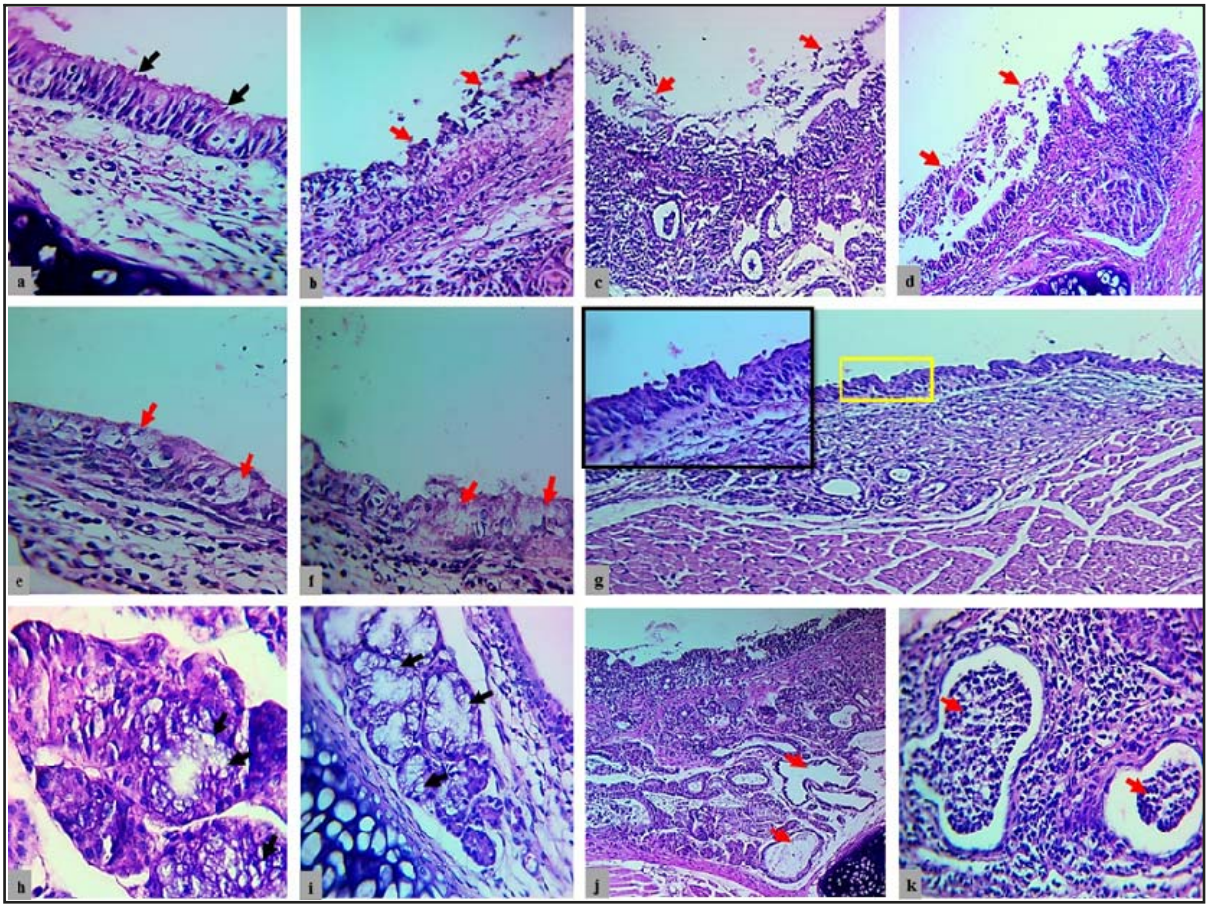

Figure 1 Microscopic tracheal section in rat. **a:** Control negative group showed intact epithelial tissue histology with normal goblet cellularity (400x). **b:** Mild epithelial necrosis with score 1, (400x) **c:** Moderate epithelial necrosis with score 2, (100x) **d:** Severe epithelial necrosis (Score 3) in control positive group, (100x); **e:** Mild goblet cells hyperplasia (Score 1), **f:** Moderate hyperplasia of goblet cells as indicated by red arrows (Score 2), (400x); **g:** Squamous metaplasia in control positive group score 1, (100x); **h:** Normal histological tracheal gland structures in control negative group with score 0 (400x), **i:** Hydropic degeneration as shown by black arrows (Score 1, 200x), **j:** Distention of tracheal gland obstructed by large amount of mucin as indicated by red arrows with score 2, (100x), **k:** Presence of purulent exudate within dilated tracheal gland as showed by red arrows in control positive group (Score 3), (H&E stain, 400x).

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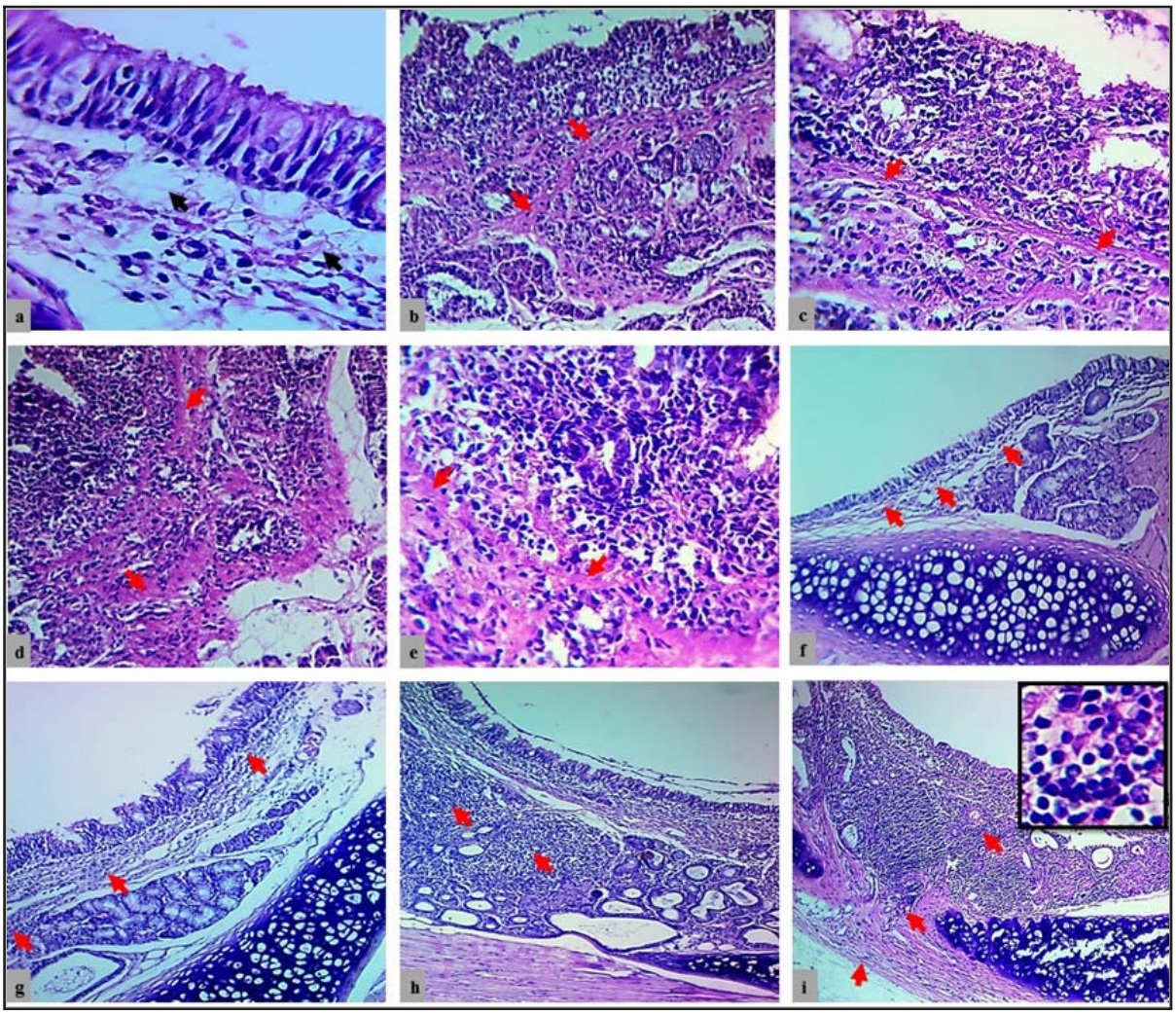

Figure 2: Microscopic tracheal section in rat. **a:** Normal sub-mucosal structures of connective tissue (Score 0, 400x), **b** and **c:** Mild fibrosis in the sub-mucosa (Score 1), **d and e:** Moderate fibrosis in the tracheal sub-mucosa with score 2 in control positive group, (100x and 400x); **f:**Normal cellular compartment in control negative group (Score 0), **g:** Mild inflammation (Score 1), **h:** Moderate inflammatory reaction (Score 2), **i:** Severe inflammation (Inset, score 3), (H&E stain, 100x).

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Histomorphometric result of tracheal epithelial thickness (TET)

There was a showed highly significant (*P <0.001*) increase in the thickness of tracheal epithelium (12.47±0.85 µm) in control positive group that thickened about 2.07 folds more than tracheal epithelium in the control negative group, which had normal histological thickness (6.01±0.39 µm) and demonstrating the correlation between effect of formaldehyde vapor and increasing TET (*rpearson*=-0.921, *P <0.001*).

Immunohistochemistry of S-100 expression

According to H-score system from figure 3, S-100 expression revealed a high scores in the lining epithelial cells, chondrocytes and inflammatory cells including fibroblast, macrophages, lymphocytes, and plasma cells as a brownish nuclear and cytoplasmic staining in the control positive group in comparison to the control negative group (Figure 4), also in the control positive group the S-100 expression showed the higher percentages in all types of cells (epithelial cells, chondrocytes and inflammatory cells) which were recorded as strong intensity (3+), then moderate intensity (2+), and followed by weak intensity (1+) that showed minimum percentages in comparison to the control negative group in which the weak intensity (1+), showed the highest percentages followed by moderate intensity (2+) and lastly strong intensity (3+), as seen in Figure 5.

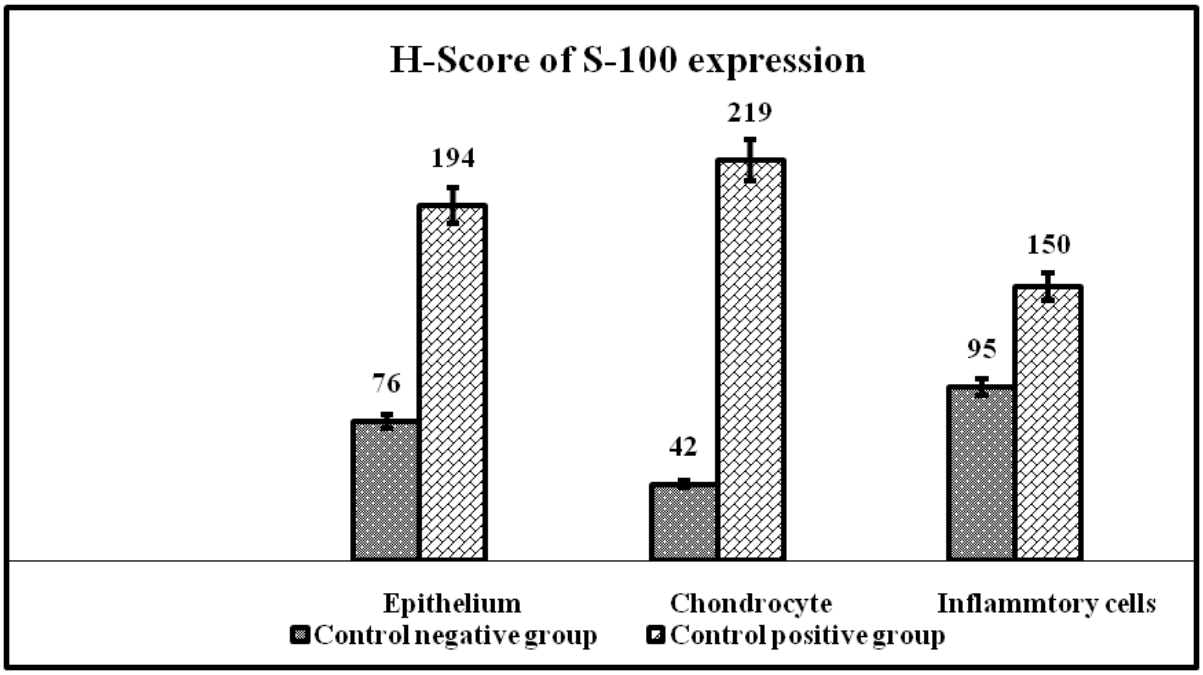

Figure 3 H-Score system for the S-100 expression among different types of cells in both groups.

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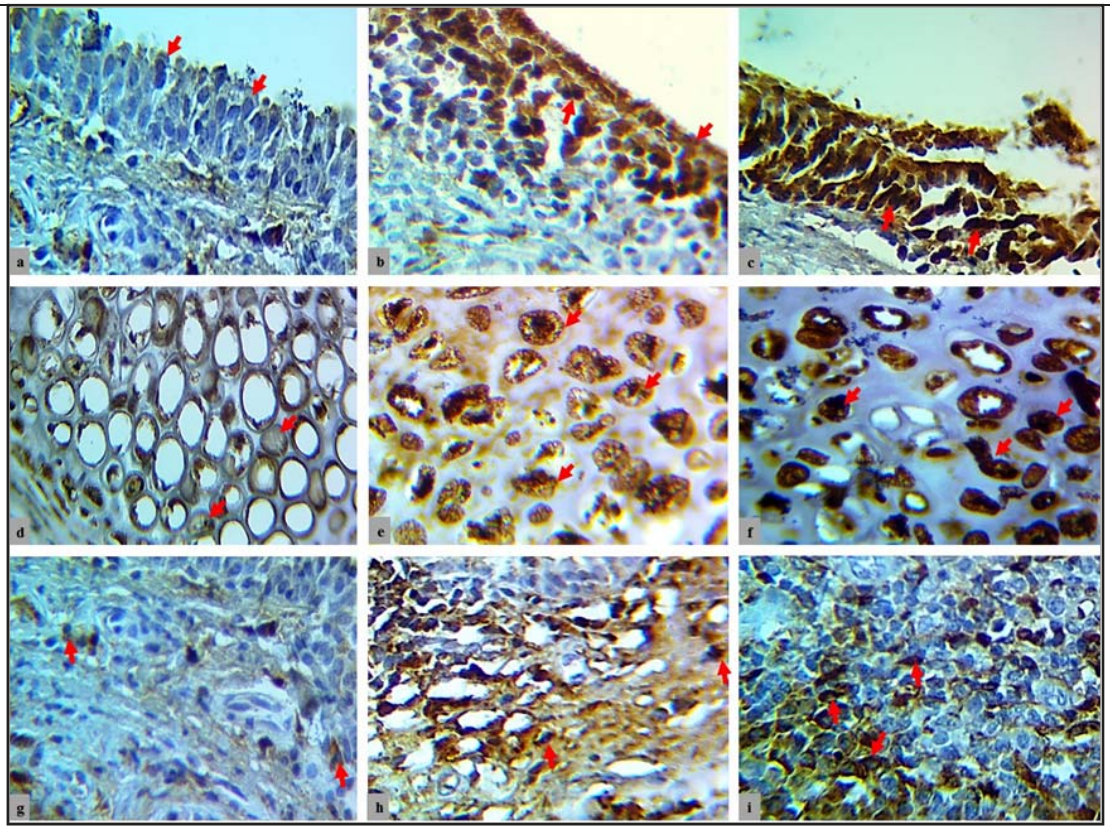

Figure 4 Immunohistochemical staining of S-100 expression in various cells, in the epithelium, **a:** Weak intensity (1+), **b:** Moderate intensity (2+), **c:** Strong intensity (3+) as indicated by red arrows (400x); in the chondrocytes, **d:** Weak intensity (1+), **e:** Moderate intensity(2+), **f:** Strong intensity (3+) as indicated by red arrows (400x); in the inflammatory cells, **g:** Weak intensity (1+), **h:** Moderate intensity (2+), **i:** Strong intensity (3+) as indicated by red arrows (400x).

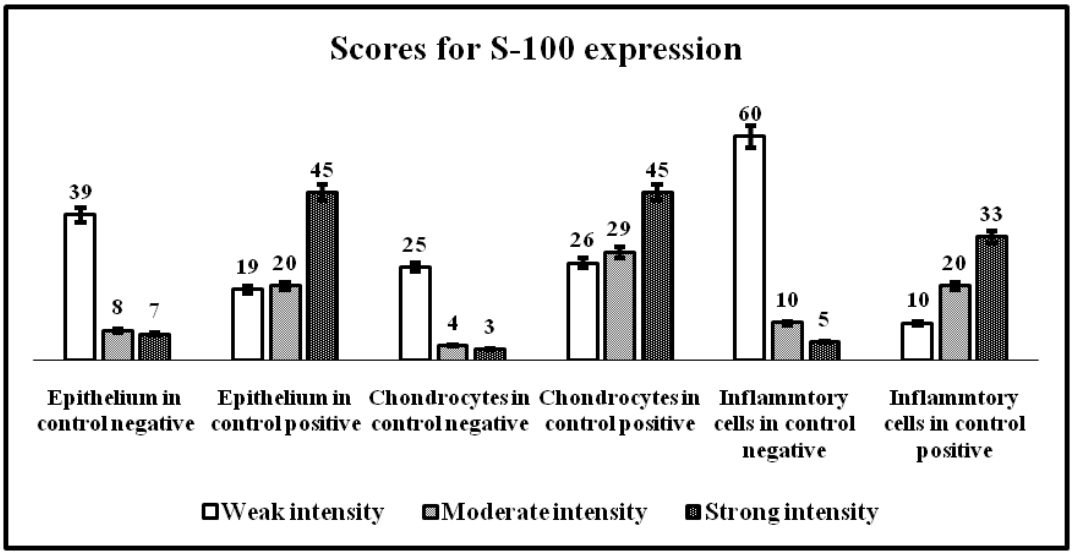

Figure 5 Percentages of different scores among various cells in control negative and positive group.

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Discussion

Inhalation of formaldehyde causes various damage to many organs, comprising not only the respiratory system but also the numerous organs of living bodies such as testis,[16] brain,[17] kidney and liver.[18]

Formaldehyde inhalation may cause irritation in the respiratory system as formaldehyde may be absorbed into the mucus of the nose and throat.

Formaldehyde reacts directly to tissue components and cytotoxicity is likely a function of this reactivity.[19]

The present study demonstrated that the tracheal histopathological changes in the experimental group exposed to 40ppm formaldehyde showed considerable alterations in epithelium cytoarchitecture such as; Epithelial necrosis with different degrees and scores, goblet cells hyperplasia ranged from mild-moderate hyperplasia, with severe alteration in the tracheal epithelium that characterized by squamous metaplasia, This is in agreement with Sapmaz *et al*. (2017), who showed that rat exposed to formaldehyde produced necrotic lesions and desquamation in the lung and tracheal epithelium, which could be affect the functional ability of the studied tissues.[6] Moreover, this research is also supported by Ouies and Abd El-Naeem. (2020), which indicated respiratory and transitional epithelium to be more vulnerable than other types of epithelium to formaldehyde damage.[20]

Also the current study is in consistent with the previous study, which found that 40% w/v of formaldehyde vapor was inhaled for 6 weeks using a rabbit model, produced squamous metaplasia and documented that goblet cell hyperplasia could be triggered by mucociliary function inhibition.[21] In addition, chemical reactions have been reported to be one of the causes of oxidative stress and the product of free radicals that destroy the cells.[22]

In comparison to the control negative group, the experimental group showed various pathological lesions in the tracheal gland such as; Hydropic degeneration in the secretory portion of the lining epithelium, distention of tracheal gland containing a large quantity of mucin, with or without purulent exudate within the distended tracheal gland, the reason for such lesion is supported by a previous study, which mentioned that the mucociliary function must be intact in the trachea to mitigate the carcinogenic effect.[23] In this research, the tracheal epithelial metaplasia was described and resulted in the loss of cilia required for the continuous movement of glandular secretions to the pharynx as stated in the Swenberg *et al*. (2013) study by inhibiting mucociliary activity, formaldehyde has been shown to increase its own toxic effect[24] and therefore the mucus material formed by the tracheal gland is not moved by cilia and stayed in the lumen of the gland leading to distention of the gland.

The other tracheal changes evident in the current study included, infiltration of mononuclear inflammatory cells, mild-severe inflammatory reaction were found in the different layers from mucosa and in few cases reached to the muscularis layer, this is confirmed previously in the rabbit lungs after exposure to 40% formaldehyde,[21] also detected in rats,[20] and in children.[24]

The mechanism of infiltration of inflammatory cells that has been induced by formaldehyde inhalation documented by Kimura *et al*. (2010), formaldehyde rapidly increases vascular permeability in the airways of rats and develops microvascular leakage by stimulating tachykinin NK1 receptors from sensory nerves.[26] Moreover, formaldehyde can induce inflammation of the airway through various signaling pathways such as the mitogen-activated protein kinase (MAPK) and NF-κB pathways.[27] In addition, an increase in the production of intracellular reactive oxygen species (ROS) has been reported.[28]

S-100 is a family of proteins of small molecular weight. Members of the S-100 family are involved in transduction of calcium signal, cytoskeletal-membrane

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interaction, cell growth and differentiation.[29] Acute/chronic inflammatory disorders and various cancers have been associated with subgroups of the S-100 family (S-100A12, S-100A8 and S-100A9).[30] In the present study S-100 expressed by moderate-strong intensity (2+ to 3+) in the nuclear and cytoplasm of the inflammatory cells in the experimental group in comparison to the control group that showed weak or negative staining (score 0), also in this study S-100 member act as pro-inflammatory mediator and modulate inflammation, these findings are in accordance with a study, which demonstrated that elevated S-100 protein levels are associated with chronic inflammation and it is involved in the regulation of cellular reaction and cancer development.[31,32]

The present study showed an increase (*P <0.001*) in the thickness of tracheal epithelium (12.47±0.85 µm) in the experimental group which was thickened about 2.07 folds more than tracheal epithelium of control negative group (rpearson = -0.921, *P <0.001*), this result is in agreement with study Sapmaz *et al*. (2017), which demonstrated the development of tracheal epithelium thickness or hyperplastic trachea in rats exposed to 10 ppm for 13 weeks.[5] The positive S-100 cells were found in the hyperplastic epithelium from moderate to strong intensity (2+ to 3+), this is in line with previous data, which showed that over expression of S-100A6 leads to an increase in the cell proliferation and development of hyperplasia.[11,33,34]

S-100 protein was studied primarily as a marker for chondrocytic and/or chondrogenic origin.[35] Additional studies, indicated that the intensity of S-100 protein in chondrocytes near cartilage lesions increases and suggests that S-100 protein may be involved in cartilage repair.[36] In the current study, the S-100 immunoreactivity increased in the experimental group in comparison to the control group, for instance, negative expression was found in the control group, while weak-strong expression was found in the experimental group, and this is in accordance with a previous study, which found that S-100 protein was colored differently by chondrocytes from different regions in cartilage lesions[37], other studies have postulated that extracellular S-100B activates the RAGE signaling pathway in chondrocytes, leading to activation of ERK and NF-κB signaling molecules and increased production of MMP-13, making S -100B a pro-catabolic, pro-inflammatory factor promoting cartilage degradation.[38]

Conclusion

Exposure to high concentrations and long-term formaldehyde (40% formaldehyde for 21 consecutive days), severe histological changes that may increase cancer risk, S-100 proteins encourage airway inflammation and act as a pro-inflammatory marker, Therefore, expression of S-100 protein is associated with hypertrophy of chondrocytes and is identified as proliferative marker

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Competing interests

The author declares that she has no competing interests.

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