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Creation of Myeloma Model in BALB/c Mice: Experimental Study

BALB/c Farelerde Miyeloma Modelinin Oluşturulması: Deneysel Çalışma

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ABSTRACT Objective: Multiple myeloma (MM) disease is characterized by clonal proliferation of malignant plasma cells in the bone marrow. It represents approximately 1% of all cancers in the world. Individual dissolution foci or diffuse osteopenia are common in the bones. In approximately 3% of the cases, non-bone marrow (extramedullary) involvement may occur and this solid involvement outside the bone marrow is defined as plasmacytoma. Experimental animal studies are widely used to elucidate metabolic and physiological mechanisms. It is more advantageous to use experimental animal models since there are many systemic organ involvement, especially in hematological cancers. We aimed to create a standard animal model for MM which is suitable for new experiments and drug trials. Seven experimental animal groups were created apart from the control group. Different amount of MOPC315 cells (1-2-5x10^6/200-500 µL) applied to three different regions (subcutaneous right abdominal, intravenous to the tail vessel, intraperitoneal right abdominal), in male (n=10) and female (n=7) BALB/c mice. Insulin injection should not be used. Different waiting times were applied for tumor development (20-30 days). **Material and Methods:** The control group consisted of 1 male and 1 female mouse. Pathological examination was performed after hematoxylin and eosin staining of organs and suspicious tissues of mice killed by cervical dislocation. **Results:** According to our study, after cells were injected, pneumonia, which is one of the late symptoms of MM, developed in females, but no tumor was formed. Whereas, after the cell was injected, tumor formation was observed in males. **Conclusion:** According to our results, we suggest that the standard MM animal model can be created by intraperitoneally injecting of 2x10^6/500 µL MOPC315 cells into male BALB/c mouse. Our work has been a pioneer in the use of animal experiments in MM disease in Turkey.

Keywords: Multiple myeloma; animal model

ÖZET Amaç: Multipl miyelom (MM) hastalığı, kemik iliğindeki malign plazma hücrelerinin klonal çoğalması ile karakterizedir. Dünyadaki tüm kanserlerin yaklaşık %1'ini temsil eder. Kemiklerde, tek tek erime odakları ya da diffüz osteopeni sık görülür. Olguların yaklaşık %3'ünde kemik dışı (ekstramedüller) tutulum olabilir ve kemik iliği dışındaki bu katı tutulum, plazmasitom olarak tanımlanır. Deneysel hayvan çalışmaları, metabolik ve fizyolojik mekanizmaları aydınlatmak için yaygın olarak kullanılmaktadır. Özellikle hematolojik kanserlerde, çok sayıda sistemik organ tutulumu olduğu için deneysel hayvan modellerinin kullanılması daha avantajlıdır. MM için yeni deneyler ve ilaç denemelerine uygun standart bir hayvan modeli oluşturmayı amaçladık. Kontrol grubunun dışında, 7 deneysel hayvan grubu oluşturuldu. Farklı sayıda erkek (n=10) ve dişi (n=7) BALB/c farelere, 3 farklı bölgeden (subkütan sağ karın, kuyruk damarına intravenöz, intraperitoneal sağ karın) farklı miktarda MOPC315 hücresi (1-2-5x10^6/200-500 µL) uygulandı. İnsülin enjektörü kullanılmamalıdır. Tümör gelişimi için farklı bekleme süreleri uygulandı (20-30 gün). **Gereç ve Yöntemler:** Kontrol grubu, 1 erkek ve 1 dişi fareden oluşmaktaydı. Servikal dislokasyonla öldürülen farelerin organları ve şüpheli dokuların, hematoksilen-eozin boyanması sonucunda patolojik incelemesi yapıldı. **Bulgular:** Çalışmamıza göre hücreler enjekte edildikten sonra MM'nin geç semptomlarından biri olan pnömoni, dişi farelerde gelişti, ancak tümör oluşmadı. Oysa hücre enjekte edildikten sonra erkek farelerde tümör oluşumu gözlendi. **Sonuç:** Sonuçlarımıza göre standart MM hayvan modelinin, 2x10^6/500 µL MOPC315 hücresinin erkek BALB/c fareye intraperitoneal yoldan enjekte edilmesi ile oluşturulabileceğini önermekteyiz. Çalışmamız, Türkiye'de MM hastalığında deney hayvanı kullanılması konusunda öncü olmuştur.

Anahtar Kelimeler: Multipl miyelom; hayvan modeli

With the advancement of age, the accumulation of cellular and molecular changes increases, which causes susceptibility to cancer. Approximately 60% of all cancers and 70% of deaths from cancer occur at the age of 65 and over. As in the world, Turkey is increasing rapidly day by day rate for the elderly population. According to records of Turkey Statistical Institute, the elderly population ratio is expected to

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be 10.2% in 2023. It is estimated that it will increase to 27.7% in 2075.[1] This may increase the risk of hematological malignancy.

Multiple myeloma (MM) is characterized by clonal proliferation of malignant plasma cells in the bone marrow.[1] MM is the second most common hematological neoplasia, accounting for about 10-15% of hematological malignancies.[2,3] It represents approximately 1% of all cancers in the world.[3]

MM is characterized by a recurrent course. Despite significant improvements in patient outcomes after immunomodulatory drugs and proteasome inhibitors in primary care, eventually relapse is shown in most patients.[4] New treatment options are needed to overcome impaired immune surveillance.[5]

As is known, many steps from the molecular biological mechanism of the disease to the recognition of new biomarkers, the discovery and development of new treatments require the use of pre-clinical cancer models.

Primary cultures made using human cell lines or using tissue samples taken directly from the patient are widely used in pre-clinical studies. However, although cell cultures are suitable for molecular and genetic studies, since these cells or cell culture conditions are homogeneous and do not fully reflect the natural tumor biology and microenvironment, it may be insufficient to predict how a drug applied to the cultured cells will act in the patient.

Especially in hematological cancers, since there are many systemic organ involvement, animal models should be used in in-vivo conditions where metabolic activities gain more importance than in-vitro cell culture.[6] Therefore, standardized animal models are needed for pre-clinical studies on cancer development and treatment.

When the literature is scanned, it is seen that there is no study for the creation of the myeloma animal model in Turkey. This causes new drug development trials to remain in-vitro. Therefore, in the presented study, it was aimed to create a standardized myeloma model in animals by using different application methods on animals.

MATERIAL AND METHODS

CELL LINES, CULTURE CONDITIONS

The MOPC315 cell line was obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured in Dulbecco modified Eagle medium/F12 supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

Next, cultured MOPC315 cells in different concentrations were prepared depending on the region to be injected.

Our study was conducted in line with the "Guide for the Care and Use of the Laboratory Animals Principles" (http://www.nap.edu/catalog/5140.html) and animal rights were protected.

ETHICS STATEMENT

Ethical approval was obtained from Erciyes University Animal Experiments Local Ethics Committee (Approval number: 19/035, 13.2.2019). 19 BALB/c mice, 11 male and 8 female, were provided from the Betül Ziya Eren Genome and Stem Cell Center.

Transfer of the MOPC315 cells to BALB/c mice: BALB/c mice were preferred for reasons such as being easy to produce, easy to reach both female and male genders, and being inexpensive. Mice were adult, 2 months old, weighing 28-40 g, male and female, BALB/c mice. Mice were kept in cages with nocturnal feeding, corn cob pads, 12 hours light on, 12 hours light off, 23 °C room temperature.

Cells of different dosages (1-2-5x10^6/200-500 µL) were given to mice of different sexes in different ways (subcutaneous right abdominal, intravenous to the tail vessel, intraperitoneal right abdominal). Tumor development was examined at different waiting times (20-30 days). The quality of life of the animals was monitored daily.

After the MOPC315 cells were given to the mice, those whose waiting period was completed were killed by cervical dislocation.

TAKING TISSUE SAMPLES FROM BALB/C MICE

It was investigated whether there was a tumor in the subcutaneous region and intraperitoneal region of the killed mice.

TABLE 1: Experimental groups and results (Group 4-1st male mouse died on day 18) (The control group consisting of a female and a male mice is not shown).

Group	Method of application	Mouse number	Gender	x10⁶ cell	Duration (Day)	Tumor development
1st group	Subcutaneous, right abdomen	3	Female	1-2-5/(500 µL)	20	None developed a tumor (Pneumonia detected)
2nd group	Subcutaneous, right abdomen	3	Male	1-2-5/(500 µL)	30	Tumor developed in the mouse given 2x10⁶ cells (Inflammatory foci was detected)
3rd group	Intravenous, from tail vein	3	Male	1-2-5/(500 µL)	30	None developed a tumor
4th group	**Intraperitoneal, right abdomen**	**2**	**Male**	**2/(500 µL)**	**18 30**	**Tumors developed in both mice**
5th group	Subcutaneous, right abdomen	2	Male	2/(500 µL)	30	Tumors developed
6th group	Intraperitoneal, right abdomen	2	Female	2/(500 µL)	30	None developed a tumor (Inflammatory foci was detected all)
7th group	Subcutaneous, right abdomen	2	Female	2/(500 µL)	30	None developed a tumor

Tissue samples were taken from abnormal structures, tissues that are likely to be tumors, lump, spleen, peritoneum, stomach, intestine, kidney, liver, lung, bone marrow, or any suspicious mass and lymphoreticular masses.

Tissue samples were taken into 10% formaldehyde and immunohistochemical and histopathological examinations were done in Erciyes University Medical Faculty Medical Pathology Laboratory. Thus, it was determined whether the MM experimental animal model was created.

RESULTS

When the current literature is scanned, there were no in-vivo studies on MM in Turkey. With this project, it is aimed to fill this gap. In the future, an experimental animal model suitable for drug applications was tried to be created.

In our study, 7 experimental animal groups were created apart from the control group. In the experimental groups, different cell dosages, different administration methods and different waiting times were applied to create an experimental animal model that best mimics MM disease. Experimental groups and results are shown in the Table 1.

The 1st group; 3 female mice; $1x10^6$, $2x10^6$ and $5x10^6$ MOPC315 cells were delivered to the subcutaneous, right abdomen. After 20 days, hematoxylin and eosin (H&E) staining of tissue samples taken from stomach, kidney, spleen, intestine, liver, lung, bone and peritoneum was performed. As a result of H&E staining, the presence of neutrophils-rich areas in the lung alveoli showed that pneumonia developed (Figure 1B, C, D). Lung cells of the control group are shown too (Figure 1A). No abnormality was observed in the other organs examined.

The 2nd group; 3 male mice were given $1x10^6$, $2x10^6$ and $5x10^6$ MOPC315 cells subcutaneously to the right abdomen. 30 days later, the male animal, which received only $2x10^6$ cells, developed a 3x2 cm tumor under the skin (Figure 2A, B, D). Vascularization of the tumor was quite clear (Figure 2C). H&E staining was performed on tissue samples taken from stomach, kidney, spleen, intestine, liver, lung, bone, tumor, and peritoneum. Inflammation areas were seen in the lungs (Figure 3). Atypical mitotic plasma cells with an eccentric nucleus, eosinophilic cytoplasm were observed by H&E staining of the tumor (Figure 2E).

The 3rd group; 3 male mice were given $1x10^6$, $2x10^6$ and $5x10^6$ MOPC315 cells from the tail vein. Waited 30 days. H&E staining was performed on stomach, lung, liver, spleen, kidney, intestine, testicle, bone and peritoneal tissue samples. As a result of staining, no abnormal finding was observed with staining in organs.

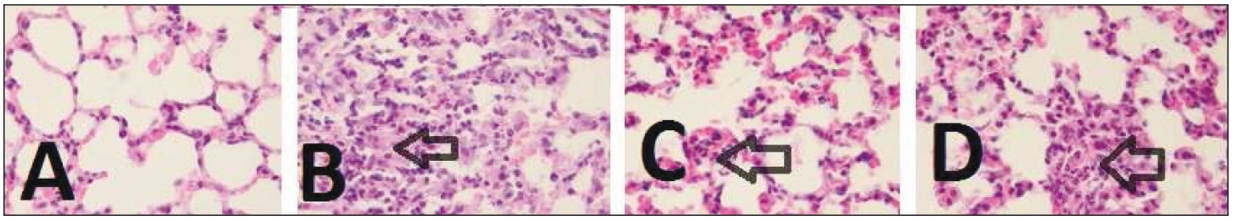

FIGURE 1: A) Healthy control female lung and Group 1 (subcutaneous) pneumonia in the lungs, B) 1st female, C) 2nd female, D) 3rd female. Black arrows: neutrophils are intensely seen (haematoxylin and eosin stain, original magnification: X 200).

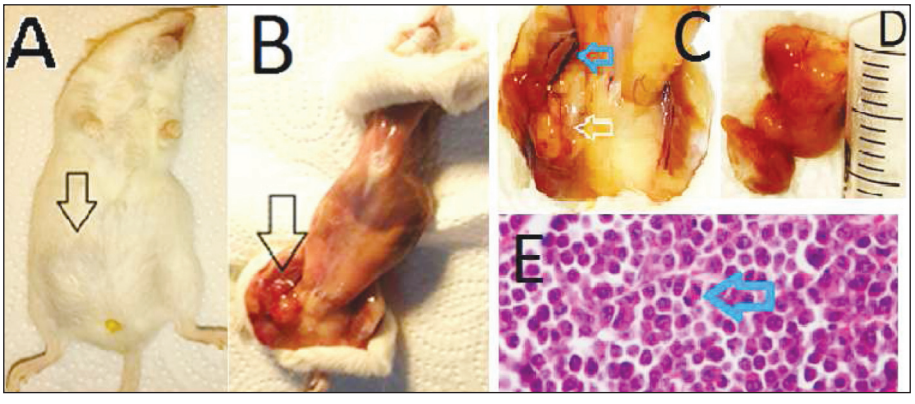

FIGURE 2: Group 2 (subcutaneous) 2nd male animal; tumor development in the subcutaneous male animal given 2x106 MOPC315 cells. A) View of the tumor from the outside, B) Tumor, C) Close view of the tumor (white arrow), (Blue arrow: indicates tumor vein), D) Tumor size 3x2 cm, E) H&E staining of tumor (Blue arrow: atypical mitotic plasma cells) (haematoxylin and eosin stain, original magnification: X 200).

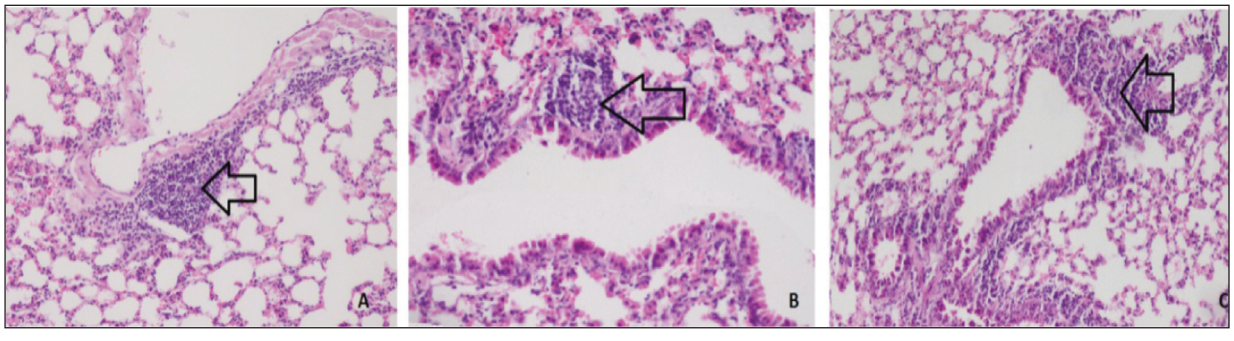

FIGURE 3: Group 2 (Subcutaneous) focal inflammation in the peri brochial area. A) 1st male, B) 2nd male, C) 3rd male (haematoxylin and eosin stain, original magnification: X 100).

The 4th group; 2 male mice were given, $2x10^6$ MOPC315 cells to the intraperitoneal right abdomen.

One of the animals in this group died on the 18th day without completing 30 days. When it was noticed that he was dead, the animal was examined immediately (Figure 4A). Approximately 5x5 mm sized tumor was seen in the adipose tissue (Figure 4B,C). H&E staining was performed on lung, intestine, bone, stomach, liver, spleen, kidney, tumor and peritoneal tissue samples. Cancer cells were demonstrated by H&E staining (Figure 4D). No abnormal finding was found in staining in other organs. A very large, swollen, approximately 2.5x2 cm urinary bladder was seen (Figure 4B). While a normal kidney is 7-11 mm, a large kidney with a size of about 10x15 mm has been observed (Figure 4E,F).

The other animal from the fourth group has completed the 30-day waiting period. A large tumor was developed (Figure 5A,B,C). After 30 days, H&E staining was performed on stomach, spleen, kidney,

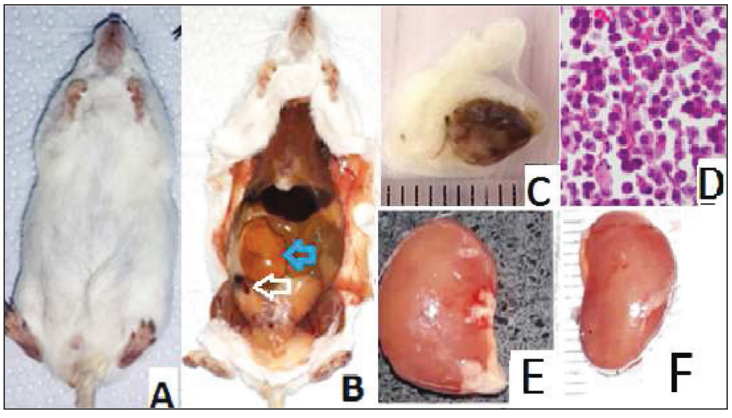

FIGURE 4: Group 4 (Intraperitoneal) A) 1st male who died on the 18th day, B) Tumor in cell application area (white arrow), very large urinary bladder (blue arrow), C) 5x5 mm tumor in fat tissue, D) H&E staining of the tumor cells, E) Kidney, F) Healthy control mouse kidney (haematoxylin and eosin stain, original magnification: X 200).

liver, lung, intestine, bone, tumors and peritoneal tissue samples. As a result of staining, no abnormal finding was observed with staining in organs.

When the peritoneum was opened, three tumors were seen. First tumor of about 3x3 cm size was seen, in adipose tissue, next to the intestine, in the right abdomen (Figure 6A,B,C). Second tumor of about 1x2 cm size was seen in the left abdomen, near the spleen (Figure 6D,E,F). Third tumor of about 1.5x1.5 cm size was seen in the right abdomen, near the testicle (Figure 6G,H,I).

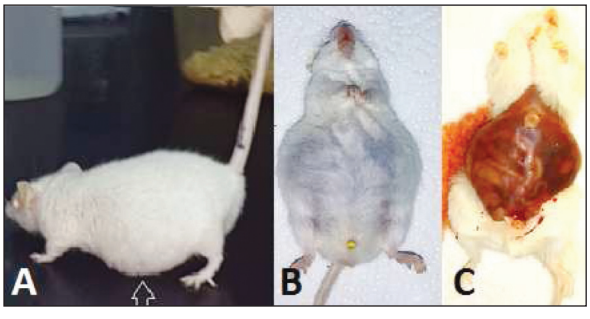

FIGURE 5: Group 4 (Intraperitoneal) 2nd male, A) Tumor, B) Cervical dislocation, C) Peritoneal appearance by skin cutting.

The 5th group; 2 male mice were given $2x10^6$ MOPC315 cells subcutaneously in the right abdomen. H&E staining was performed on stomach, spleen, kidney, liver, lung, intestine, bone and peritoneal tissue samples.

In one mouse, two tumors, approximately 5x7 mm in size, were seen near the kidney and in the right adipose tissue (Figure 7A,B,C,D,E,F). The other mouse had no tumor.

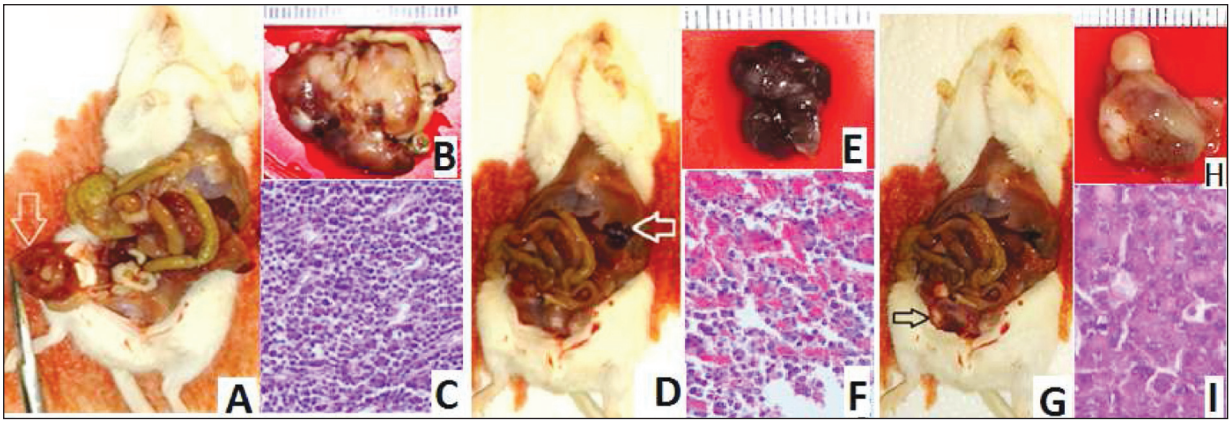

FIGURE 6: Group 4 (Intraperitoneal) 2nd male. A) Tumor near the intestine, B) 3x3 cm tumor, C) H&E staining of tumor cells, D) Tumor near spleen, E) Tumor 1x2 cm size, F) H&E staining of tumor cells, G) Tumor beside testicle, H) 1.5x1.5 cm tumor side testis, I) H&E staining of tumor cells (haematoxylin and eosin stain, original magnification: X 100).

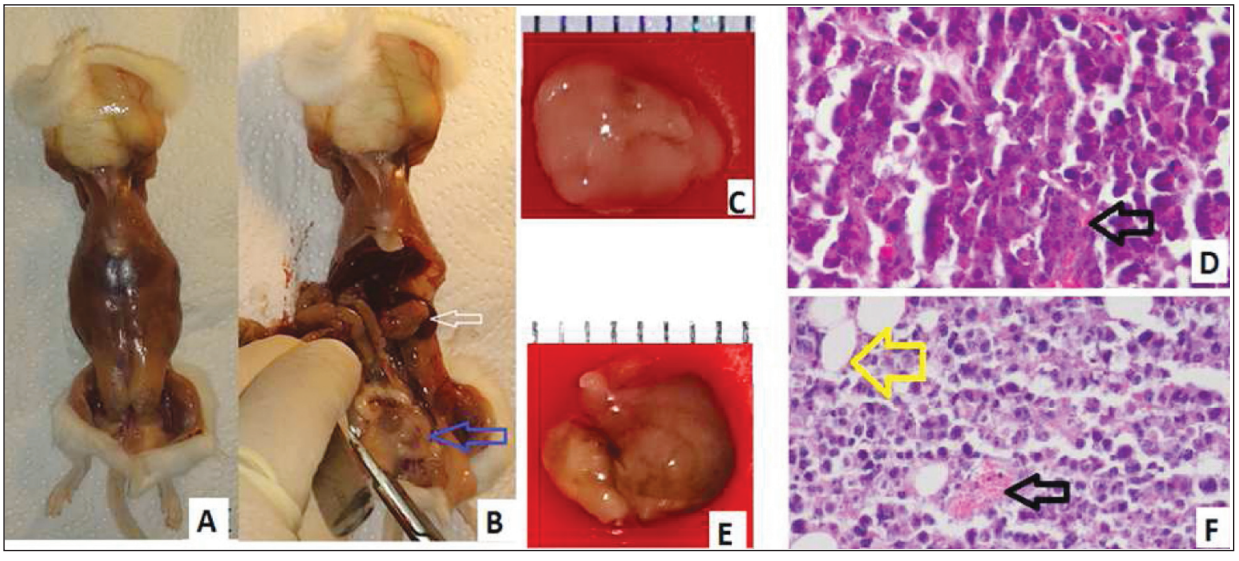

FIGURE 7: Group 5 (Subcutaneous) 1st male. A) Skin cut, B) Tumors seen with peritoneum cut (White arrow: tumor near kidney, blue arrow: tumor near intestine), C) 7x5 mm sized tumor near the kidney, D) H&E staining of tumor cells near the kidney (Black arrow: indicates veining), E) 7x5 mm sized tumor near intestine, F) H&E staining of tumor cells near the intestine (Black arrow: veins, yellow arrow: fat cells) (haematoxylin and eosin stain, original magnification: X 200).

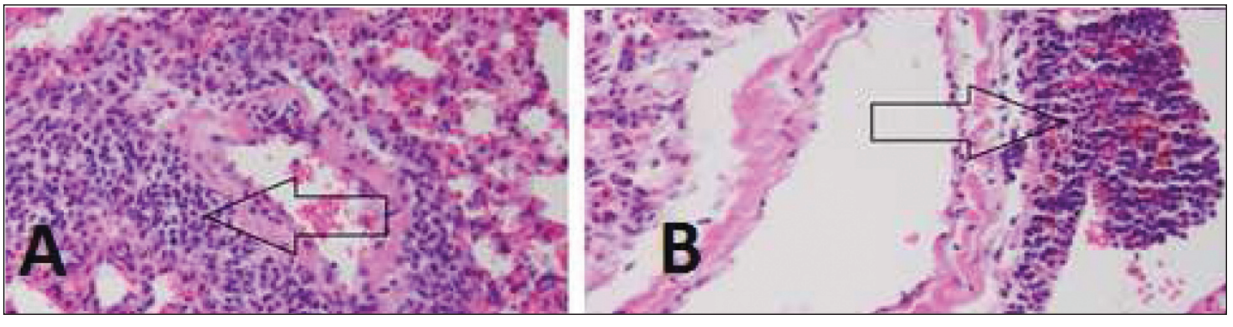

FIGURE 8: Group 6 (Intraperitoneal). Inflammation focus in peribronchial areas. A) 1st female, B) 2nd female (haematoxylin and eosin stain, original magnification: X 100).

The 6th group; 2 female mice were given 2x10^6 MOPC315 cells intraperitoneally in the right abdomen. H&E staining was performed on stomach, spleen, kidney, liver, lung, intestine, bone and peritoneal tissue samples. As a result of staining, inflammation foci were observed in the peri-bronchial area of the lungs (Figure 8A, B). No abnormal finding was found in other organs. There were no abnormal masses in the body like a tumor.

The 7th group; 2 female mice were given 2x106 MOPC315 cells subcutaneously in the right abdomen. 30 days later, H&E staining was performed on stomach, spleen, kidney, liver, lung, intestine, bone and peritoneal tissue samples. No abnormal finding was found as a result of staining. No abnormal mass like tumor was seen in the body.

DISCUSSION

In some sources, the frequency of male/female incidence is shown as 7/4.5, 3/2.[7,8] Estrogen is known to be a negative regulator of normal hematopoiesis and lymphopoiesis.[9] In MM, the mortality rate is 3.3 per 100,000 in males and 2.3 per 100,000 in females.[10]

The MOPC315 cell line is a specialized cell line used to generate MM in BALB/c mice.[11] In some studies, MOPC315 cells were delivered directly into bone.[12] It is essential to create an experimental animal model in order to better describe the metabolic effects that develop in MM disease.[13]

Estrogen is known to be a negative regulator of normal hematopoiesis and lymphopoiesis.[9] Therefore, it is thought that estrogen has a protective effect

in women, and due to estrogen level the rate of MM in women may lower than that of men. Even, a study reported that female MM patients were shown to have more low oestogenes compared to healthy females.[14] Another study, demonstrated that MM risk observed among those who had been on estrogen replacement therapy was significantly decreased.[15] Similar to other studies, in our study, it suggests that absence of tumor formation in female mice which was injected MOPC315 cells is due to the protective effect of estrogen. Therefore, according to our findings, it was concluded that it was appropriate to use male mice to create a myeloma model.

Pneumonia is one of the late symptoms of MM disease. It is known that MM increases the susceptibility to infection. In some of the our experiments, mice had pneumonia, although the tumor did not develop. It was thought that late MM symptoms were seen early by injecting MOPC315 cells at high concentrations from outside (at least $2x10^6$/500 µL). No tumors occurred in male mice injected with MOPC315 cells from the tail vein. It was thought that the cancer cells that were given to the circulation were destroyed by the immune system. Therefore, it was found that transferring cells from the tail vein was not an appropriate method to create a MM model.

In our study, a large kidney and an enlarged bladder were seen in a mouse that died on the eighteenth day after intraperitoneal $2x10^6$/500 µL MOPC315 cells were administered to the right abdomen. In MM, protein can accumulate in the kidney. It was thought that the protein could block the urinary tract and cause the bladder to grow and the mouse to die.

CONCLUSION

As a result, our data showed that MM was formed, 30 days after the intraperitoneal $2x10^6$/500 µL MOPC315 cells were applied to male mice. In preclinical studies especially in drug trials, we can suggest that this technique may be suitable for creating a MM animal model.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Mustafa Çetin;* ***Design:*** *Zühal Hamurcu;* ***Control/Supervision:*** *Zühal Hamurcu;* ***Data Collection and/or Processing:*** *Selda Taşdemir;* ***Analysis and/or Interpretation:*** *Kemal Deniz, Özlem Canöz;* ***Literature Review:*** *Selda Taşdemir;* ***Writing the Article:*** *Selda Taşdemir;* ***Critical Review:*** *Zühal Hamurcu;* ***References and Fundings:*** *Zühal Hamurcu;* ***Materials:*** *Zühal Hamurcu, Nesrin Delibaşı Kökçü.*

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