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Effect of Various Bedding Materials on Rat Skin: An Investigation into Secretion of IL-1 Beta and TNF-Alpha and Its Relation to Apoptosis

Kubra Asena Terim Kapakin[1*], Serdar Altun[1], Samet Kapakin[2], Fatih Yildirım[3]
[1]Department of Pathology, Atatürk University, Faculty of Veterinary Medicine, Yakutiye, Erzurum, Turkey
[2]Department of Anatomy, Atatürk University, Faculty of Medicine, Yakutiye, Erzurum, Turkey
[3]Department of Animal Science, Atatürk University, Faculty of Veterinary Medicine, Yakutiye, Erzurum, Turkey

Original Research Article

***Corresponding author**
Kubra Asena Terim Kapakin

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Abstract: In this study, we aimed to investigate the effects of different bedding materials such as perlite, corncob, and wood shavings on skin of rats using histopathological examination, the expression of the Interleukin-1 beta (IL-1β), and tumor necrosis factor-α (TNF-α) immunohistochemically, and evaluating the apoptosis with terminal deoxynucleotidyl transferase-mediated (TdT) dUTP nick end labeling (TUNEL) method. In this study, 24 male Sprague-Dawley rats, randomly allocated into three groups consisting of eight animals in each cage, and wood shavings, perlite, andas bedding materials were used. The bedding material in each cage was changed twice a week during a period of 30 days. At the end of the experiment, all rats were sacrificed, and the skin samples taken from foot, abdomen, neck regions of all animals were collected for histopathological, immunohistochemical, and apoptotic evaluation. When skin tissues of all the groups were examined histopathologically, allergic contact dermatitis was observed. Dermatitis was the more severe in perlite group as compared with other groups. All group rats showed immunopositivity for IL-1β and TNF-α expressions in the, epidermal, inflammatory cells, and epithelial cells of the skin glands. Apoptosis was also observed in these cells. These data suggested that perlite bedding material use in labaratory animals housing should be limited, wood shavings and corncob use as bedding materials should be encouraged especially in rat hausing.
Keywords: Apoptosis, Bedding Material, Cytokine, Rat.

INTRODUCTION

Bedding is an essential component of rodent housing, directly having an effect on the health and wellbeing of laboratory animals [1]. Several bedding materials exist, including hardwood chips, wood shavings, contact paper, corncob, and perlite. Each of them has advantages and disadvantages. The quality of the bedding material is dependent on its moisture, microbial, traumatic and aeration components [1,2]. Recent studies have reported that the selection of a good bedding material for laboratory animals are of great importance in the health of the animals [3,4]. Perlite, an amorphous volcanic glass, is used to absorb moisture and to reduce bacteria and hazardous gases consisting of carbon dioxide and ammonia derived from urine and feces [5]. Despite these benefits of perlite, recent studies have shown that it has some toxicities to laboratory animals [5-7] and animal caretakers and researchers [5,8]. Although the corncob is used as bedding material in experimental animals due to its dust-free, non-allergic and low ammonia level, its resting surface is hard for experimental animals [9-13]. Wood shavings are cheap and, consisting of dust and small pieces of wood [1]. However, it has been revealed that it has some adverse effects such as allergic reactions in current studies [14].

Cytokines are small proteins produced by a broad range of cells, including skin epithelia, endothelial cells, macrophages, lymphocytes, mast cells and fibroblasts. IL-1β and TNF-α are proinflammatory cytokines playing important roles in immunity, inflammation, and apoptosis [15,16]. Apoptosis, programmed cell death, is controlled physiologically and genetically and step-wise series of reactions in response to external and internal stimuli [15].

To the best of our knowledge, there is no study reporting the role of cytokines and apoptosis in the effect of bedding materials used in rats on the skin. Therefore, in this study, we aimed to investigate the effects of following bedding materials; perlite, corncob, and wood shavings on skin inflammation in rats, specifically by skin histopathology, IL-1β and TNF-α expression analyses, and TUNEL method.

MATERIALS AND METHODS

Animals and experimental design

The experiments were performed according to the ethical conditions confirmed by the Ethics Committee of Experimental Animal Teaching and Researcher Center, Ataturk University, Erzurum, Turkey (Decision No: 2013:256). The study was carried out on 24 male, 65± 5 g one-month Sprague-Dawley rats. The rats were housed in a standard environment with a room temperature of 21±2 °C, a humidity level of 50-70% and 12/12 hours of a light-dark cycle. Before starting the experimental procedure, the rats were acclimated to our facilities for one week. Standard rat chow and tap water were provided ad libitum. The rats used in the experiment were randomly divided into three groups of eight animals. Group Control: The group used wood shavings as bedding material. It was accepted as a control group. Group Corncob: The group used corncob as bedding materials. Group Perlite: The group used perlite as bedding materials. The bedding material in each cage was renewed twice a week during a period of 30 days. At the end of the experiment, after all of the rats had been sacrificed under thiopental sodium (5 mg/kg) anesthesia by cervical dislocation, we conducted complete necropsies on the rats. The skin samples taken from foot pad, abdomen, neck regions of all animals were collected for histopathological, immunohistochemical, and apoptotic evaluation.

Bedding materials

Three types of bedding materials were utilized in this study: expanded perlite, (Kaleblokbims®, Erzurum, Turkey) corncob (M.B.D. Food Company, Kocaeli, Turkey), and wood shavings (ATADEM, Erzurum, Turkey).

Histopathological examination

The skin tissue samples (foot pad, abdominal and neck) were fixed in 10% buffered formalin and routinely processed for histological examination by embedding in paraffin wax. Tissue sections were cut 4 µm in thickness and stained with Haematoxylin-Eosin and examined under a light microscope [17].

Immunohistochemical examinations

After the deparafinizing process, antigen retrieval (pH 6.0) was applied in the microwave for 15 min. Then the sections were incubated in 3% H_2O_2 for 10 min to prevent endogenous peroxidase activity. The sections washed with phosphate buffered saline (PBS) were incubated with polyclonal rabbit IL-1β antibody (Catalog No. ab9722, dilution 1/200; Abcam, UK) and rabbit TNF- α antibody (Catalog No: NBP1-19532, dilution 1/200; Novus Biologicals) at room temperature for 30 min. Sections rewashed with PBS were stained with expose mouse and rabbit specific horse radish peroxidase/diaminobenzidine (HRP/DAB) detection IHC kit (Catalog No: ab80436, Abcam, UK) as recommended by the manufacturer. 3,3-diaminobenzidine (Dako Cytomation) was used as the chromogen. Slices which were passed through alcohol xylol series following counterstaining with Mayer's hematoxylin were examined under light microscope [18].

Detection of apoptotic cells

Apoptotic cells were detected by Terminal deoxynucleotidyl transferase-mediated (TdT) dUTP nick end labeling (TUNEL) stain using a commercial ready-to-use kit (In Situ Cell Death Detection Kit, POD, Roche, Mannheim, Germany). Briefly, paraffin sections were mounted on silanized slides. After deparaffinization and rehydration, sections were digested with proteinase K (20 µg/ml, 30 min) and quenched with 3% hydrogen peroxide in methanol. Sections were incubated in a humidified chamber in 200 µl of TUNEL (TdT and label solution) at 37 °C for 60 min and with conjugated with peroxidase (POD) converter at 37 °C for 30 min. The sections were then treated with Diaminobenzidine (DAB) as a chromogen for 5 min, washed with PBS and counterstained with Mayer's hematoxylin (Dako Cytomation) [18].

Image Analysis

Tissue sections were evaluated by high-power light microscopic examination using an Olympus Bx51 with a DP72 camera system. Each specimen was examined in 10 randomly selected areas of approximately an X20 objective. Histopathological and immunohistochemical all slides were evaluated by two independent pathologists (KATK and SA) in a blinded fashion. In the cases of disagreement**,** the slide was re-evaluated. The scores were derived semi-quantitatively using light microscopy on the preparations from each rat and were reported as follows: Grade 0 = – (negative); Grade 1 = +1 (mild); Grade 2 = +2 (moderate); Grade 3 = +3 (severe); Grade 4 = +4 (most severe) [18,19].

STATİSTİCAL ANALYSİS

Histopathological and immunohistochemical findings were carried out using SPSS statistical software (SPSS for Windows, version 20.0). All data were presented in mean (±) standard error. The differences in the measured parameters among the three groups were analyzed with a nonparametric test (Kruskal–Wallis). Dual comparisons among groups exhibiting significant values were evaluated with a Mann-Whitney U-test. $P<0.05$ value was accepted to be significant [18].

RESULTS

Bedding material effects on moisture percentage

The moisture percentages of perlite were lower than those of corncob and wood shavings throughout the study. These results were reported in our previous study [7].

Macroscopic findings

There was no significant difference ($p > 0.05$) between the body weights of the animals in all groups at the end of the experimental duration according to the data obtained when the experiment ended. Rats in all experimental groups, in particular perlite group, macroscopically, erythema, edema, swelling, reddening at the skin of the foot pad were observed. These findings seen in the foot pad were observed very mildly in skin of the abdominal and neck regions. At the same time, these lesions were accompanied by an itching that began on the skin of foot pad and then spread other regions of the skin. (Figures. 1A-B).

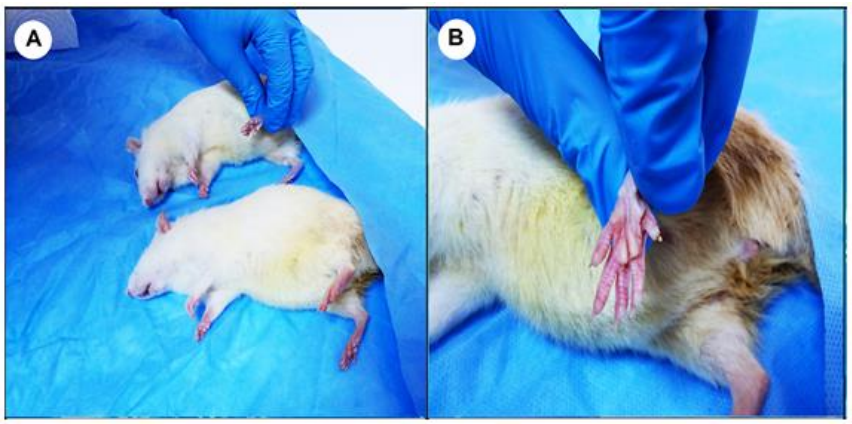

Fig-1: A-B. Hyperemia on the skin of foot pat

Histopathological findings

Acanthosis and spongiosis in the epidermis were observed when the skins of all groups were examined histopathologically. In addition to edema in the dermis, mononuclear cells infiltration consisting of a large number of lymphocytes as well as mast cells were noticed (Table 1). Moreover, hyperplasia was observed in the sebaceous and sweat glands. Rats in all experimental groups demonstrated similar lesions except hyperkeratosis that was observed in perlite group. The spongiosis and inflammatory changes were more severe in perlite group (Figure 2A), whereas these lesions were the mildest in control (Fig. 2B) and corncob (Fig. 2C) groups, respectively. No differences (Tables 1, 2) were observed between corncob and control groups in terms of the severity of the lesions ($p<0.05$). These lesions, which were observed histopathologically at the skin of foot pad, were very lightly shaped in skin of the neck and abdomen regions.

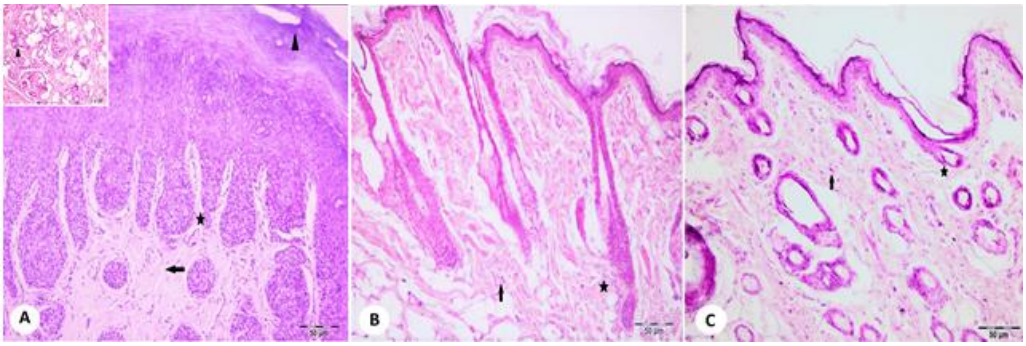

Fig-2: Hyperkeratosis (arrow head), Acanthosis and spongiosis in the epidermis (star), lymphocytes and mast cells in the dermis (arrow) A. Severe (Perlite group),

B. Weak (Control group), **C.** Weak (Corncob group) HE., Bar: 50 µm.

Above small plate: Hyperplasia in the skin glands (arrow head) (Perlite group), HE., Bar: 20 µm.

Immunohistochemical findings

All groups showed immunopositivity for IL-1β and TNF-α expressions in the epidermal cells, inflammatory cells (lymphocytes, macrophages, fibroblast, fibrocytes, and mast cells) and epithelial cells of the hair follicles, sebaceous and sweat glands. When groups were compared with each other, the expression of IL-1β and TNF-α were observed to be the highest in perlite group (Figures. 3A-B) and the mildest in control (Figures 5 A-B) and corncob (Figures 4 A-B) groups, respectively ($p<0.05$). Statistically, no difference was found between corncob and control groups (Table 3).

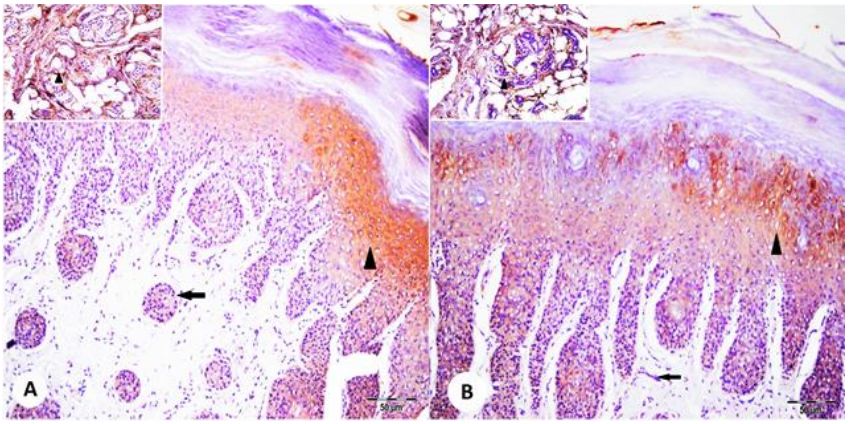

Fig-3: Indirect immunoperoxidase staining; intense cytokines expression in the keratinocyte (arrow head), inflammatory cells (arrow), (Perlite group), A. IL-1β, B. TNF-α, Bar: 50 µm. Above small plate: Epithelial cells of the skin glands (arrow head) Bar: 20 µm

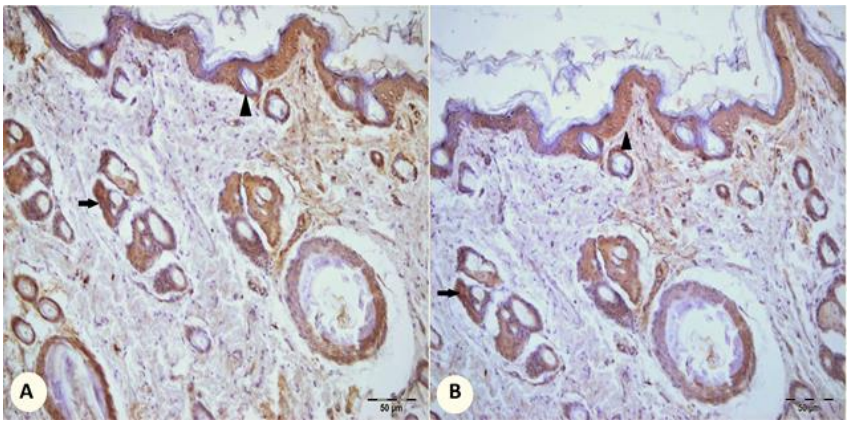

Fig-4: Indirect immunoperoxidase staining; mild cytokines expression in the keratinocyte (arow head), epithelial cells of the hair and the sebaceous and sweat glands (arrow) (Control group), A. IL-1β, B. TNF-α, Bar: 50 µm

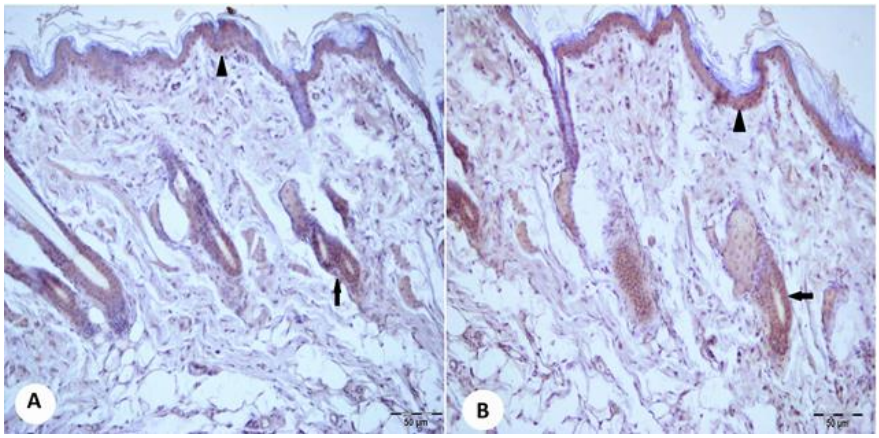

Fig-5: Indirect immunoperoxidase staining; mild cytokines expression in the keratinocyte (arow head), epithelial cells of the hair and the sebaceous and sweat glands (arrow) (Corncob group), A. IL-1β, B. TNF-α, Bar: 50 µm

Evaluation of apoptotic cells by TUNEL method

The apoptosis was observed in the epidermal cells, inflammation cells and epithelial cells of the glands. While the number of apoptotic cells was high in perlite group (Figure 6A), it was lower in control (Figure 6B) and corncob (Figure 6C) groups, respectively ($p<0.05$). However, no statistically significant difference was found between corncob and control groups (Table 3).

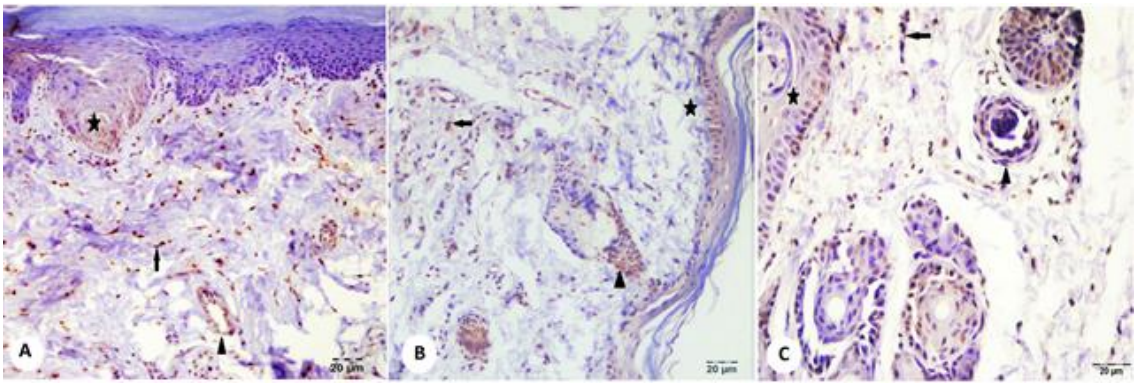

Fig-6: Tunel test; apoptosis in the keratinocyte (star), lymphocytes and mast cells (arrow), epithelial cells of the hair and the sebaceous and sweat glands (arrow head), A. Intensive (Perlite group), B. Mild (Control group), D. Mild (Corncob group), Bar: 20 µm

Table-1: The scoring values of histopathological features of skin lesions

Group	Acanthosis	Spongiosis	Lymphocytes	Mast cell
Perlite n:8	2/8 (++++) 5/8 (+++) 1/8 (++)	2/8 (++++) 4/8 (+++) 2/8 (++)	1/8 (++++) 5/8 (+++) 2/8 (++)	1/8 (++++) 4/8 (+++) 3/8 (++)
Corncob n:8	2/8 (++) 4/8 (+) 2/8 (-)	2/8 (++) 4/8 (+) 2/8 (-)	3/8 (++) 4/8 (+) 1/8 (-)	2/8 (++) 4/8 (+) 2/8 (-)
Control n:8	2/8 (++) 3/8 (+) 3/8 (-)	2/8 (++) 3/8 (+) 3/8 (-)	2/8 (++) 4/8 (+) 2/8 (-)	1/8 (++) 4/8 (+) 3/8 (-)

Table-2: Analysis of histopathological changes for each group

Bedding materials	Acanthosis	Spongiosis	Inflammation
Corncob	1,50±0,22[a]	1,66±0,33[a]	1,50±0,22[a]
Perlite	2,50±0,22[b]	3,33±0,42[b]	1,66±0,21[a]
Wood shavings	1,50±0,22[a]	1,83±0,30[a]	0,33±0,21[b]
Significance	(p<0.05)	(p<0.05)	(p<0.05)

The different letters (a,b) in the same column indicate differences between the groups (P<0.05)

Table-3: The relationship between expression of IL-1β, TNF-α, and apoptosis according to the groups

Bedding materials	Apoptosis	IL-1β	TNF-α
Corncob	1,50±0,22[a]	1,33±0,21[a]	1,32±0,21[a]
Perlite	2,52±0,22[b]	2,50±0,22[b]	2,52±0,22[b]
Wood shavings	1,50±0,22[a]	1,35±0,21[a]	1,38±0,21[a]
Significance	(p<0.05)	(p<0.05)	(p<0.05)

The different letters (a,b) in the same column indicate differences between the groups (P<0.05)

DISCUSSION

Laboratory animals should be confined under the optimum physical, physiological and psychological conditions [1]. Choosing the bedding material, one of the significant components of captivity conditions, is very important in terms of both animals and staff health [2-4]. Perlite has low thermal conductivity, low density, and a high surface area, it is used as a bedding material in animals and also used for various industrial purposes [5]. Although perlite has many advantages, it has been reported that it can have hazardous effects on both animals [5-7], and humans [5,8] health. Corncob as a bedding material is commonly preferred due to its many advantages [9]. However, it has been stated that it induces stress and chronic pain, and therefore disrupts sleep patterns due to its hard surfaces. Some studies reported its adverse effects such as inhibiting endocrine functions, and inducing irritation in the respiratory system [10,13], either. Wood shavings is preferred as a bedding material due to its low cost [1]. However, it has been reported that it causes allergic reactions and even cancer in the human respiratory system [1,14].

In the study, which we did previously, biochemical, microbiological and histopathological effects of different bedding materials in both male and

female rats were evaluated. In our study mentioned, both the wood shavings and corncob groups showed mild inflammatory changes, while perlite group showed more severe lesions [7].

In this study, histopathological changes observed in the skin were consistent with earlier ones. Although all of the three bedding materials caused contact dermatitis in the present study, this was more prominent in perlite group.

Contact dermatitis is defined as a reactive eczematous inflammation of the skin that takes place after direct contact with a chemical but occasionally due to biological or physical agents. The severity of the contact dermatitis depends on the type, concentration and contact time of the substance with the skin. According to the pathophysiological mechanisms contact dermatitis is defined in two different ways: irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD). Although their clinical appearances are similar, their pathophysiological mechanisms are different from each other. Although it is diffucult to distinguish between ACD and ICD, previous studies have reported that both macroscopic and microscopic distinctions can be made. It has been reported that lesions accompanied by erosions and ulcers is mostly localized only in one region of the skin in ICD while redness, edema, swelling and itching are macroscopically observed in different regions of skin in ACD [20,21].

In our study, skin lesions were macroscopically observed in the foot pad, abdomen, and neck in animals, and the absence of erosions and ulcers in these lesions were consistent with findings of ACD. The itch finding that supports ACD was also present in our study.

Previous histopathological studies have reported that ICD brings about the erosions, ulcers, and acantholisis in epidermis and inflammatory reactions in dermis while ACD leads to acanthosis and spongiosis in epidermis and inflammation consisting largely of lymphocytes and mast cells in dermis [22]. In our study, edema in epidermis and dermis, acantosis and spongiosis in the epidermis pointed to ACD. At the same time, previous studies have focused on the presence of mast cells in inflammatory reactions in allergic contact dermatitis, unlike ICD. In our study, the presence of inflammatory changes in the dermis, consisting of lymphocytes and mast cells, coincided with findings of ACD reported in the literature.

Laboratory animals can not be housed without bedding materials. Therefore we don't have a control group without any bedding materials. However the group using wood shavings bedding materials can be accepted as the control group. Because wood shavings bedding material has been used routinly for a long time in housing the experimental animals [1]. In our study, allergic contact dermatitis was observed in all groups, but it was the most severe in the perlite group.

When irritant agents, such as physical and chemical agents, make contact with the skin, they initiate a distinct inflammatory process [20-22]. They lead to tissue damage and the expression of various cytokines. Cytokines are the primary mediators of this inflammatory response to contact dermatitis. They play important roles in immune and inflammatory responses to infection and tissue injury. IL-1β and TNF-α are involved in this process and they are produced during the early cellular response to inflammatory stimuli. Both cytokines are expressed by fibroblasts, keratinocytes, Langerhans cells, lymphocytes, neutrophils, endothelial, macrophages and mast cells [23, 25]. A few studies have been carried out on the release of cytokines in different skin diseases in laboratory animals. In these studies, it has been observed that the increase in the amount of cytokines, including TNF-α [26], IL-31 [27], IL-4, IL-13 [28], and IL-18 [29] is proportional to the severity of lesions occurring on the skin.

In the present study, expressions of IL-1β and TNF-α in epithelial gland cells, inflammatory cells, epidermal cells were observed in all groups. In perlite group which severe dermatitis was observed, the level of expression of IL-1β, TNF-α were the highest. The levels of expressions of IL-1β and TNF-α were the lowest in corncob and control groups having mild dermatitis. No statistical difference was found between control and corncob groups in terms of the expression of these cytokines and also dermatitis. The data obtained from this study supported that the increases in the expressions of IL-1β and TNF-α were in proportion to the severity of inflammation as reported in other studies associated with skin.

It has been reported that mast cells and lymphocytes, play an important role in allergic contact dermatitis and causes release of various proinflammatory cytokines such as IL-1β and TNF-α [20-22,30].

In our study, the presence of mast cells and lymphocytes in the inflammation and the release of IL-1β and TNF-α in these cells coincided with the findings of allergic contact dermatitis. There is a synergetic interaction between IL-1β and TNF-α. When one increases, the other increases or when one decreases, the other decreases [24,31]. There was no statistically significant difference in the relationship between IL-1β and TNF-α in each group.

Apoptosis, which is characterized by programmed cell death, is responsible for the deletion of cells. Apoptosis occurs in the skin physiologically, at the same time, It plays a critical role in the pathogenesis of many skin diseases. Pathological apoptosis can be

triggered by a wide variety of stimuli such as cytokines, hormones, drugs, and viruses. The presence of apoptosis has been immunohistochemically demonstrated especially in the spongiosis and its around in epidermis, epithelial cells of the skin glands, and inflammatory cells [15,16]. Our findings were in consistent with the other studies in the literature. In addition, apoptosis in the perlit group was more severe than the corncob and control groups.

IL-1β and TNF-α are significant cellular mediators that are involved in various cellular responses including proliferation, differentiation**,** and apoptosis. In previous studies, it was stated that there was an increase in the number of apoptotic cells as the expression of IL-1β and TNF-α increased (15,16). In recent years, preventing apoptosis by reducing the secretion of cytokines such as IL-1β and TNF-α has been preferred in the treatment of some skin diseases [24,25,31].

In this study, it was observed that the increase in the number of apoptotic cells accompanied the increase in the release of IL-1β and TNF-α. This study indicated that perlite used as bedding materials for rats led to allergic contact dermatitis and increased expression of IL-1β and TNF-α and also the number of apoptotic cells.

The choice of a good bedding material is of a great importance for laboratory animals to live healthily. The choice of a good bedding material, which has the least negative impact on health, is of utmost importance for the yield of experimental animals. This study is the first report showing that apoptosis can be connected with the release of IL-1β and TNF-α in allergic contact dermatitis caused by different bedding materials in rats by using histopathological, immunohistochemical and TUNEL methods.

In conclusion, perlite bedding material use in labaratory animals housing should be limited, wood shaving and corncob use as bedding materials should be encouraged especially in rat hausing. We believe that these results will shed light on the subject for future studies to cure or reduce of side effects that may result from using of bedding materials.

Conflict of interest

The authors declare that they have no competing interests.

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