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## **The Role of Progesterone in Cellular Apoptosis of Skin and Lung in a Bleomycin-injured Mouse Model**

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### **ABSTRACT**

Systemic sclerosis is a female predominant, a fibrotic autoimmune disease in which disturbance in tissue homeostasis and cell turnover including cell apoptosis are central events in pathogenesis. Sex hormones are known as the important players in sexual dimorphism of autoimmune diseases, and in tissue homeostasis. Progesterone influences autoimmune disease via its immunomodulatory effect or by its direct action on parenchymal cell function. On the other hand, this hormone impacts tissue homeostasis by acting on cell apoptosis in a different situation. The objective of this study was to examine the effect of progesterone on cellular apoptosis of skin and lung tissues in a mouse model of scleroderma.

Four groups of mice were involved in this study with 10 mice in each. The fibrotic model was induced by daily subcutaneous injection of bleomycin for 28 days. One week after initiation of fibrosis induction, mice received subcutaneous progesterone alone or with bleomycin for 21 days. Control group received only Phosphate buffered saline PBS. After 28 days, under lethal anesthesia skin and lung tissues were harvested for histological assessment and hydroxyproline measurement. Apoptosis in tissue sections was detected by TUNEL assay technique.

Bleomycin administration induced fibrosis in skin and lung tissues. Severe apoptosis was seen in skin and lung tissues of the bleomycin-treated group ( $p < 0.001$  in the skin and  $p < 0.05$  in the lung). Progesterone injection either in the skin ( $p > 0.05$ ) or in the lung ( $p > 0.05$ ) did not alter apoptosis in bleomycin-treated animals.

Our data confirm the role of apoptosis in the pathogenesis of fibrosis in this model; however, progesterone does not affect cellular apoptosis in skin and lung tissues of bleomycin-injured animals.

**Keywords:** Apoptosis; Bleomycin; Progesterone; Systemic sclerosis

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## INTRODUCTION

Systemic sclerosis (SSc) is a chronic connective tissue disorder with immunological abnormalities, microvascular injury and progressive fibrosis of the skin and internal organs.<sup>1</sup> Progressive fibrosis compromises organ functions through the continuous accumulation of extracellular matrix (ECM) components, especially collagen, and the replacement of normal tissue architecture with permanent scar tissue.<sup>1,2</sup> The exact etiology of the disease is unknown. B cell, T cell dysregulation and production of autoantibodies especially anti-endothelial cell antibodies (AECA) and anti-nuclear antibodies are important events in the pathogenesis of systemic sclerosis (SSc). Anti-endothelial cell antibodies cause endothelial cell apoptosis via Fas-mediated cell cytotoxicity. Endothelial and epithelial cell apoptosis initiate fibrosis by activation of innate and adaptive immune responses.<sup>3</sup> Activation of immune system results in activation of alternatively-activated of M2-type macrophages, Th2 cells, production of soluble mediators and trans-differentiation of fibroblasts to myofibroblasts.<sup>4</sup> Then TGF- $\beta$ , in the downstream of Th2 polarization and IL-13 production, enhances apoptosis in alveolar epithelial cells and promotes the function of myofibroblasts, therefore aggravates the vicious cycle of cell death, inflammation and fibrosis.<sup>5</sup>

Scleroderma is a female-predominant disease.<sup>6</sup> This emphasizes the effect of female hormones on the risk and development of the disease.<sup>7</sup> Female hormones influence autoimmune disease by their immunomodulatory effects and contribute to immune dimorphism.<sup>8</sup> On the other hand, these hormones impact tissue homeostasis by acting on cellular turnover, thus influencing organs in health and diseases.<sup>9-12</sup>

Most of our knowledge concerning the action of hormones in disease stem from studies scrutinized estrogens, and a few data exist about the action of progesterone (P) as a major hormone of luteal phase and pregnancy. Progesterone by induction of Th2 immune responses and polarization of M2-type macrophages affects the autoimmune disorders.<sup>13,14</sup> On the other hand Progesterone causes cell proliferation and is an anti-apoptotic agent in most organs.<sup>9,10,12</sup> Excessive apoptosis in ischemia-associated injuries of the cardiomyocytes and neuronal cells was inhibited by injection of progesterone in animal models.<sup>15,16</sup>

Collectively, based on the role of autoimmunity, ischemia, and apoptosis in the pathogenesis of systemic sclerosis and effect of progesterone on the immune system and ischemia-induced apoptosis, we thought to investigate the role of progesterone on cellular apoptosis in a murine model of systemic sclerosis.

Subcutaneous injection of bleomycin for 4 weeks described by Yamamoto et al, induces a mouse model of scleroderma.<sup>17</sup> In this model, skin fibrosis is accompanied by lung fibrosis, inflammation, and production of autoantibodies. Th2 cytokine profile is predominant in sera of mice and TGF- $\beta$  as a central mediator in fibrosis increased in affected tissues. These finding all are very similar to human systemic sclerosis including autoimmunity, vascular dysfunction, and organ fibrosis.<sup>17</sup>

## MATERIALS AND METHODS

### Animal Model of Systemic Sclerosis and Progesterone Treatment

Six to eight weeks old female BALB/c mice were purchased from Pasteur Institute (Tehran, Iran) and kept at "Center of Experimental and Comparative Studies". Mice were allowed to acclimate to the laboratory conditions for at least 2 weeks prior to the beginning of the experiments. Mice were housed in the standard laboratory cages at room temperature and ambient humidity under a 12-hour reverse light-dark cycle with water and rodent laboratory chow, ad libitum. All animal experiments were approved by the Animal Care Committee of Iran University of Medical Sciences (N.: IR. IUMS.FMD.REC1394.25723). Mice were randomly divided into the 4 following groups with 10 mice in each group: 1) Control ((Phosphate buffered saline group, PBS) PBS), 2) Bleomycin (BLM), 3) Bleomycin+Progesterone (BLM+P), 4) Progesterone for 21 days (P).

Induction of SSc in mice was performed according to Yamamoto et al.<sup>17</sup> Bleomycin (BLM) (Nippon Kayaku, Tokyo, Japan) was dissolved in PBS at a concentration of 1.5 U/mL and 100  $\mu$ L was injected subcutaneously daily for 4 weeks into the shaved back skin of mice. In order to study the effect of progesterone, mice received daily 1 mg/body Progesterone<sup>18</sup> (dissolved in 40  $\mu$ L sesame oil) subcutaneously for 21 days (started on day 8 in addition to bleomycin or alone) in (BLM+P) or (P) respectively. The injection of bleomycin and

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progesterone were performed using a 30 and 29 gauge needle respectively in separate areas of mice back skin.

Mice from Control group (PBS) received daily 100  $\mu$ L PBS subcutaneously in the same position with bleomycin.

### Tissue Harvesting and Histological Evaluation

Under deep anesthesia, the chest was opened and lungs were removed. The skin in bleomycin or PBS injection site was excised. The left lungs and half of the excised injured skins were fixed overnight in 10% neutral formalin buffered. Paraffin-embedded lung and skin sections of 4 $\mu$ m were stained with hematoxylin and eosin for morphological evaluation and with Masson's trichrome to assess the degree of fibrosis. Quantification of pulmonary fibrosis was determined as described by Jiang et al.<sup>19</sup> In brief, trichrome stained slides were examined under a light microscope at 200X magnification (Olympus BX50, Japan). For each specimen, 10 random fields were selected and images were taken (Olympus DP12, Japan) then with image j software pixels of fibrotic and total area were measured and summed. Finally, the percentage of fibrotic area per each lung was calculated. The dermis thickness was defined as the distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction.<sup>20</sup> After image acquisition from trichrome sections under the light microscope at 100X magnification, the thickness was measured by ImageJ software in 5 different skin field of each specimen. Tissue microscopic evaluations were performed by two independent observers.

### Hydroxyproline Assay

Collagen deposition was estimated by measurement of hydroxyproline. After tissue excision, two upper lobes from right lungs and the other half of the skins immediately were snap frozen and stored at -80°C until used for hydroxyproline assay. Hydroxyproline was measured with hydroxyproline assay kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer protocol.

### Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling (TUNEL) Assay

TUNEL is a method for detecting double-stranded low molecular weight DNA fragments in apoptotic cells. TdT enzyme catalyzes attachment of deoxynucleotides tagged with a marker to 3'-hydroxyl

termini of DNA double-strand breaks. TUNEL assays were performed using In Situ Cell Death Detection Kit (Roche Applied Science, 68298 Mannheim, Germany) according to the manufacturer's instructions. In brief, paraffin-embedded lung and skin tissue sections were deparaffinized and rehydrated. Permeabilisation of the samples was done with protease treatment (Proteinase K, 30 min, 37°C). Then TUNEL reaction mixture and Converter-POD were added. Finally, sections were stained with DAB (3, 3'-Diaminobenzidine tetrahydrochloride), then counterstained with hematoxylin.

### Statistics

Experiments were repeated at least twice. Statistical analyses were performed by using GraphPad Prism software. Groups greater than two were analyzed with One-Way ANOVA, tukey multiple comparisons test. All data are expressed as Mean $\pm$ SEM. Differences were considered significant when  $p < 0.05$ .

## RESULTS

### Bleomycin-induced Fibrosis in Skin and Lungs

Collagen deposition in tissues was used as a detection marker for fibrosis. Hydroxyproline and trichrome staining were applied for evaluation of collagen. In this model, bleomycin injection caused fibrosis in skin and lung. The dermal thickness of skin from BLM treated mice were approximately three times greater than Control group (276.4  $\mu$ m $\pm$ 35.51 vs. 94.63  $\mu$ m $\pm$ 8.348,  $p < 0.001$ ) (Figure 1A, B).

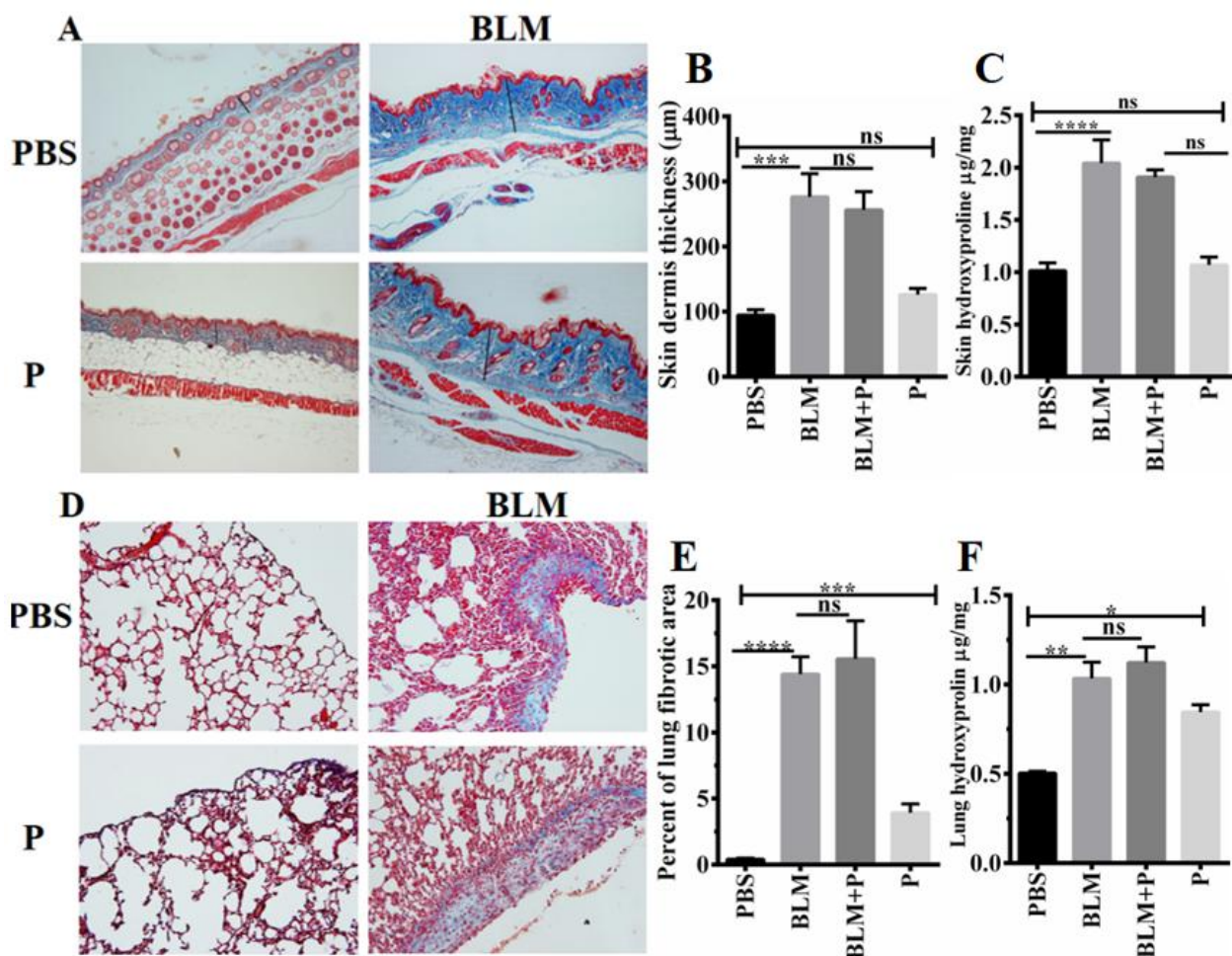
Hydroxyproline content in the skin of bleomycin-treated animals increased two-fold compared to the control group (2.043  $\mu$ g $\pm$ 0.2214 vs. 1.014  $\mu$ g $\pm$ 0.07390,  $p < 0.001$ ) (Figure 1C). Dermal thickness and hydroxyproline content of skin did not change in BLM+P group compared to bleomycin group ( $p > 0.05$ ). Progesterone injection alone did not affect the dermal thickness and hydroxyproline content compared to the PBS group ( $p > 0.05$ ) (Figure 1B, C).

Trichrome staining showed that subcutaneous injection of BLM for four weeks caused pulmonary fibrosis. The pulmonary fibrosis was started from pleura and subpleural area and extended to the lung parenchyma, however in this model central areas of the lungs were not affected (Figure 1D)

Percentage of fibrotic area in the BLM group increased significantly in comparison to the control

group ( $p < 0.0001$ ). The amount of hydroxyproline in lung tissues of mice received bleomycin increased two times as compared to the Control group ( $0.5220 \mu\text{g} \pm 0.03584$  vs.  $0.2616 \mu\text{g} \pm 0.004737$ ,  $p < 0.0001$ ) (Figure 1E, F). Progesterone injection did not change

fibrotic areas and hydroxyproline content in the lung of bleomycin-treated animals ( $p > 0.05$ ). Progesterone injection alone increased fibrotic area ( $p < 0.001$ ) and hydroxyproline content ( $p < 0.05$ ) in lung tissue compared to the control group (Figure 1D, E).



Subcutaneous injection of bleomycin caused skin and lung tissues fibrosis in mouse model of systemic sclerosis. Fibrosis induced in mice by injection of bleomycin subcutaneously for 28. After 28 days, under lethal anesthesia skin and lung tissues were harvested for histological assessment and hydroxyproline measurement. (A) Masson's trichrome staining of the skin tissues. 4 µm of paraffin- embedded skin tissue sections were stained with Masson's trichrome and examined under a light microscope. Original magnification was 100X. (B) Dermis thickness. Dermis thickness was measured from epidermal-dermal junction to dermal-subcutaneous fat junction in the skin. (C) Skin hydroxyproline content. (D) Masson's trichrome staining of the lung tissues. 4 µm of paraffin- embedded lung tissue sections were stained with Masson's trichrome and examined under a light microscope. Original magnification was 200X. (E) Percent of fibrotic area in the lung. Lung fibrosis quantitated in trichrome staining sections and presented as a percentage. (F) Lung hydroxyproline content. Data were analyzed with One-way ANOVA, Tukey multiple comparisons test. Results are presented as Mean ± SEM. N = 9-10 mice in each group. BLM = Bleomycin, P = Progesterone, PBS= Control, BLM+P = Bleomycin + Progesterone, \*\*\*\* =  $p < 0.0001$ , \*\*\* =  $p < 0.001$ , \*\* =  $p < 0.01$ , \* =  $p < 0.05$ , ns =  $p > 0.05$

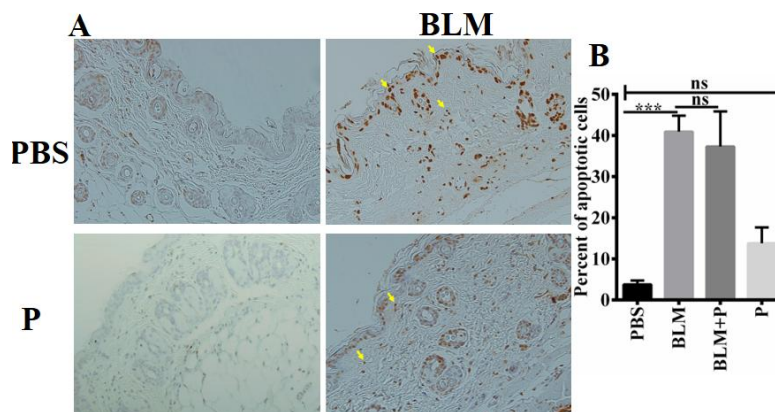
### Apoptotic Cell Count in Skin and Lung

TUNEL test was used for the detection of apoptotic cells (Figure 2A, 3A). Quantification of apoptotic cells in stained sections of lungs and layers of skins showed that bleomycin caused significant apoptosis in the skin ( $p < 0.001$ ) and lungs of mice ( $p < 0.05$ ) (Figure 2B, 3B).

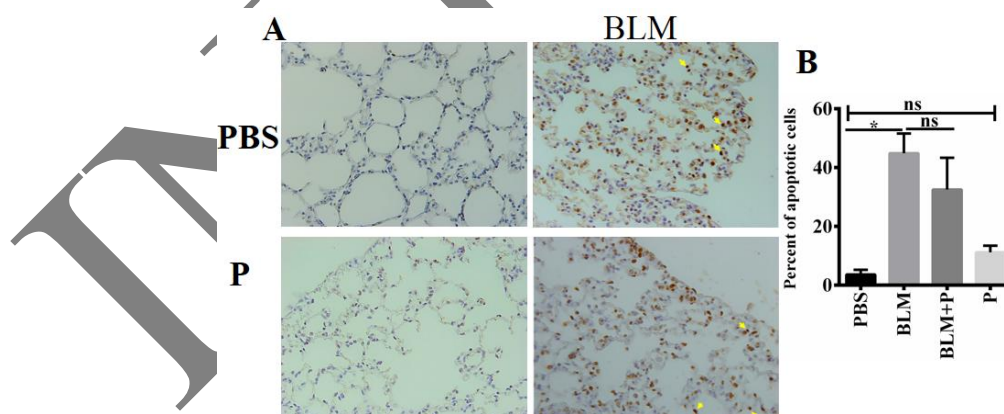
Progesterone injection in group BLM+P reduced apoptotic cells in skin compared to bleomycin group in a non-significant manner ( $37.33\% \pm 8.524$  vs

$40.93\% \pm 3.873$ ,  $p > 0.05$ ). Progesterone injection alone did not change the number of apoptotic cells in skin compared to PBS group ( $p > 0.05$ ) (Figure 2A, B)

Progesterone injection reduced the number of apoptotic cells in the lung of bleomycin-treated animals in a non-significant manner ( $32.51\% \pm 10.84$  vs  $44.8\% \pm 6.703$ ,  $p > 0.05$ ). Progesterone alone did not alter the apoptotic cells in normal lung tissue ( $p > 0.05$ ) (Figure 3A, B).



**Figure 2.** Progesterone does not affect apoptosis in skin tissues of bleomycin injured- mouse model. After induction of model and intervention of progesterone, under lethal anesthesia injured skin tissues were harvested. Paraffin-embedded skin tissue sections were deparaffinized, rehydrated and Permeabilized with Proteinase K. Then TUNEL reaction mixture and Converter-POD were added. Finally, sections were stained with DAB and counterstained with hematoxylin. (A) TUNEL staining of skin sections. (B) Percent of apoptotic cells in layers of skin. Apoptotic and normal cells in layers of skin were counted then the percent of apoptotic cells were calculated. Data were analyzed with One -way ANOVA, Tukey multiple comparisons test. Results are presented as Mean±SEM, N=5 mice in each group. Original magnification was 200X. BLM=Bleomycin, P=Progesterone, PBS=Control, BLM+P=Bleomycin+Progesterone, ns =  $p > 0.05$ , \*\*\* =  $p < 0.001$



**Figure 3.** Progesterone does not affect apoptosis in lung tissues of bleomycin injured - mouse model. After induction of model and intervention of progesterone, under lethal anesthesia lung tissues were harvested. Paraffin-embedded lung tissue sections were deparaffinized, rehydrated and Permeabilized with Proteinase K. Then TUNEL reaction mixture and Converter-POD were added. Finally, sections were stained with DAB and counterstained with hematoxylin. (A) TUNEL staining of lung tissue sections. (B) Percent of apoptotic cells in the lung. Apoptotic and normal cells in lung were counted then the percent of apoptotic cells were calculated. Data were analyzed with One -way ANOVA, Tukey multiple comparisons test. Results are presented as Mean±SEM, N= 5 mice in each group. Original magnification was 200X. BLM= Bleomycin, P=Progesterone, PBS=Control, BLM+P=Bleomycin+Progesterone, ns= $p > 0.05$ , \* =  $p < 0.05$

## DISCUSSION

Our results show that bleomycin causes massive cellular apoptosis in skin and lung tissues, however, progesterone at supra-physiological (pregnancy maintenance) concentrations exerts no effect on skin and lung tissue cell apoptosis in bleomycin-induced fibrosis.

Apoptosis is a component of normal cell turnover and important for physiological cell loss in most tissues. Several regulatory stimuli such as hormones influence programmed cell death. Different studies proposed that alteration of cell survival and death can contribute in pathologies of cancers, viral infections, autoimmunity, and neurodegenerative diseases.<sup>21,22</sup>

Systemic sclerosis is a lethal fibrotic autoimmune disease in which tissue homeostasis is disturbed. Cell damage with aberrant healing is accepted as important pathology in the initiation of disease.<sup>23</sup> Apoptosis of endothelial and epithelial cell is confirmed as a central event in the pathogenesis of skin and lung fibrosis.<sup>24-26</sup> Anti-endothelial cell antibodies in sera of SSc patients caused apoptosis of microvascular endothelial cells and leaky vessels which induced the initiation of fibrosis cascade.<sup>3,27</sup> Clinical study revealed that injury of lung epithelial cells may occur as an early event in asymptomatic patients.<sup>28</sup> Apoptosis of type 2 pneumocytes was detected in the pathogenesis of idiopathic pulmonary fibrosis.<sup>25</sup> Some studies used bleomycin for induction of fibrosis showed that lung epithelial cells are the main targets of induced injuries in the lungs. Increased ROS production by bleomycin and pursued mitochondrial leakage causes apoptosis in epithelial cells<sup>29</sup> whereas in endothelial cells bleomycin activates the extrinsic apoptotic pathway.<sup>30</sup> Therefore one of the actions from bleomycin as a fibrogenic factor could be the induction of cell death and fibrosis, as is confirmed by our data.

Our study showed that subcutaneous injection of bleomycin for 28 days caused significant apoptosis in skin and lungs according to other studies.<sup>31</sup> In this model, significant cell death in lung tissues occurred in the periphery while central areas were protected. The localization of dead cells was according to fibrotic areas in the lung, thus confirms, the cellular damage as a central event in the pathogenesis of fibrosis. Despite the constant turnover of cells through apoptosis, apoptotic cells are rarely seen under physiological conditions, even in tissues with high rates of

apoptosis.<sup>32</sup> Therefore presence of great amount of apoptotic cells in our study showed disturbance in tissue homeostasis and this may contribute in tissue fibrosis.

Since controversial results obtained from different studies, the effect of progesterone on cells apoptosis is obscure and further investigations are necessary. Progesterone acts as an anti-apoptotic agent in ovarian and endometrial cells by induction of BCL-xl but, prevents the proliferation of ovarian cancer cells.<sup>33-35</sup> Whereas progesterone with induction of anti-apoptotic agents in injured cardiomyocytes, protects women from cardiovascular disease however, progesterone can induce pregnancy-related diabetes through the induction of apoptosis in insulin-secreting cells.<sup>15, 36</sup>

Anti-apoptotic effect of progesterone causes lung cancer cells to become resistant to the action of cisplatin. Cisplatin such as bleomycin causes tumor cells to be arrested in the G2 phase of the cell cycle and mRNA synthesis was inhibited in cancerous cells. Inhibition of transcription induces apoptosis in these cells.<sup>37</sup> Despite the fact, that bleomycin and cisplatin are both chemotherapeutic agents and act similarly in cell apoptosis, our obtained results suggest that progesterone cannot alter cell death induced by bleomycin. We cannot explain this bias in action of progesterone in lung tissue; however, the behavior of cancer cells would be different than normal cells of the lung. In addition, we can suggest, progesterone could not alter the action of bleomycin in chemotherapy but this idea needs to be checked in cancerous cells.

Progesterone, in addition to its reproductive functions, has been shown to exhibit the potent neuroprotective effect.<sup>38</sup> After cortical contusion injury, the high level of progesterone in female rat reduced cerebral edema and these animals had better cognitive recovery than males. This event is through an increment of anti-apoptotic genes and decrement of pro-apoptotic genes expression in neuronal cells.<sup>16</sup>

Progesterone receptors mediate the effects of progesterone by cooperation with a range of coregulatory proteins and binding to specific target sequences in progesterone-regulated gene promoters. Scattering of Progesterone receptors, their coregulatory proteins, and targeted gene sequences, differ in various cell types and between normal cells with cancer cells. Thus this hormone action is specific for each tissue and is different in health and disease.<sup>39</sup>

In this study we did not evaluate the effect of

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progesterone on apoptosis after the induction of scleroderma, also for confirming the results, additional methods would be required.<sup>40</sup> Progesterone affects the autoimmune disease by acting on parenchymal cell function and by action on immune cells. Therefore for complete determining the effect of progesterone on systemic sclerosis, other aspects of hormone actions such as Th2 induction, M2-type macrophage polarization and anti-inflammatory effects should be investigated.<sup>14</sup>

In the present study, we demonstrate that progesterone exerts no effect on skin and lung tissue cell apoptosis in bleomycin-induced fibrosis.

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All animal works were done according to approval Number IR. IUMS.FMD.REC1394.25723

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