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# Immunohistochemical Evidence of Melatonin Protection on Lung Tissue During Chemotherapy

## Evidencia Inmunohistoquímica de la Protección de la Melatonina en el Tejido Pulmonar Durante la Quimioterapia

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**SUMMARY:** The present study investigated the possible protective effects of melatonin on Bleomycin, Cisplatin and etoposide (BEP) chemotherapy regimens using immunohistochemistry. Forty male Wistar rats were divided into four groups of ten as; group 1 as untreated control; group 2 as BEP group which received the three cycles of 21 days' regimen each of 0.5¥ dose levels ofBEP (bleomycin 0.75 mg/kg, etoposide 7.5 mg/kg and cisplatin 1.5 mg/kg). Rats in the group 3 (MEL group) received 10 mg/kg/day melatonin once daily. Group 4 received the melatonin (30 min before the BEP injections) and BEP as in groups 2. Proliferating cell nuclear antigen (PCNA) staining was used to detect cell proliferation and caspase-3, caspase-9 and Caspase-8 were detected to investigate apoptosis. PCNA immunostaining in alveolar epithelium, alveolar macrophages and bronchus was weak to moderate in BEP group. However, diffuse and strong caspase immunoreactions for caspase-3, caspase 8- and caspase-9 were detected in the bronchioles epithelium, vascular endothelium, alveolar luminal macrophages in the BEP group. PCNA and caspase immunoreactivities in MEL and Mel + BEP groups were close to the control one. The surface are in the BEP group was significantly reduced as compared to the control one (($P<0.05$). However, in MEL and Mel + BEP groups, it was close to the control value ($P>0.05$). It can be concluded that BEP regimen can affects negatively on lung tissue and melatonin inhibits lung tissue injuries during BEP chemotherapy.

**KEY WORDS: BEP chemotherapy; Stereology; Melatonin; Antioxidant; Apoptosis.**

## INTRODUCTION

Bleomycin, Cisplatin and etoposide (BEP) constitute a known and common chemotherapy regimens in treating testicular cancer and many other types of cancer (Hanna & Einhorn, 2005). However, this chemotherapy regime can result in damages and severe toxicities on vital organs such as kidneys, liver, heart, lungs, and testes (Kilarkaje *et al*., 2013; Altun *et al*., 2014). The bleomycin is concentrated in skin and lung, and these two tissues have been the major sites of bleomycin toxicity. It was reported that bleomycin can introduced pulmonary toxicity in patients with germ cells tumors (Simpson *et al*., 1998). Furthermore, individuals older than 40 years and those with smoking habits are remarkably susceptible to the bleomycin-induced pneumonitis during chemotherapy (Haugnes *et al*., 2015). The precise pathophysiological pathways of oxidants actions on the lung tissue are poorly understood (Ciencewicki *et al*., 2008).

Melatonin that is a main produced hormone of the pineal gland, has a broad-spectrum effect such as anti-apoptotic, anti-inflammatory, and anti-aging (Oxenkrug *et al*., 2001; Reiter *et al*., 2007; Ji *et al*., 2012; Chen *et al*., 2016).

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It has been well documented that melatonin can be effective for treating some pathological conditions such as cancer, neural toxicity, hepatotoxicity and metabolic disorders. Melatonin exerts its therapeutic effects via modulating endoplasmic reticulum stress and controlling autophagy and apoptosis (Fernández *et al*., 2015).

The mechanism of action of melatonin in detoxifying free radicals is independent from its membrane receptors (Tan *et al*., 2003; Reiter *et al*., 2007; Jockers *et al*., 2016). Therefore, this compound can scavenge harmful oxidants and in parallel, enhances cellular antioxidant defense (Reiter *et al*., 2001; Purohit & Rao, 2014).

In the respiratory tract, alveolar sacs are naturally exposed to reactive oxygen species (ROS) present in air. Therefore, beside the surfactant metabolism, which is a unique characteristic of the lung tissue, agents with antioxidant properties can protect the lungs against ROS-induced damages (Schmidt *et al*., 2004; Messent *et al*., 1997).

The present work was aimed to investigate whether melatonin have protective effects on lung damage caused by BEP chemotherapy. PCNA, caspase-8, caspase-9 and caspase-3 expression were assessed immunohistochemically to detect cell proliferation, DNA damage and apoptosis. The change in the surface area of the alveoli was estimated using unbiased stereological methods.

## MATERIAL AND METHOD

**Animals.** Forty Wistar rats (220-300 g weight) were used for this study. All rats were kept in a polypropylene cage with a relative humidity of 50-60 %, dark/light cycle 12:12 at 22±2 °C, with free access to water and food (standard rat pellet) in accordance with standard laboratory animal care conditions. All institutional and national standards for the animal welfare were followed in line with the protocol of Institutional Animal Care and European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethics Committee of King Khalid University, Abha, KSA (Approval no; R.G.P.2021/725).

**Experimental design.** The subjects were randomly divided into four groups, each containing 10 rats. Group 1 treated with 1ml of 0.9 % saline solution intraperitoneally and served as control group. Group 2 treated with three cycles of BEP regimen (bleomycin 0.75 mg/kg, etoposide 7.5 mg/kg and cisplatin 1.5 mg/kg) that each cycle was 21 days (3 weeks). Bleomycin was administrated on second day of each week, while cisplatin and etoposide were injected on days 1–5 of each week with a 30 min interval. Rats in the group 3 received 10 mg/kg/day melatonin once a day intraperitoneally. Group 4 received the melatonin (Sigma-Aldrich, M5250) (30 min before the BEP injections) and BEP regimen similar to the groups 2 and 3, respectively. Melatonin treatment in this group was started 7 days before the onset of BEP regimen and continued in parallel with three cycles of the BEP therapy. The total duration of the experiment was 63 days.

**Tissue sampling.** On the last day of the experiment, the animals were anesthetized with ketamine10 % (10 mg/kg) and xylazine 2 % (100 mg/kg). Whole lung tissue was removed and fixed in 10 % neutral formalin solution for one week. After routine tissue processing and blocking in paraffin, sections with 5 μm thickness were obtained from paraffin blocks using a microtome (Leica RM2125 RTS, Leica Microsystems Inc, United States), and were placed on glass slides.

**Immunohistochemistry.** For investigating apoptosis, three involved antibodies including caspase-3, caspase-8 and caspase-9 were used. Also, proliferating cell nuclear antigen (PCNA) antibody was used for showing cell proliferation.

PCNA primary antibody was used to detect cell proliferation, and caspases primary antibodies were used to detect apoptosis. For immunohistochemical protocol, all samples incubated overnight at 65 °C, deparaffinized and rehydrated. For antigen retrieval all slides incubated in tris buffer (95 °C for 20 min in EDTA- (0.4 g EDTA+ 2 g tris dissolved in 1 liter of distilled water, PH=9). Then for blocking the endogenous peroxidase, the slides were placed in 5 % BSA and incubated in 3 % $H_2O_2$ for 15 min. The slides were then incubated in Biotinylated rabbit anti-mouse IgG primary antibodies. The biotinylated secondary antibodies, (10 min at room temperature), streptavidin-horseradish peroxidase (for 10 min) and 1, 3 -diaminobenzidine (DAB) tetrahydrochloride as evasion solution (for 10 min) were used for visualizing antigenic sites. Finally, hematoxylin counterstain was used. All slides were analyzed by selecting 50 random fields/slides at X400 magnifications under a light microscope (Olympus IX71 microscope, Japan) equipped with a KEcam (KEcam Technologies, Lekki Lagos, Nigeria) and Top view software (Version 3.7). All commercial primary and secondary antibodies used are described in Table I.

The obtained tissue sections were investigated using semi-quantitative method to reveal the values of expression of the examined immunohistochemical markers. For this purpose, eight fields of view were selected randomly from each tissue slide and an H-score was determined as follows:

$$\text{H-score} = \Sigma Pi\ (i + 1)$$

Table I. The list of primary and secondary antibodies applied for immunohistochemical staining.

| Primary antibody | Company | Number | Clonality | Host | Dilution |
|---|---|---|---|---|---|
| PCNA | abcam | ab18197 | Polyclonal | Rabbit | 1:4000 |
| Caspase-3 | abcam | ab179517 | Monoclonal | Rabbit | 1:1000 |
| Caspase-8 | abcam | ab25901 | Polyclonal | Rabbit | 1:2000 |
| Caspase-9 | abcam | ab184786 | Monoclonal | Rabbit | 1:1000 |
| **Secondary antibody** | | | | | |
| PCNA | abcam | ab150077 | | Goat anti-rabbit IgG | 1:5000 |
| Caspase-3 | abcam | ab150077 | | Goat anti-rabbit IgG | 1:1000 |
| Caspase-8 | abcam | ab205718 | | Goat anti-rabbit IgG | 1:2000 |
| Caspase-9 | abcam | ab97051 | | Goat anti-rabbit IgG | 1:100000 |

In this formula, ''Pi'' was intensity of labeling. Weak staining was determined with digit 1, moderate labeling designated with digit 2 and intense labeling allocated with digit 3. Also, ''i'' was the percentage of immunopositive cells for each intensity (Gürgen *et al*., 2013).

**Stereological estimation**

A linear test probe and the following formula were applied for estimating the surface density (Sv) and surface (S) of the lung alveoli (Fig. 1):

$$S_v = \frac{2.\sum l}{\sum P.l/p}$$

$$S = S_v.V$$

Where  was the total intersections between the outer surface of the alveoli and test lines,  was sum of the points hitting the epithelium and  was the length of test lines of the probe (Howard & Reed, 2005).

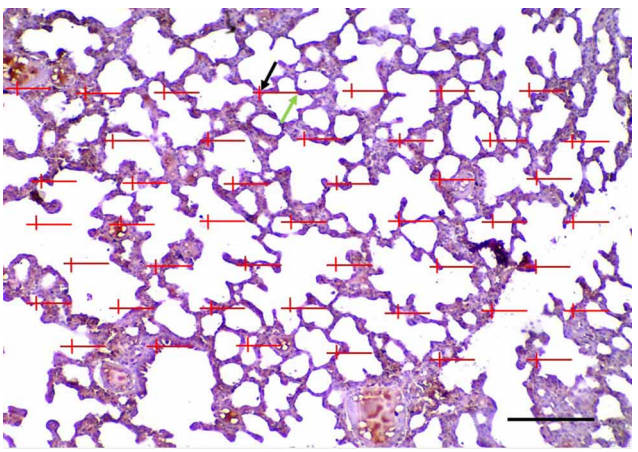

Fig. 1. Line probe for estimating the alveolar surface density. The black arrow shows a point hitting the epithelium, and the green arrow indicates the intersection of a test line with inner surface of the alveoli (scale bar =300 mm).

**Statistical analysis.** At first, the normality of data was determined using Shapiro-Wilk's test. Data are presented as means ± SD. All data were analyzed using SPSS 21.0 (SPSS Inc., Chicago, IL). The statistical differences between the examined groups were evaluated using one-way ANOVA. All statistical analysis was performed using SPSS software, V. 21.0 (SPSS Inc., Chicago, IL) and a p value ≤ 0.05 was considered statistically significant.

## RESULTS

**PCNA immunostaining.** was observed moderated in interstitial connective tissue cells in the control group (Fig. 2A). BEP group showed stronger positive PCNA staining in epithelial cells nuclei and alveolar luminal macrophages as compared to the control group (Figs. 2B-D). The PCNA immunoreactivity in MEL group was similar to that observed in the control group (Fig. 2E). Weaker staining was observed in a few numbers of interstitial cells and alveolar epithelial cells in BEP + MEL group in comparison to the BEP group (Fig. 2F, Table II).

**Caspase 3 immunostaining.** In the control group, immunopositive cells for Caspase-3 marker were limited to a small number of alveolar pneumocytes, smooth muscle cells, and vascular endothelial cells (Fig. 3A). In BEP group, immunostaining was significantly stronger in the bronchial epithelium and alveolar pneumocytes ($p < 0.05$) (Table III, Figs. 3B-D). Notably, the intensity in the MEL and BEP + MEL groups was weaker as compared to the BEP group (Figs. 3E,F), and was similar to the control .

**Caspase 8 immunostaining.** Infrequent epithelial cells in the bronchus of the control group were immunopositive for Caspase-8 marker. Also, a few numbers of alveolar pneumocytes and interstitial connective tissue were weakly positive (Fig. 4A). Considerably, heavy and intense immunostaining was detected in the lung tissue of the BEP

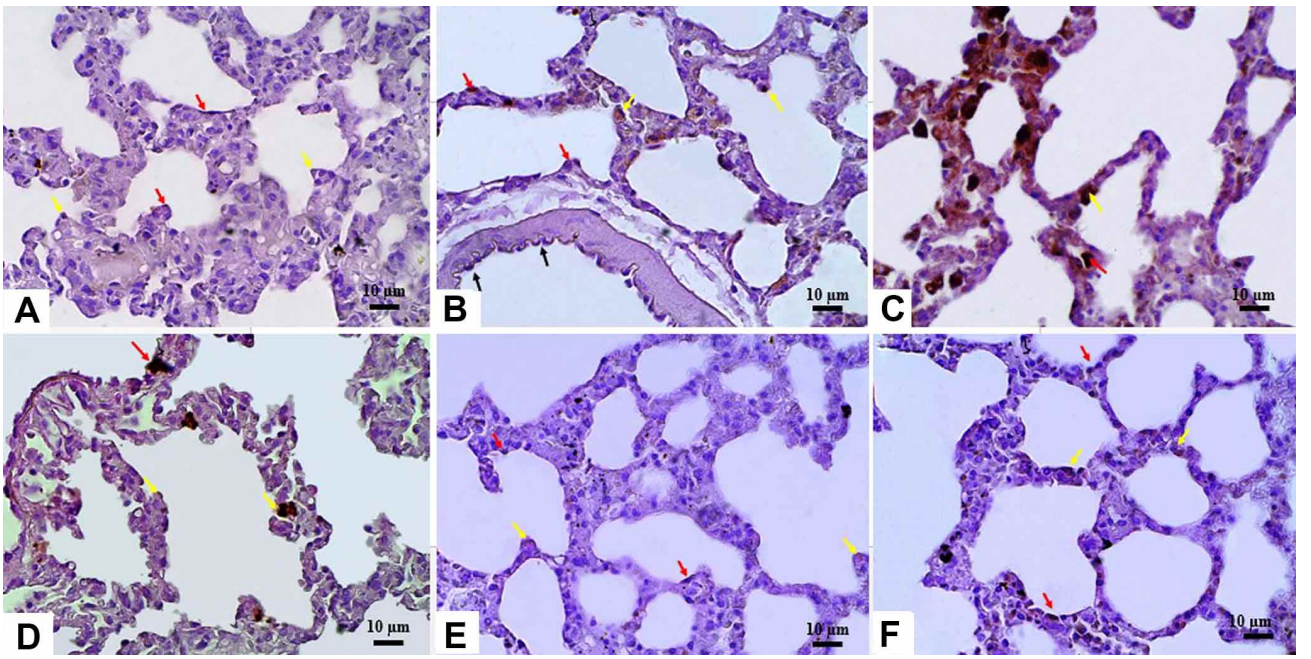


Fig. 2. PCNA immunostaining in the control group (A), BEP group (B, C and D), MEL group (E), and BEP+MEL (F). Red arrow: type 1 alveolar pneumocyte; yellow arrow: type 2 alveolar pneumocyte, black arrow: vascular endothelium (× 400).

Table II. Immunostaining of PCNA in the control and experimental groups (n=10).

| Group | Mean | SD | Max | Min |
|---|---|---|---|---|
| Control | 24.55[a] | 4.75 | 31.06 | 18.55 |
| BEP | 31.17[a] | 5.58 | 37.92 | 20.76 |
| MEL | 22.48[a] | 2.70 | 27.33 | 18.54 |
| BEP+MEL | 20.66[a] | 1.31 | 22.53 | 18.13 |

Different letters indicate significant difference between groups.

Table III. Immunostaining of Caspase-3 in the control and experimental groups (n=10).

| **Group** | **Mean** | **SD** | **Max** | **Min** |
|---|---|---|---|---|
| **Control** | 11.25[a] | 1.14 | 13.19 | 9.22 |
| **BEP** | 34.59[b] | 3.75 | 39.18 | 28.09 |
| **MEL** | 18.91[a] | 1.99 | 22.71 | 16.44 |
| **BEP+MEL** | 21.45[a] | 2.32 | 25.79 | 17.63 |

Different letters indicate significant difference between groups.

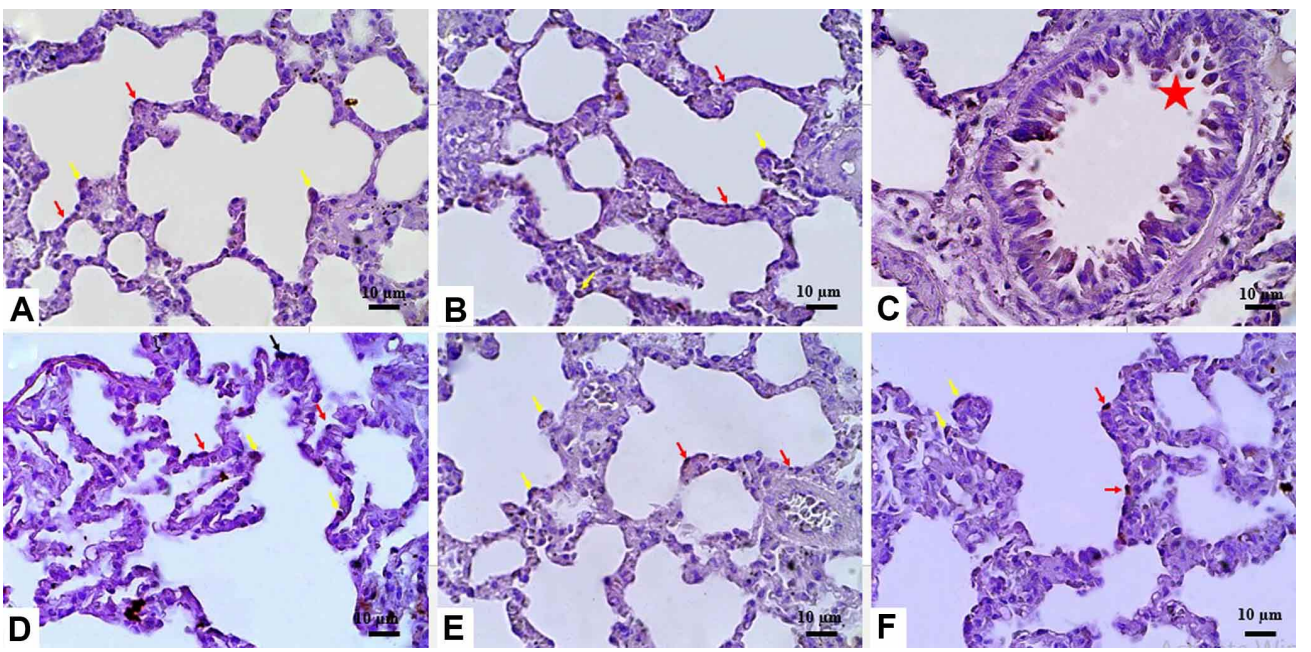


Fig. 3. Caspase- 3 immunostaining in the control group (A), BEP group (B, C and D), MEL group (E), and BEP+MEL (F). Red arrow: type 1 alveolar pneumocyte; yellow arrow: type 2 alveolar pneumocyte, red star: bronchiolus epithelial cell nucleus; black arrow: pleural cell nucleus (× 400).

group with remarkable coloration in the bronchial epithelium and alveolar pneumocytes as compared with the other experimental groups ($p<0.01$) (Figs. 4B-D). MEL group exhibited weak immunoreactivity in alveolar wall and connective tissue cells similar to the control one (Fig. 3C). Melatonin administration significantly reduced the Caspase-8 expression in the BEP + MEL group as compared to the BEP group ($p<0.01$) (Table IV, Figs. 4E,F).

Table IV. Immunostaining of Caspase-8 in the control and experimental groups (n=10).

| Group | Mean | SD | Max | Min |
|---|---|---|---|---|
| **Control** | 15.35[a] | 3.14 | 18.33 | 10.09 |
| **BEP** | 124.77[b] | 10.51 | 144.01 | 109.75 |
| **MEL** | 22.96[a] | 2.46 | 26.16 | 18.51 |
| **BEP+MEL** | 35.39[a] | 4.66 | 43.83 | 28.61 |

Different letters indicate significant difference between groups.

**Caspase 9 immunostaining.** We observed negative or weak caspase-9 immunoreactivity in the control group (Fig. 5A). However, in the BEP group, caspase-9 expression was intense and remarkable in the lung tissue components including bronchioles epithelium, vascular endothelium, alveolar luminal macrophages and alveolar pneumocytes (Figs. 5B-D). On the other hand, diminished immunostaining was observed in the MEL group and BEP + MEL group as compared to the BEP group (Figs. 5E,F). These findings indicated that melatonin could significantly reduce BEP induced caspase-9 immunostaining ($p< 0.001$) (Table V).

Quantitative evaluation revealed that after BEP regimen administration, PCNA immunostaining in the BEP group was higher than the control group, however, there was no significant difference between the all experimental groups ($p>0.05$) (Table II). The highest values for caspase-9, caspase-3, and caspase-8, were found in the BEP group. Those groups which received melatonin exhibited values close to the control one ($p>0.05$) (Fig. 6).

Table V. Immunostaining of Caspase-9 in the control and experimental groups (n=10).

| Group | Mean | SD | Max | Min |
|---|---|---|---|---|
| **Control** | 27.51[a] | 3.812 | 33.09 | 21.49 |
| **BEP** | 154.83[b] | 20.99 | 185.19 | 106.08 |
| **MEL** | 22.94[a] | 4.60 | 31.45 | 17.43 |
| **BEP+MEL** | 76.92[c] | 15.38 | 98.65 | 45.93 |

Different letters indicate significant difference between groups.

**Lung surface area.** The values of surface estimation was shown in Table VI. The surface area was decreased significantly in the BEP group as compared to the control one ($p<0.05$). However, in the experimental groups which received melatonin, alveolar surface area was close to the control value ($P>0.05$).

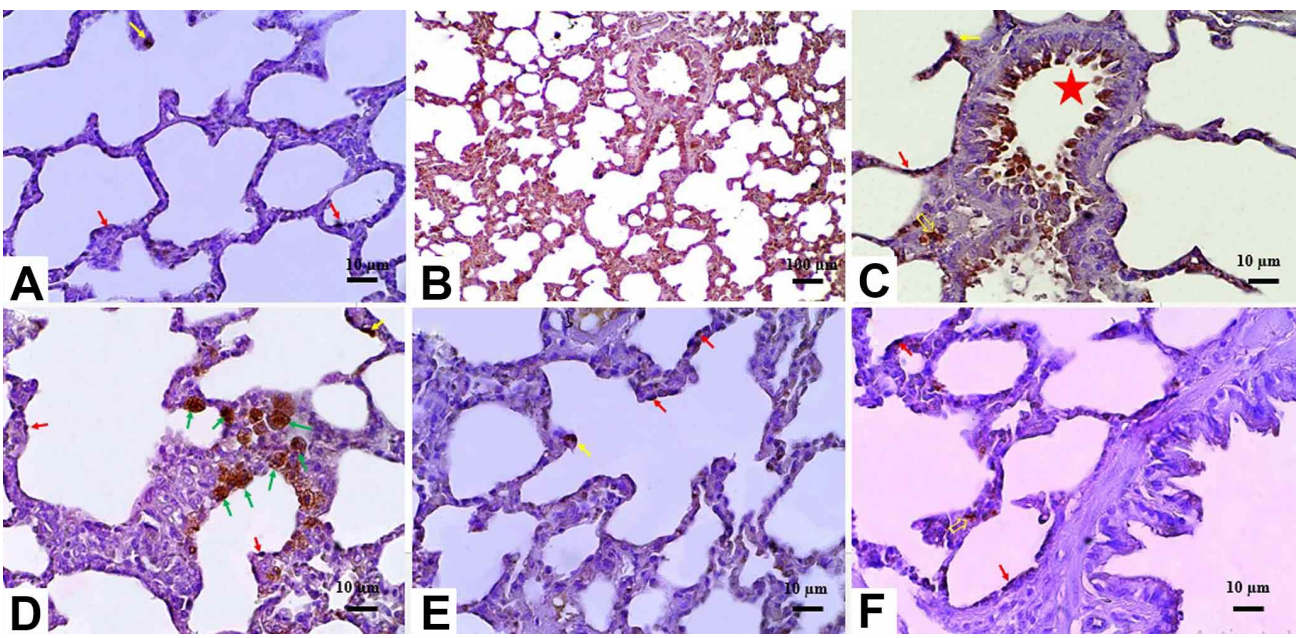


Fig. 4. Caspase- 8 immunostaining in the control group (A), BEP group (B, C and D), MEL group (E), and BEP+MEL (F). Red arrow: type 1 alveolar pneumocyte; yellow arrow: type 2 alveolar pneumocyte, red star: bronchiolus epithelial cell nucleus; green arrow: alveolar macrophage (A, C, D, E × 400, B × 100).

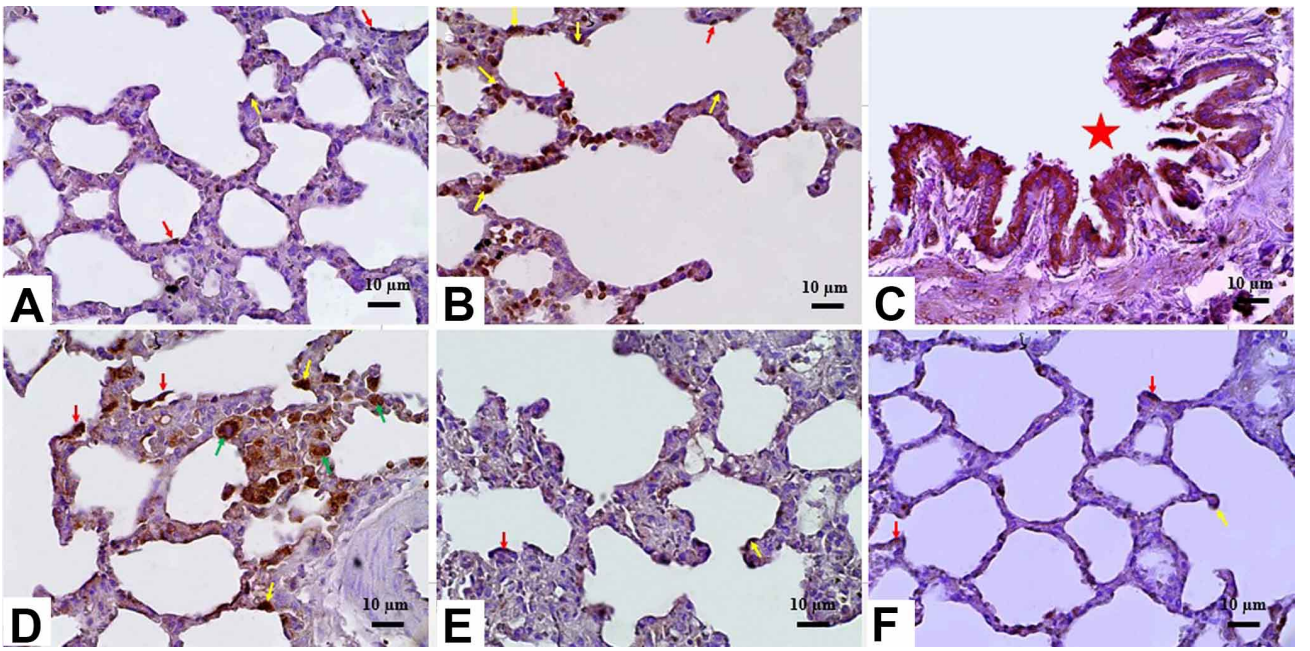


Fig. 5. Caspase- 9 immunostaining in the control group (A), BEP group (B, C and D), MEL group (E), and BEP+MEL (F). Red arrow: type 1 alveolar pneumocyte; yellow arrow: type 2 alveolar pneumocyte, red star: bronchiolus epithelial cell nucleus; green arrow: alveolar macrophage (× 400).

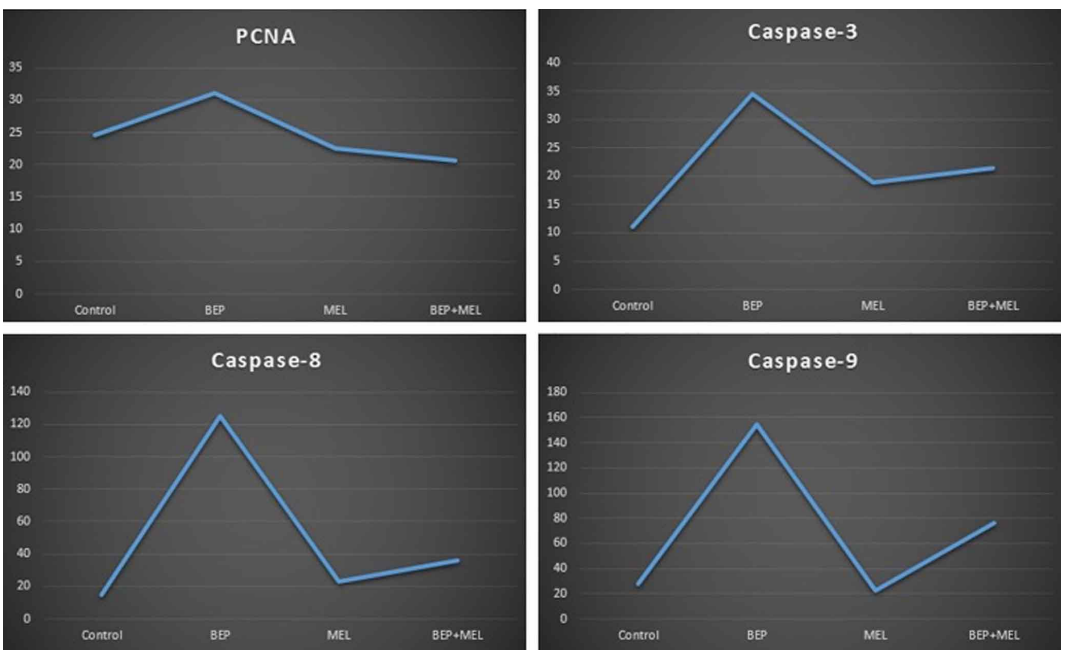


Fig. 6. Semi-quantitative analysis (H score) of the examined markers in the four experimental groups.

Table VI. Surface area / volume ($cm^2/cm^3$) unit in the control and experimental groups (n=10).

| Group | Mean | SD | Max | Min |
|---|---|---|---|---|
| **Control** | 18.08[a] | 1.31 | 20.35 | 15.29 |
| **BEP** | 8.31[b] | 1,91 | 12.83 | 5.48 |
| **MEL** | 16.56[a] | 3.38 | 22.71 | 12.57 |
| **BEP+MEL** | 15.25[a] | 2.63 | 20.79 | 11.48 |

Different letters indicate significant difference between groups.

## DISCUSSION

The results of the present work show that treatment with melatonin is protective against BEP-induced lung injury and toxicity. Three cycles of BEP chemotherapy regimen resulted to a significant increase in the expression of caspase-family proteins including caspase-3, capsase-8 and capase-

9 as well as reduction in the surface area density of the alveoli. Melatonin pretreatment inhibited of caspase-genes expression and cells apoptosis, thereby maintained the surface area density of alveoli close to the normal value.

Apoptosis the alveolar epithelial cells is highly depended on the Caspase-family proteins. For instance, the activation of caspase-9, caspase-3 and ultimately DNA damage can be initiate with mitochondrial damage in the alveolar cells. It has been reported that caspase-9 plays an important role in the development of lung cancer and variation in the caspase-9 gene can increase the risk of this disease (Park *et al*., 2009).

Alveolar cells, especially type 2 pneumocytes, are very susceptible to apoptosis, and a slight increase in their apoptosis rate can lead to extensive disruption in the repair of alveolar epithelial tissue. These events will eventually lead to pulmonary fibrosis. It has been shown that bleomycin can induce epithelial cell apoptosis and ultimately pulmonary fibrosis in rat and mice (Wang *et al*., 2000; Barbas-Filho *et al*., 2001). In the present study, due to the decrease in the alveolar surface area, it seems that pulmonary fibrosis was occurred following BEP regimen.

Various inhibitory proteins are contributed in regulating and controlling cellular apoptosis. These proteins can directly or indirectly inactivate caspase proteins (Konopleva *et al*., 1999). There are two different mechanisms for induction of apoptosis in alveolar pneumocytes. Apoptosis of Type-1 pneumocytes needs caspase-8 activation, however, type-2 pneumocytes apoptosis is depended on the degradation of the mitochondrial membrane permeability (Constantini *et al*., 2000). In addition, DNA fragmentation and mutation which led to apoptosis, are mediated by free radicals during oxidative stress process. Potent antioxidants prevent oxidation caused by free radicals. Melatonin was found as a broad-spectrum antioxidant that scavenges a variety of reactive oxygen and nitrogen species (Tan *et al*., 2002). Therefore, it can be assumed that melatonin exerts its alleviate effects due to its antioxidant properties. The present results revealed that melatonin with dose 10 mg/kg could effectively protect the lung tissue in 3 cycle of BEP chemotraphy regimen. In addition to melatonin, other antioxidant agents such as a-tocopherol, selenium and ascorbic acid protect rat lung against pulmonary toxicity of cyclophosphamide (Yazici *et al*., 2020).

In parallel, previous research indicated that melatonin exerts positive effects on the alleviation of acute lung injuries, and acute respiratory distress syndrome. All experimental studies have been demonstrated that melatonin reduces lung injury via antioxidative effects and upregulation of anti-inflammatory cytokines, profibrotic cytokines and chemokines in lung tissues, which have a pivotal role in the pathophysiology of lung injury (Wang & Gao, 2021). The present findings also indicated that co-administration of melatonin could efficiently have decreased BEP-induced strong caspase-9 immunoreactivity throughout the lung tissue. Furthermore, high immunoreactivity of caspase 8 and caspase- 3 which were found in the epithelial cells, alveolar macrophages of BEP group, diminished significantly in BE P + MEL group compared to BEP group. In a study, Yazici *et al*. (2020) have shown that a-tocopherol as a strong antioxidant have protective effects on rat lung following cyclophosphamide-induced degeneration.

In another part of the study, plausible changes in the surface area of alveoli was investigated stereologically. In confirmation of immunohistochemical findings, surface area of alveoli was diminished significantly in BEP group as compared to the control one. This indicated a remarkable decrease in the cell population of the alveolar epithelium due to the BEP-induced pulmonary toxicity. It is noteworthy, the equivalent cellular apoptosis and proliferation is important for tissue homeostasis. As the obtained results showed, the PCNA immunostaining in BEP group was not significantly higher than control one. Therefore, diminish in the surface area of alveoli can be attributed to the enhanced apoptosis and slight increase in proliferation of alveolar cells. On the other hand, anti-apoptotic feature of melatonin led to cellular survival and maintaining the alveolar surface area close to the normal values.

Our results showed dense immunoreactivity for capspase-8 and caspase-9 in entire bronchioles and alveolar epithelium after BEP regimen administration which was an indicator of degenerative changes. However, alveolar structure was similar to that observed in control following co-administration of melatonin and BEP, which indicated that melatonin prevent the degeneration induced by BEP chemotherapy.

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**MAO, Y.; MA, H.; EL-KOTT , A. F.; BIN-MEFERIJ, M. M.; SHALDOUM, F. & MASSOUD, D.** Evidencia inmunohistoquímica de la protección de la melatonina en el tejido pulmonar durante la quimioterapia. *Int. J. Morphol., 41(1)*:167-174, 2023.

**RESUMEN:** El presente estudio investigó los posibles efectos protectores de la melatonina en los regímenes de quimioterapia con bleomicina, etopósido y cisplatino (BEP) mediante inmunohistoquímica. Cuarenta ratas Wistar macho se dividieron en cuatro grupos de diez: grupo 1, control sin tratar; grupo 2, quimioterapia con una dosis de 0,5x de BEP (0,75 mg/kg de bleomicina, 7,5 mg/kg de etopósido y 1,5 mg/kg de cisplatino) con tres ciclos de 21 días cada uno. Las ratas del grupo 3 (grupo MEL) recibieron 10 mg/kg/día de melatonina una vez al día. El grupo 4 (Mel + BEP) recibió melatonina (30 minutos antes de las inyecciones de BEP) y BEP, como en los grupos 2. Se usó la tinción del antígeno nuclear de células en proliferación (PCNA) para detectar la proliferación celular y, caspasa-3, caspasa-9 y caspasa-8 para investigar apoptosis. La inmunotinción de PCNA en el epitelio alveolar, los macrófagos alveolares y los bronquios varió de débil a moderada en el grupo BEP. Sin embargo, se detectaron inmunorreacciones difusas y fuertes para caspasa-3, caspasa 8- y caspasa-9 en el epitelio de los bronquiolos, endotelio vascular y macrófagos luminales alveolares. Las inmunorreactividades de PCNA y caspasa en los grupos MEL y Mel + BEP fueron similares a las del control. El área de superficie en el grupo BEP se redujo significativamente en comparación con el control (P<0,05). Sin embargo, en los grupos MEL y Mel + BEP, estuvo cerca del valor del control (P>0,05). Se puede concluir que la quimioterapia con BEP puede afectar negativamente al tejido pulmonar y la melatonina inhibe las lesiones durante la quimioterapia.

**PALABRAS CLAVE: Quimioterapia BEP; Estereología; Melatonina; Antioxidante; Apoptosis.**

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