Original Article

Evidence for Mast Cells Activation in the Lung of Propionic Acid-Induced Autism-Like Rat Model (Histological and Immunohistochemical Study)

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ABSTRACT

Introduction: Autism spectrum disorder (ASD) is a global health problem. Growing evidence suggests that high prevalence rates of different allergic conditions are associated with autism.

Aim of the Work: As mast cells are the main cells included in the pathophysiology of allergic reactions and anaphylaxis, therefore, this study aimed to find scientific evidence for the association between propionic acid-induced ASD and mast cell activation in the lung.

Materials and Methods: Twenty rats (Two weeks-old) were randomly divided into two equal groups ten rats each; control group: rats were given SC injection of phosphate buffer saline (1ml) for five successive days and Propionic acid (PPA) treated group: rats were given (500 mg/kg/ day) SC for five successive days. By the end of two-months-old lungs were dissected and examined by histological and immunohistochemical methods.

Results: A significant increase in mast cell density, intact mast cells and degranulated cells were observed in PPA treated group compared with the control group. Thickened interalveolar septum with inflammatory cellular infiltration and congested blood vessels were observed. Most of mast cells were degranulated. Mast cells were found within the smooth muscle layers of respiratory bronchioles. A statistically significant increase in area percent of collagen was detected in PPA treated group in comparison to the control group. A significant increase in area percentage of IL-6 was also detected in PPA treated group in comparison to the control group.

Conclusion: The results are indicative that an increase in mast cell density was detected in PPA treated group. It was associated with lung fibrosis and increased area percentage of IL-6.

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Key Words: Autism spectrum disorder, Mast cells, propionic acid.

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INTRODUCTION

Propionic acid (PPA) is a fatty acid that can be produced in the gut from metabolism of fatty acid and as well from fermentation end product of antibiotic-resistant enteric gut bacteria such as clostridia[1]. Interestingly, excessive exposure to antibiotics was proved to altered microbial biogeography and appearance of resistant enteric gut bacteria[2], in addition to the use of food preservative that contains PPA as wheat and dairy products. As a result of increased PPA level, the body became unable to correctly convert amino acids to sugars. These consequences result in a toxic propionic acid increase in the bloodstream. It can pass through the blood-brain barrier causing up-regulation of central nervous system (CNS) pro-inflammatory cytokine levels, persuade a varied series of neurophysiological processes capable of changing brain function and activities and development of ASD[3,4,5].

Plentiful lines of evidence signifying that PPA metabolites formed from microbial fermentation of foods could disturb both the immune system and the CNS of patients with ASD[6,7]. Numerous researches have signified that gut metabolites persuade ASD. Besides, numerous original models of ASD have been established in the animal by modifying the level of gut metabolite[8,9]. Several previous works specify that administration of PPA in experimental animals produces many changes related to human ASD[1,10,11,12].

ASD is a global public health problem that has been increasing progressively over the past few years. It is a neurodevelopmental condition due to developmental or environmental causes characterized by a defect in verbal and nonverbal communications[13].

IL-6 is a pro-inflammatory and immune-regulating cytokine that was proved to be released from mast cells and other cells during acute inflammation. Recently, there is great evidence that ASD is accompanied with deregulated immune procedures that influence the pathogenesis of autism[14,15,16]. Evidence revealed that autistic patients usually have disturbed levels of cytokines, inflammatory markers and immunoglobulin[17,18]. A high prevalence rates of different allergic conditions including; asthma, rhinitis and food allergy were documented in autistic children than the control ones[18,19].

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Mast cells (MCs) are usually dispersed through vascularized tissues, chiefly that in close connection with the atmosphere such as; the skin, airways and gastrointestinal tract[20]. Previous reports indicated that there was a convincing relation between mast cell and the pathophysiology of allergic reactions and anaphylaxis. Recently, mast cells are also evident to have a perilous role in various other disease processes including, tissue remodeling that are accompanying with chronic allergic inflammation, wound healing[21,22,23], maintenance of tissue homeostasis[24], revascularization[25] and pathological fibrosis[26].

Environmental and genetic aspects can stimulate mast cell proliferation, survival, and vulnerability to stimulation by different stimuli produced during immune responses[27]. Changes in the arrangement of mast cells in the tissue and increases in their numbers were evident in allergic conditions, tissue inflammation and tissue remodeling[20,28].

The goal of this work was to find a scientific evidence for the association between propionic acid-induced ASD and mast cell activation in the lung through histological and immunohistochemical methods.

MATERIALS AND METHODS

2.1- Chemicals

Sodium propionate (PPA) was obtained from Sigma-Aldrich. St. Louis, MO, USA and liquefied in 0.1 M phosphate buffer saline (PBS).

2.2- Animals and experimental protocol

The protocol of this study was reviewed and permitted by (IRB) Institutional Review Board of Faculty of Medicine, Mansoura University (Code: R.19.11.671). In this study 20-Sprague–Dawley male rats (two-weeks-old) weighing 60-80 gm were used. Animals were kept in a well-prepared animal house for one week before the experiment for acclimatization. The animals were freely allowed to tap water and the ordinary rodent diet. Animals were kept under average temperature (24 °C), usual humidity (55%) and under a consistent light/ dark cycle (12:12 hours). Animals were classified to two equal groups (10-rats/group):

Group I (control): rats administrated PBS (1 ml) by subcutaneous injection for five successive days

Group II (PPA treated group): rats were given PPA (500 mg/kg/ day) SC for five successive days. This dose was cautiously designated according to preceding studies[1,5].

All rats were kept in their cages until the age of two months. At this age all rats were anesthetized and the lungs were dissected under a strict sterile condition and specimens were obtained and processed for histological examination.

2.3- Histological procedure

- Light microscopic study

The lungs specimens were used to prepare paraffin blocks. Rotary microtome was used for obtaining sections of 5 microns thickness. The following stains were used:

- Hematoxylin and eosin (H&E) to identify the histological details[29].
- Toluidine blue stain: to verify mast cell

Sections were deparaffinized and gradually rehydrated then stained with 1% toluidine blue, mounted with dibutyl phthalate xylene. Microscopic identification of mast cells was done by the brilliant red/purple appearance of the granules (metachromasia)[30].

- Masson's trichrome stain: for demonstration of collagen fibers[31].

2.4- Immunohistochemistry (IHC) for detection of IL-6

Five µm thickness sections were deparaffinized followed by rehydration. IHC was done using streptavidin-peroxidase immunohistochemistry kit. IL-6 antibodies (polyclonal anti-rabbit, Gene Tex, Inc, North America. Cat No. GTX110527) (1:100) was used as primary antibodies. Biotinylated anti-mouse IgG (LSAB 2 Kit; Dako) was used as secondary antibodies. Hematoxylin was used as a counterstain and then the slides were dehydrated. Negative control sections were done in a similar way but without adding the primary antibodies[32].

2.5- Morphometric study

Morphometric measurements were done according to what we formerly reported[33]. Objective lens ×40 were used to examine the slides. The mean values of 3 non-overlapping microscopic fields/rat for 5 different rats in each group were estimated. The images acquired were assessed on Intel Core 13(Toshiba Satellite A5055 computer, UK) based computer using VideoTest Morphology software (Russia, Saint-Petersburg). The values were calculated and expressed as mean ± standard deviation.

- Assessment of mast cell density: total Mast cells were calculated using toluidine blue-stained sections (the total mast cell count/optical field)[34].
- Intact mast cells: the mast cells that displayed metachromasia and dense granules obscuring the nucleus[35].
- Degranulated mast cells: the cells that displayed less metachromasia and a distinct nuclear outline[35].
- Area percent of collagen: was done using sections stained with Masson's trichrome-stain.
- Immunohistochemical assessment of area percentage of IL6 using immunohistochemical stained slides.

2.6- Statistical analysis

Statistical analysis was done using the Statistical Package Social Sciences (SPSS) version 22 for windows® (IBM SPSS Inc) quantitative data were tested for normality and were expressed as mean ± SD or median (range). Ordinarily disseminated numbers between the two examined groups were compared via independent sample t-test (expressed as t). Non-parametric data compared by the Mann-Whitney test (expressed in Z).

P <0.05 was considered to be statistically significant.

RESULTS

3.1. Histological results

Sections stained with H&E from control specimens demonstrated the ordinary architecture of the lung. The sections comprised bronchi, bronchioles, blood vessels, lung alveoli and alveolar sacs separated by thin interalveolar septa (Figure 1a). The bronchi revealed folded mucosa lined by respiratory epithelium, smooth muscle (SM) layer surround the mucosa and adventitia containing cartilage plates (Figure 1b).The bronchioles showed folded mucosa covered with simple columnar ciliated epithelium, spirally arranged SMs, and adventitia of areolar connective tissue (CT). Alveoli spaces were covered mostly by flat cells with flattened nuclei (pneumocytes Type-I), and cuboidal shape cells with rounded nuclei mainly at the angles (pneumocytes type-II) (Figure 1c).

Examination of lung sections of PPA treated group revealed thick interalveolar septa with narrowing of alveolar space. The septa contained numerous inflammatory cells. Congested blood vessels and blood capillaries were noticed in all sections (Figures 2 a,b).

Examination of Toluidine blue stained sections from the control group showed few numbers of granulated metachromatic MCs. They were apparent in CT of bronchioles and blood vessels (Figures 3 a,b). Whereas, PPA treated group demonstrated an obvious increase in the count of MCs. Mast cells were distributed within the SML around bronchioles, in the CT nearby the blood vessels and in the septa between the lung alveoli. The majority of MCs were degranulated (Figures 4 a,b,c).

Examination of control sections stained with Masson's trichrome stain showed the ordinary arrangement of collagen fibers in the connective tissue of the lung. Fine collagen fibers were distributed mainly in the CT of bronchioles and blood vessels, and few thin fibers were seen in the thin interalveolar septa (Figures 5 a,b). Sections from PPA treated group revealed an increase in the collagen deposition around bronchioles, blood vessels in addition to the thick interalveolar septa (Figures 6 a,b).

3.2. Immunohistochemical results

IL-6 immunohistochemical stained sections of control specimens demonstrated weak positive reaction in the bronchiolar epithelium, connective tissue around bronchioles and blood vessels (Figures 7a,b). on the other side, PPA treated group showed strong positive reaction in the bronchiolar epithelium, connective tissue around bronchioles, blood vessels and in the thick interalveolar septa (Figures 8 a,b,c).

3.3. Statistical and Morphometric results

Mast cell density, intact MCs and degranulated MCs were significantly increased in PPA treated group in comparison with group I (control) (Table 1, Histogram 1). A statistically significant rise in the area % of collagen in the lung of the PPA treated group in comparison with the control group (Table 2, Histogram 2). A significant rise in the area percentage of IL-6 immunohistochemical stain in the PPA treated group was also detected (Table 3, Histogram 3).

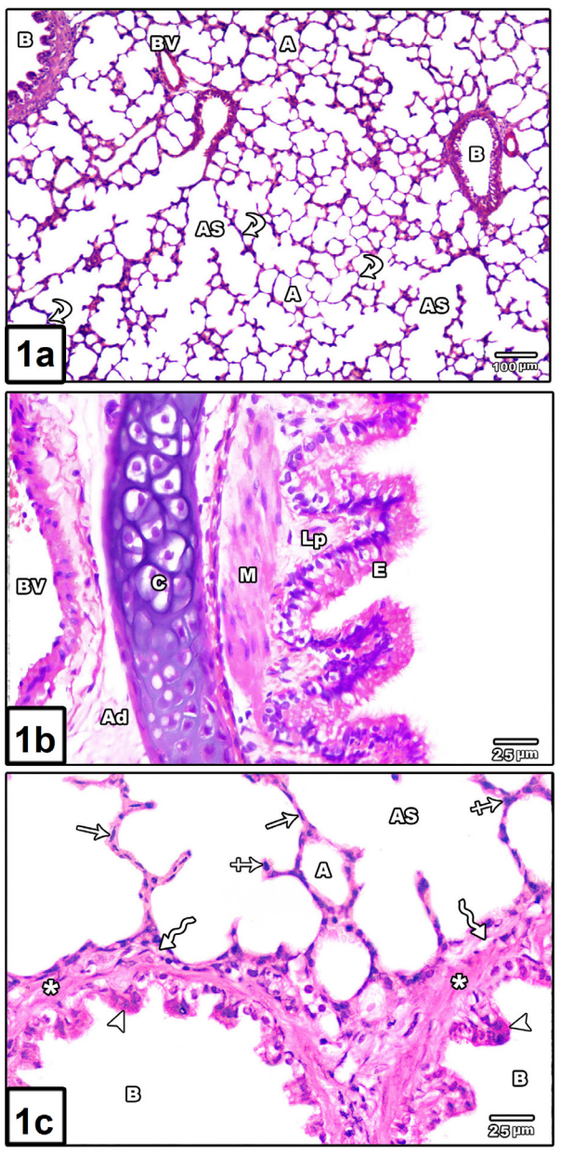


Fig. 1: Photomicrograph of sections in the lung of a control rat. 1a) Displaying the normal lung construction; expanded alveoli (A) and alveolar spaces (AS) separated by thin interalveolar septa (curved arrow), bronchioles (B) and blood vessels (BV). 1b) the bronchus showing respiratory epithelium (E), lamina propria (Lp) a SML (M) surround the mucosa and adventitia (Ad) containing hyaline cartilage plate (c) note blood vessel is present (Bv). 1c) showing two adjacent bronchioles (B) lined by simple columnar partially ciliated epithelium (arrow head), a thin smooth muscle layer (*) and adventitia (zigzag arrow). Expanded alveoli (A) separated by thin interalveolar septa are seen. The alveoli lined by pneumocytes type I (arrows) and pneumocytes type II (crossed arrows). (H&E; a X100, b,c X 400)

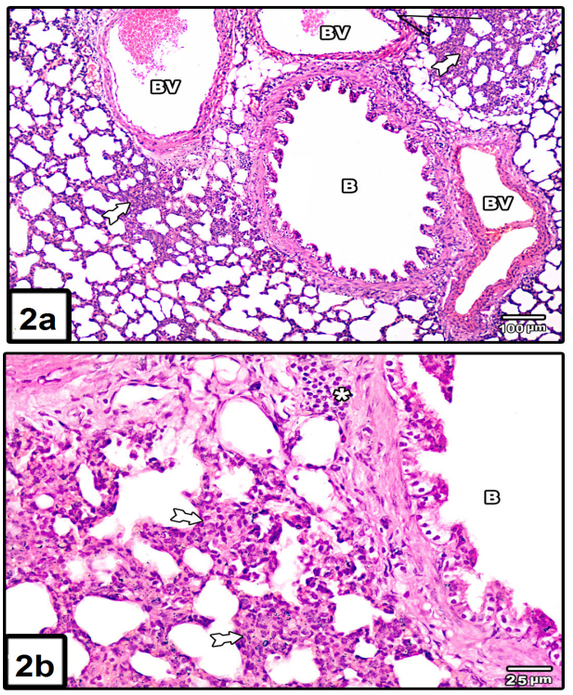


Fig. 2: Photomicrographs of sections in the lung of PPA treated group showing bronchiole (B) congested blood vessels (Bv) and thickened interalveolar septum (thick tailed arrow) with inflammatory cellular infiltration (*). (a X 100, bX 400)

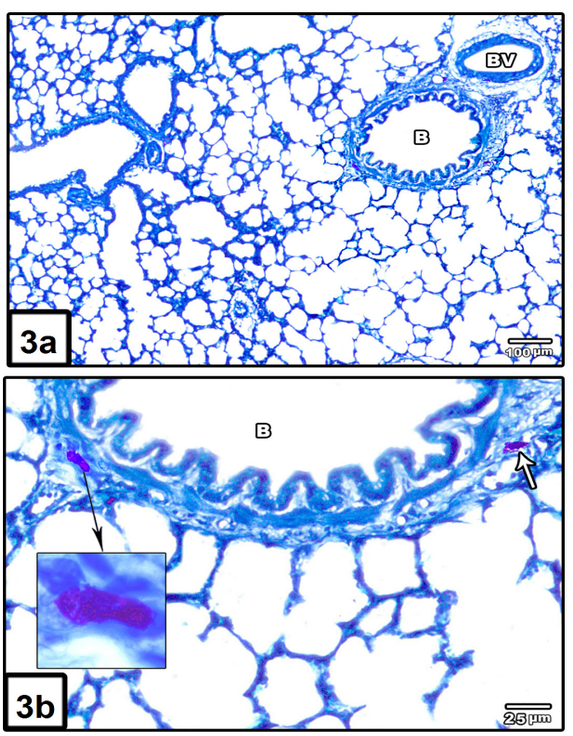


Fig. 3: Photomicrographs of lung sections from the control group showing granulated metachromatic mast cells (arrow) in the CT around the bronchiole (B) and blood vessels (Toluidine blue; a X 100, bX 400, inset X1000)

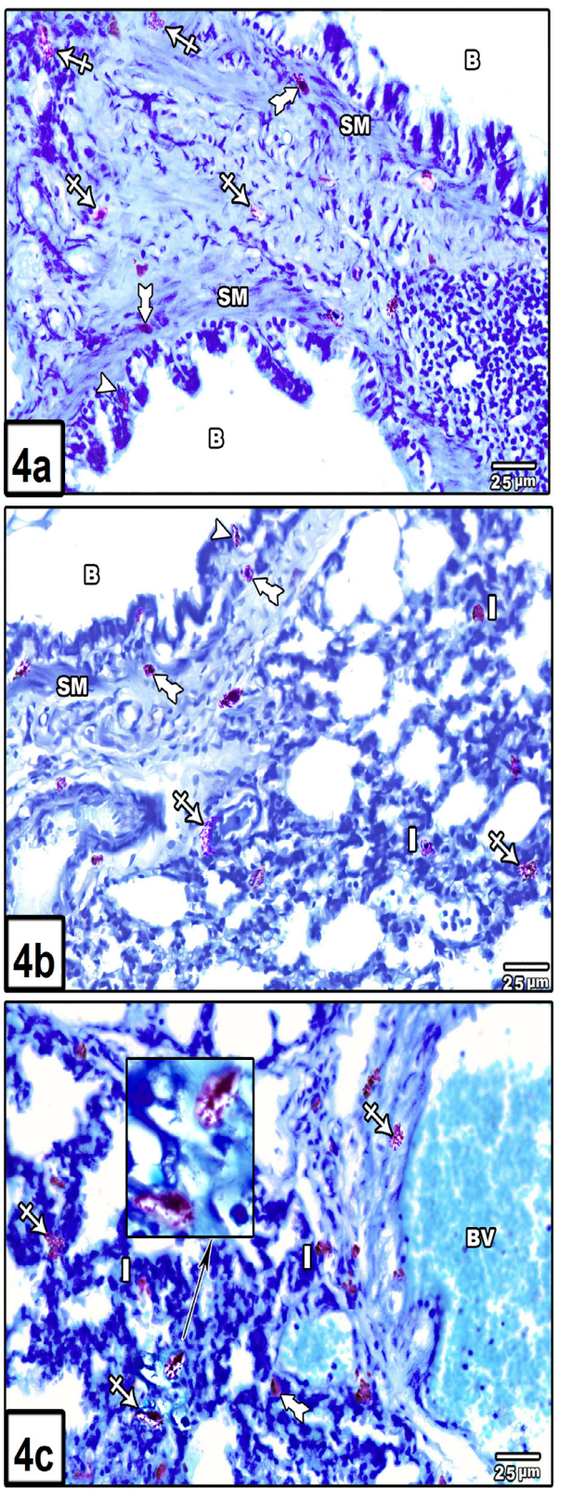


Fig. 4: Photomicrographs from PPA treated group showing numerous mast cells in the smooth muscle layer (SML) around bronchiole (B), in the epithelial (arrow head) lining of the bronchiole, in the thick interalveolar wall (I) and in the CT around the blood vessels (Bv). The majority of mast cells are degranulated (crossed arrow). Few intact mast cells are also seen (thick tailed arrow). (Toluidine blue; a,b,c X 400, inset X1000)

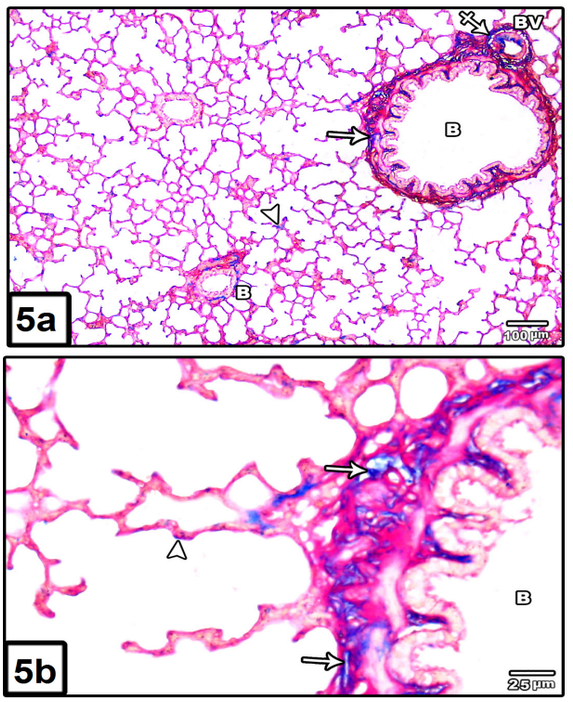


Fig. 5: Photomicrographs of lung sections of the control group revealing collagen fiber (arrow) scattering in the walls of a respiratory bronchiole (B) and around the blood vessels (Bv). Fine fibers are seen in the interalveolar septa (arrow head). (Masson's trichrome; a X 100, bX 400)

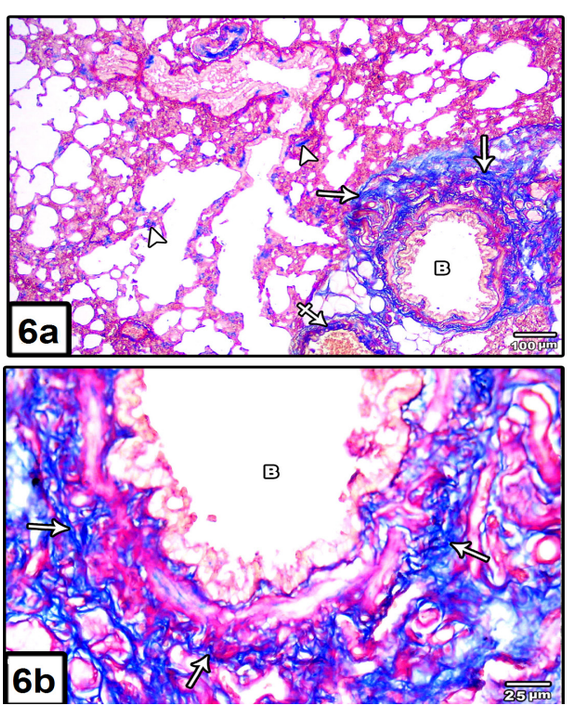


Fig. 6: Photomicrographs of lung sections from PPA treated group displaying an obvious rise in collagen fibers (arrow) deposition in the wall of a respiratory bronchiole (B), blood vessel (crossed arrow) and apparent increase in collagen fibers in the thick interalveolar septa. (Masson's trichrome; aX 100 bX400)

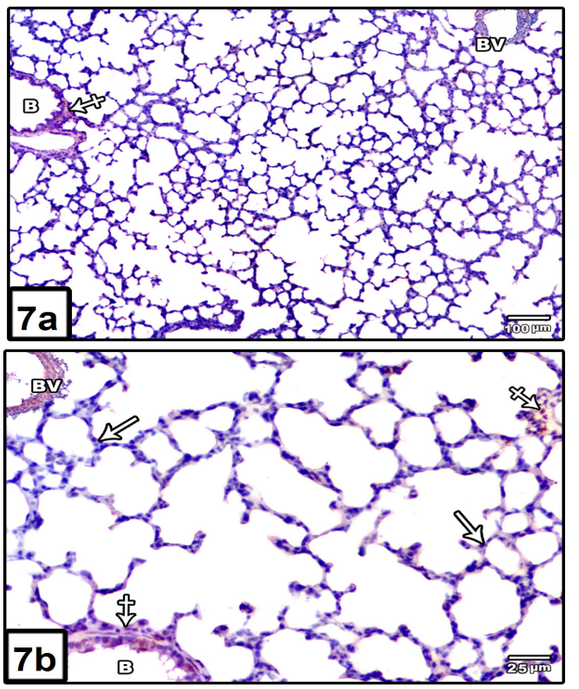


Fig. 7: Photomicrographs of sections from the lungs of the control group showing positive reaction in the epithelial cell and CT of bronchiole (crossed arrow) and around the blood vessels (BV). Absence of the reaction in the interalveolar wall was observed (arrow). (IL-6 IHC; a X 100, bX 400)

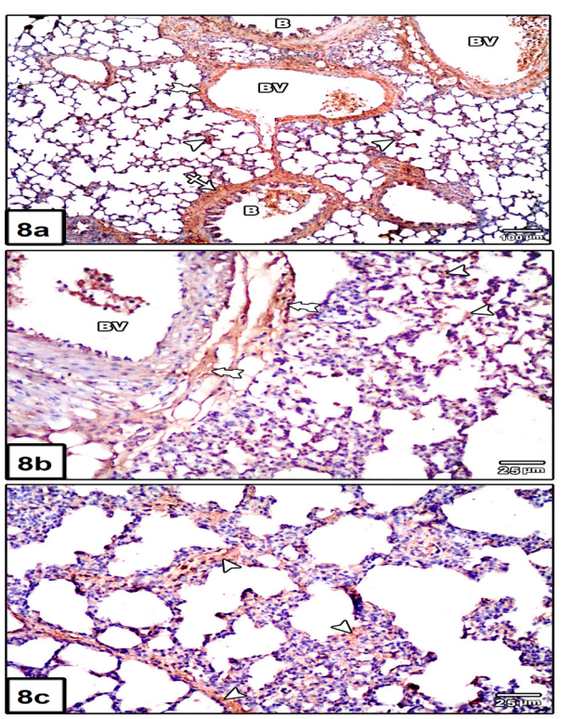


Fig. 8: Photomicrographs of lung sections of PPA treated group displaying obvious increase in IL-6 positive reaction (crossed arrow) in the bronchiole (B), around blood vessel (tailed arrow) and in the thick interalveolar wall (arrow head) (a X 100, b,c X 400)

Table 1: Mast cell analysis in the two study groups

	Groups		Test of significance
	Control group	PPA treated group	
Total number of Mast cells	2.33 ± 0.82 (1-4)	10.27 ± 1.80 (7-15)	t= - 15.606 $P < 0.001^{**}$
Number of intact Mast cells	2.07 ± 0.70 (1-3)	4.07 ± 0.96 (3-6)	t= -6 .502 $P < 0.001^{**}$
Number of degranulated Mast cells	0.33 ± 0.48 (0-1)	6.20 ± 1.27 (4-9)	z= - 4.789 $P < 0.001^{**}$

P: probability.
Continuous data expressed as mean±SD and (minimum-maximum)
T= independent samples t-test z: Mann-Whitney U test
*: significant ($p< 0.05$) **: highly significant ($p\leq 0.001$)

Table 2: analysis of area percentage of collagen/ high power in the two study groups

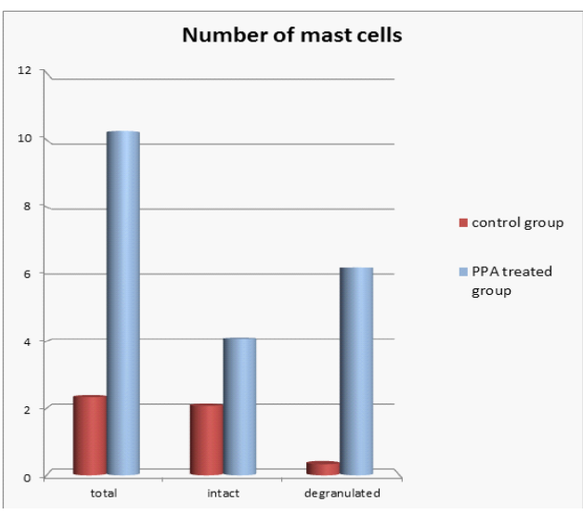
	Groups		Test of significance
	Control group	PPA treated group	
Area percentage of collagen/ high power (%)	2.42 ± 0.45	9.36 ± 0.4	t= - 7.064 $P < 0.001^{**}$

P: probability.
Continuous data expressed as mean±SD and (minimum-maximum)
T= independent samples t-test z: Mann-Whitney U test
*: significant ($p< 0.05$) **: highly significant ($p\leq 0.001$)

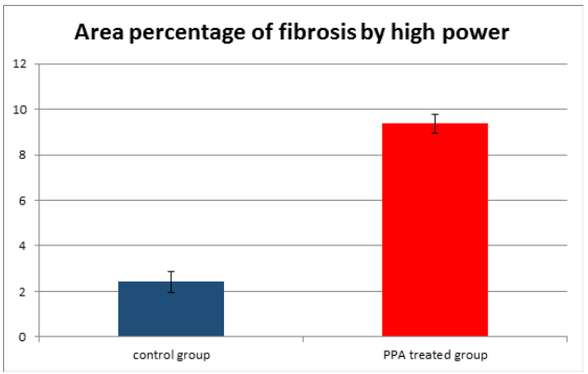
Table 3: analysis of Area percentage of IL-6/ high power in the two study groups

	Groups		Test of significance
	Control group	PPA treated group	
Area percentage of IL-6/ high power (%)	0.68 ± 0.12	4.55 ± 0.43	t= - 9.726 $P < 0.001^{**}$

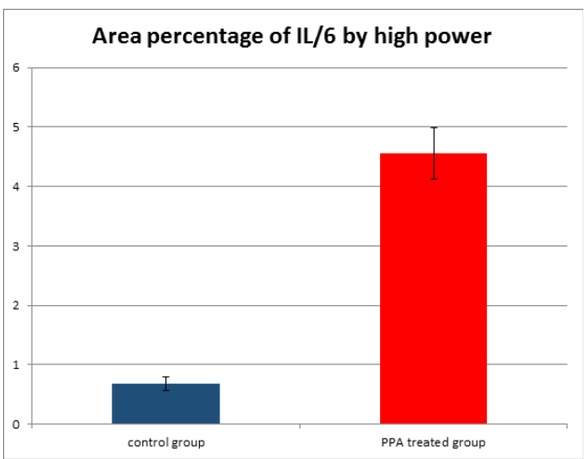
P: probability.
Continuous data expressed as mean±SD and (minimum-maximum)
T= independent samples t-test z: Mann-Whitney U test
*: significant ($p< 0.05$) **: highly significant ($p\leq 0.001$)



Histogram 1: The number of mast cells (total, intact and degranulated) in studied groups



Histogram 2: The area percentage of fibrosis in studied groups



Histogram 3: The area percentage of IL-6 in studied groups

DISCUSSION

Previous studies have found that PPA was beneficial in lowering cholesterol levels and improving insulin sensitivity only at a proper PPA level[36,37]. On contrary, abnormal high PPA exposure was thought to be one of the most important environmental triggers of the brain and changes in behavior detected in ASDs[8,9].

Recently, ASD has been pronounced disparity in levels of cytokines, immunoglobulin and inflammatory processes[17]. The occurrence of immune-mediated illness; asthma, rhinitis, skin allergy being frequently comorbid in autism[18,19,38, 39].

As Mast cells were documented to play a fundamental role in inflammatory and allergic reactions[40], therefore; the purpose of the present work was to explain the relation between propionic acid-induced ASD and mast cell activation in the lung.

Our results revealed a statistically significant rise in mast cell count in the lung of PPA treated group in comparison to the control group. A similar finding was previously reported

in asthmatic lungs and other allergic conditions[41,42].The increased number of mast cells could be due to movement of MCs or their progenitors to the site of inflammation or the proliferation of resident mast cell precursors. They added that mast cells could control reactions from allergies to inflammation, in addition to a wide range of immune regulation function.

Mast cells were existed mainly in the connective tissue (CT) of lamina propria of the bronchioles and around the blood vessels in the control lung. Whereas, they were mainly distributed within the SML of the bronchioles, interalveolar wall and around the blood vessels in PPA treated group. The migration of mast cells to the muscle layer of the airway is a fundamental abnormality in asthma and allergic conditions of the lung by affecting the severity of hypersensitivity reaction[43,44,45]. Besides, the interactions between SM and infiltrating MCs was considered as a crucial component in the development of functional airway disordered in asthma[43].

The existence of MCs in the CT of blood vessels explained their role in the release of cytokines and histamine which affect the blood vessels' permeability. This permits the inflammatory cells to be adherent to the vascular endothelium and then migrates to the nearby tissue[40].

The results of this work establish a significant rise in the density of mast cells in the SML of the bronchioles, interalveolar wall and around the blood vessels. As the majority of previous researches reported that MCs stimulate tissue fibrosis[46,47,48] therefore, this could explain the increased area percent of collagen fibers in the lungs of PPA treated group . Mast cell was considered as a profibrotic factor via the expression of TGF-β and fibroblast-attracting proteases[49]. Besides, mast cells are capable to produce mediators and enzymes that possibly will either prompt collagen deposition or destroy the excess extracellular matrix. This different reaction of mast cells may explain their capability to change their phenotype as a function of the microenvironment[20,50].

In the present work, we demonstrated the typical granules of MCs with common degranulation and pouring of their content to the surrounding. A statistically significant rise in the count of degranulating mast cells was observed. In agreement with our result, MCs degranulation have been described in asthma[51] and cases of idiopathic pulmonary fibrosis and other interstitial lung diseases[48]. MCs degranulation and release their preformed granule mediators into the extracellular space occurs when MCs are stimulated via the interaction of their surface receptors for IgE or other antigen[52].

IL-6 is an immune-regulating cytokine that was proved to be released from T-cells, macrophages, Mast cells and other cells mainly as a result to acute inflammation and in association with the pathogenesis of numerous human mast cell (HuMC) related diseases[53]. The level of IL-6 was proved to be related to the degree of severity of asthma[54], acute and chronic urticaria[55,56] and the degree of severity of disease in systemic mastocytosis[57].

Immunohistochemical result IL-6 revealed few positive reactions in the bronchiolar epithelium and the CT around bronchiole and blood vessel with negative reaction in the interalveolar septa. A similar distribution of IL-6 in the control lung was previously described[58]. On the other side, tissue samples from the PPA treated lung revealed significantly increased in the area percentage of IL-6 in comparison to the control group. Besides, a strong positive reaction in the thick interalveolar septa was also detected. Interestingly, IL-6 has been proved to be an essential inflammatory mediator that is directly correlated to the activity of the disease[59].

It has been stated that IL-6 is an important mediator affecting the nervous system and consequently the development of autism. A high level of IL-6 gene was reported in the brain of autistic patients[60]. Besides, other authors established that numerous cytokines including IL-6 and IL-8 are raised in the plasma of young children with ASD and these rises are connected with more impaired communication and abnormal performances[61,62,63].

CONCLUSION

From our results, we can conclude that allergic-like mast cell activation was evident in the lung of rats of the PPA model of ASD. An increase in mast cell concentration was shown to correlate with lung fibrosis and increased area percentage of IL-6.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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الملخص العربى

الدليل على تنشيط الخلايا البدينة في رئة نموذج الفئران الشبيه بالتوحد الناجم عن (الحمض البروبيونيك (دراسة نسيجية وكيميائية مناعية

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المقدمة: التوحد طيف الاضطراب (ASD)هو مشكلة صحية عالمية. كما تشير الدلائل المتزايدة إلى أن معدلات الانتشار المرتفعة لحالات الحساسية المختلفة ترتبط بالتوحد.

الهدف من العمل: نظرًا لأن الخلايا البدينة هي الخلايا الرئيسية المشمولة في الفيزيولوجيا المرضية للتفاعلات التحسسية والحساسية المفرطة ، فقد هدفت هذه الدراسة إلى إيجاد دليل علمي على الارتباط بين التوحد الناجم عن حمض البروبيونيك وتنشيط الخلايا البدينة في الرئة.

المواد والطرق: تم تقسيم 20 فأرًا (عمرها أسبوعان) بشكل عشوائي إلى مجموعتين متساويتين لكل منهما عشرة فئران. المجموعة الضابطة: أعطيت الفئران حقن تحت الجلد من محلول ملحي بالفوسفات (1 مل) لمدة خمسة أيام متتالية. ومجموعة حامض البروبيونيك (PPA) أعطيت الفئران (500 ملغ / كلغ / يوم) حقن تحت الجلد لمدة خمسة أيام متتالية. و بحلول نهاية شهرين تم تشريح الرئتين وفحصها عن طريق الأساليب النسيجية والمناعية.

النتائج: لوحظت زيادة كبيرة في كثافة الخلايا البدينة ، الخلايا البدينة السليمة والخلايا المتحللة في المجموعة المعالجة بحامض البروبيونيك مقارنة مع المجموعة الضابطة. ولوحظ وجود سماكة في الحاجز بين الحويصلات الهوائية مع تسلل خلوي التهابي واحتقان في الأوعية الدموية. وقد تم تفكيك معظم الخلايا البدينة مع العثور على بعض من هذه الخلايا داخل طبقات العضلات الملساء للقصبات الهوائية التنفسية. تم الكشف ايضا عن زيادة ذات دلالة إحصائية في النسبة المئوية لمساحة الكولاجين و زيادة كبيرة في النسبة المئوية لمساحة IL-6في المجموعة المعالجة بحامض البروبيونيك بالمقارنة مع المجموعة الضابطة.

الخلاصة: دلت النتائج على اكتشاف زيادة في كثافة الخلايا البدينة في المجموعة المعالجة بحامض البروبيونيك مع ارتباطه بتليف الرئة وزيادة النسبة المئوية لمساحة IL-6.