

# Cimetidine and Ibuprofen Modulate T Cell Responses in a Mouse Model of Breast Cancer

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## Abstract

Cimetidine and ibuprofen exhibit immunomodulatory effects as an antagonist of histamine H2 receptor, and a cyclooxygenase inhibitor, respectively. Here, the effects of cimetidine and ibuprofen on some effector T cell-related parameters were investigated using a breast cancer (BC) model. BC was established in Balb/c mice using the 4T1 cell line. On day 10 after tumor induction, the BC-bearing mice were classified into four groups and treated with PBS, cimetidine (20 mg/kg), ibuprofen (20 mg/kg) or a combination of “cimetidine + ibuprofen” via intraperitoneal injection (daily from days 11 to 30). The mice were sacrificed on day 31 and the frequency of splenic Th1 and Treg cells, plasma IFN- $\gamma$  and TGF- $\beta$  levels, and intra-tumoral T-bet, GATA3, FOXP3 and ROR $\gamma$ t expressions were detected using flowcytometry, ELISA and real-time-PCR, respectively. In untreated cancerous mice, the percentage of splenic Th1 cells and plasma IFN- $\gamma$  levels were lower ( $P<0.003$  and  $P<0.01$ , respectively), whereas the percentage of splenic Treg cells and plasma TGF- $\beta$  levels were higher than in healthy mice ( $P<0.04$  and  $P<0.005$ , respectively). Treatment of BC-bearing mice with cimetidine, ibuprofen or both drugs promoted the frequency of Th1 cells ( $P<0.05$ ,  $P<0.007$  and  $P<0.005$ , respectively) as well as IFN- $\gamma$  levels ( $P<0.004$ ,  $P<0.0001$  and  $P<0.03$ , respectively), while reduced the frequencies of Treg cells ( $P<0.02$ ,  $P<0.03$  and  $P<0.01$ , respectively), TGF- $\beta$  levels ( $P<0.006$ ,  $P<0.02$  and  $P<0.002$ , respectively), intra-tumoral expression of FOXP3 ( $P<0.006$ ,  $P<0.005$  and  $P<0.005$ , respectively), and intra-tumoral expression of ROR $\gamma$ t ( $P<0.04$ ,  $P<0.03$  and  $P<0.05$ , respectively) compared with untreated BC mice. The “cimetidine + ibuprofen”-treated mice displayed greater T-bet expression than the un-treated mice ( $P<0.006$ ). Cimetidine and/or ibuprofen-treated BC-bearing mice exhibited reduced intra-tumoral expression of GATA3 compared with the untreated BC mice, but the differences were not significant. Cimetidine and ibuprofen correct some effector T cell-related parameters in cancerous mice. Immunotherapeutic potentials cimetidine and ibuprofen in cancers need investigations.

**Keywords:** Breast cancer- Cimetidine- Ibuprofen- T cells- Mice

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## Introduction

Approximately 29.0% of all the newly diagnosed cancers and about 14% of all the cancer-related deaths in women are caused by breast cancer (BC) worldwide (Siegel et al., 2013). One of the most fundamental duties of the immune system is to recognize and eliminate malignant cells (Casey et al., 2014; Sheikhi et al., 2016). Both arms of the immune systems, including the innate and acquired immunities, perform an essential role in defense against malignancies (Casey et al., 2014; Sheikhi et al., 2016). T lymphocytes are the most prominent leukocytes contributing to the tumor-related

immune responses, which are classified into several major subgroups such as cytotoxic T lymphocytes (CTLs), T helper (Th) cells, and regulatory T (Treg) cells (Kursunel and Esendagli, 2016; Sun et al., 2019). Among Th cells, the Th1 cell subtype exerts anti-tumorigenic impacts, and their main transcription factor is named T-bet that induces the expression of the IFN- $\gamma$ , IL-2, and TNF- $\alpha$ , which in turn activate CTLs, natural killer (NK) cells, and natural killer T (NKT) cells (Kursunel and Esendagli, 2016; Sun et al., 2019). IFN- $\gamma$  also classically activates M1 type macrophages to produce nitric oxide and superoxide, both of which perform a key role in the destruction of cancerous cells (Muller et

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al., 2017). Conversely, Th2 cell-related cytokines (such as IL-4, IL-10 and IL-13) suppress anti-tumor immune responses by down-regulation of Th1 cells (Jafarzadeh et al., 2015b; Golubovskaya and Wu, 2016). Furthermore, Treg cells exert anti-tumorigenic impacts via a number of mechanisms, especially the secretion of IL-10, IL-35 and TGF- $\beta$  (Jafarzadeh et al., 2015a; Chen et al., 2016; Chatrabnous et al., 2018). Th17 cells produce a number of cytokines, especially IL-17A and IL-17F (Guery and Hugues, 2015; Etesam et al., 2016). There are some controversies and complexity regarding the involvement of Th17 cells in tumor immunity. The pro- or anti-tumor effects of Th17 cells may be exerted in a tumor type-dependent manner. Therefore, Th17 cells are associated with both good and bad prognoses (Guery and Hugues, 2015). Tbet, GATA3, FOXP3 and ROR $\gamma$ t are known as major transcription factors of Th1-, Th2, Treg- and Th17 cells, respectively (Jafarzadeh et al., 2018).

In addition, cancerous cells acquire capabilities to escape from the immune system (Sun et al., 2019). Increased amounts of histamine and prostaglandins have been detected in many kinds of malignancies (Pang et al., 2016; Aponte-Lopez et al., 2018). Histamine may exert differential effects on cancers, probably through binding to the various types of its receptors (Medina and Rivera, 2010). Histamine performs powerful immunosuppressive effects via binding to its H2 receptor (Frei et al., 2013; Jafarzadeh et al., 2019). Potent immunostimulatory properties are attributed to the cimetidine as an H2 receptor antagonist (Jafarzadeh et al., 2010; Jafarzadeh et al., 2013; Jafarzadeh et al., 2019). The beneficial influences of cimetidine have been indicated in several kinds of malignancies such as lung cancer, glioblastoma, melanoma, renal cell carcinoma, colorectal cancer, and gastric cancer (Kubecova et al., 2011; Faustino-Rocha et al., 2017).

Prostaglandins (PGs) such as prostaglandin E2 (PGE2) are synthesized by inflammatory cells via activation of the cyclooxygenase (COX) enzymes, especially COX-2 (Pang et al., 2016). PGs can directly stimulate cell expansion or indirectly induce cytokines such as IL-6, which serve as tumor-promoting factors (Misra and Sharma, 2014). Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen serve as anti-inflammatory agents via the inhibition of COX-1 and -2 activities (Aranda et al., 2017). The profitable impacts of ibuprofen were indicated in several kinds of the malignancies such as BC and lung cancer (Ratliff, 2005; Pennock et al., 2018). However, the elucidation of the immunomodulatory effects of cimetidine and ibuprofen in cancers needs more investigations. Here, the effects of cimetidine and ibuprofen on several parameters related to Th1- (cell frequency, plasma IFN- $\gamma$  levels, intra-tumoral expression of T-bet), Treg- (cell frequency, plasma TGF- $\beta$ , intra-tumoral expression of FOXP3), Th2- (intra-tumoral expression of GATA3), and Th17- (intra-tumoral expression of ROR $\gamma$ t) cells were investigated using a mouse BC model.

## Materials and Methods

### *Animals and tumor induction*

Totally, 70 female Balb/c mice (4–6 weeks old) were purchased from the Pasteur Institute of Iran (Tehran, Iran) and housed at a standard condition (temperature of  $21 \pm 2^\circ\text{C}$ , full ventilation, and 12 hours light/dark cycle) with free access to water and mice pellet food. An ethic committee affiliated to the Rafsanjan University of Medical Sciences also approved the investigation protocol with an assigned ethical code: IR.RUMS.REC.1394.131. For tumor induction, the 4T1 carcinoma cell line was provided from the Pasteur Institute of Iran, and expanded in RPMI-1640 medium supplemented with 15.0% of fetal bovine serum (FBS) (Gibco, Germany). The 4T1 cell line ( $7 \times 10^5$  cells in 200  $\mu\text{L}$  of RPMI) was injected in the right side flank of mice. The mice were monitored till a tumor nodule appeared.

### *Treatment program*

Treatment with cimetidine, ibuprofen or combination of cimetidine + ibuprofen was started when the tumor size reached a 100 mm<sup>3</sup> size (about 11 days after tumor induction). The drugs were dissolved in PBS (contain ethanol 10.0%) and were filtered using a 0.2 micron filter and administered intraperitoneal from days 11 to 30 after tumor induction (daily, during 21 days). The grouping of mice was done as: Group 1 was considered as a healthy control group (without tumor induction and received the same volume of the vehicle), Group 2 was subjected to tumor induction, received the vehicle and considered as an untreated group. The BC was established in mice of Groups 3-5 and were treated with cimetidine (20 mg/kg), ibuprofen (20 mg/kg) or combination of “cimetidine + ibuprofen”. Each group contained 14 mice of which 7 mice were sacrificed on day 31 after tumor induction and their blood, spleens and tumoral tissues were collected for more analysis. From each group, 7 mice were also maintained for survivorship assessment and their survival time was assessed until 200 days after the completion of the treatment program.

### *Measurement of the spleen index and tumor size*

The weighing of the mice and related spleens were done using an electronic balance and the spleen index was measured using the following formula (Aghili et al., 2014):

$$\text{Spleen index} = \frac{\text{spleen weight (g)}}{\text{body weight (g)}} \times 100$$

The tumor dimensions were measured every three days using the caliper. The tumor volume (V, mm<sup>3</sup>) compute, using the formula:  $V = 0.5 \times d^2 \times D$ , in which d and D were considered as the shorter- and the longer diameter of tumor bulk, respectively (Yazdi et al., 2015).

### *Measurement of the percentage of Th1- and Treg cells in the spleen*

The frequencies of the splenic Th1- and Treg cells were measured by counting 10,000 cells by a BD FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA) and BD CellQuest PRO software. After removing

the spleens at 31 after tumor induction, a single cell suspension was prepared from each spleen using a cell strainer (100  $\mu$ m). After that the RBCs were eliminated using an RBC lysis reagent. The cells were washed in RPMI 1640 (supplemented with 10% FBS) and their viability was determined using trypan blue staining.

A mouse multicolor flow cytometry kit (R&D Systems, Minneapolis, USA) was used to measure the frequencies of splenic Th1 cells. The reagents of the kit were conjugated antibodies, including anti-CD4-PE, anti-T-bet-PerCp, anti-IFN- $\gamma$ -fluorescein, and anti-IL-12R $\beta$ 2-APC.

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According to the manufacturer's guidelines, the cell suspensions were washed in PBS and re-suspended in fixation/permeabilization reagent and then incubated at 2–8 °C for 30 min. The cells were then centrifuged and suspended in permeabilization/wash reagent. After that, 10  $\mu$ L of each specific conjugated antibody or each corresponding isotype control antibody was added to the cells. Following the incubation, a washing step was done to remove the excess unbound antibodies. Finally, the cells were suspended in PBS for flow cytometric analysis.

#### Measurement of the plasma IFN- $\gamma$ and TGF- $\beta$ levels

Blood samples were taken by cardiac puncture on day 31 after tumor induction and the plasma specimens were separated and stored at -20°C until analyses. The plasma IFN- $\gamma$  and TGF- $\beta$  levels were measured using mouse kits (Biolegend, CA, USA) according to manufacturer's guidelines.

#### RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from tumor tissues using Trizol reagent (Bioneer, Korea). The extracted RNA was exposed to DNase I (Thermo Scientific, EU) to exclude any contaminating genomic DNA. The quantity and the purity of the RNA was estimated by detecting the optical density at 260 nm (OD260) and computing the OD260/OD280 ratio using a spectrophotometry system, respectively.

The RNA specimens were converted into complementary DNA (cDNA) using oligo (dT) and random hexamer primers provided in a cDNA synthesis kit (Bioneer, Korea). The T-bet, FOXP3, ROR $\gamma$ t and GATA3 expression was estimated by the real-time-PCR method.

Real-time PCR was performed in triplicate using a real-time PCR system (Applied Biosystems, USA) by

mixing SYBR green master mix (Biofact, Korea) with 200 ng of synthesized cDNA and 2  $\mu$ L of appropriate primers (Table S1). The procedure of the real-time PCR was designed as 40 sequential cycles of 95°C for 30 seconds and 60°C for 30 seconds, and finally 72°C for 30 seconds. The GAPDH gene was used as a housekeeping gene and the  $2^{-\Delta\Delta C_t}$  formula was used for estimation of the interested gene expression in the tumor tissues. The quantitative assessment of the data and the melting curve analysis were performed by an Applied Biosystems software version 1.1.308.111 (USA).

#### Histopathological studies

The mice were sacrificed on day 31 and their lungs were harvested, fixed in 10% formalin and embedded in paraffin. Then the tissues were sliced into 5 micron sections and mounted on slides. Hematoxylin and eosin (H&E) staining was performed for histological examination. The stained slides were examined by light microscopy concerning the existence of metastatic and histopathologic alterations.

#### Statistical analyses

The data were presented as mean  $\pm$  SEM. Statistical comparison was analyzed by a computer program (SPSS version 18, Chicago, IL, USA) using ANOVA and Student's t-test, as appropriate. Results with P values less than 0.05 were considered statistically significant.

## Results

#### The body weights in cimetidine and/or ibuprofen-treated mice

The alterations in the body weight in the healthy control group, un-treated cancer-bearing mice and treated cancer mice were demonstrated in Figure 1. The percentage increase in body weight of un-treated BC-bearing mice ( $0.53 \pm 2.5$ ) was lower than the healthy mice ( $11.98 \pm 1.07$ ,  $P < 0.0001$ ). The percentage increase in body weight of the BC-bearing mice treated with cimetidine ( $8.1 \pm 1.8$ ), ibuprofen ( $10.28 \pm 1.84$ ) or both drugs ( $7.89 \pm 1.67$ ) was enhanced compared with un-treated cancerous mice ( $P < 0.03$ ,  $P < 0.006$  and  $P < 0.03$ , respectively). No significant differences were found between cimetidine and/or ibuprofen-treated mice concerning the percentage change in body weight.

#### The tumor sizes in cimetidine and/or ibuprofen-treated mice

The tumor sizes were monitored after starting the treatment program every three days. The tumor sizes in cancerous mice treated with cimetidine, ibuprofen or both drugs at days 16, 19, 22, 25, 28 and 31 after tumor induction were markedly lower than that observed in non-treated cancer-bearing mice ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.001$ ,  $P < 0.001$ , and  $P < 0.0001$ , respectively) (Figure 2).

Moreover, the tumor tissues from BC-bearing mice were collected and weighed at days 31 after tumor induction. The tumor weights in cancerous mice treated with cimetidine ( $0.62 \pm 0.14$  gram), ibuprofen ( $0.35 \pm 0.10$  gram) or both drugs ( $0.69 \pm 0.13$  gram) were

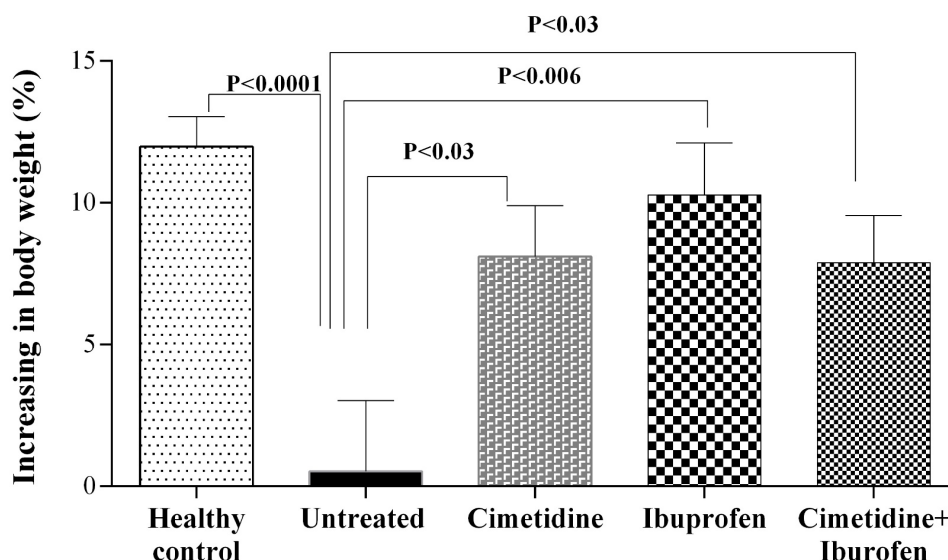


Figure 1. Effects of Treatment with Cimetidine and Ibuprofen in Mice with Breast Cancer. The percentage increase in body weight in un-treated cancer bearing mice was lower than healthy mice and the treatment of cancer bearing mice with dose 20 mg/kg of cimetidine or/and ibuprofen enhanced the percentage increase in body weight as compared with un-treated cancerous mice.

significantly smaller than in non-treated cancer-bearing mice ( $1.42 \pm 0.28$  gram;  $P < 0.02$ ,  $P < 0.001$  and  $P < 0.03$ , respectively) (Figure 3). The tumor weights in ibuprofen-treated mice were smaller than in cimetidine- and “cimetidine + ibuprofen”-treated groups, but differences were not significant ( $P = 0.14$  and  $P = 0.06$ , respectively).

The survival rates of animals were evaluated during the 240 days after tumor induction. The survival rates were also enhanced in cimetidine- and/or ibuprofen-treated mice compared with control untreated cancerous mice. Finally, the survival rate in mice treated with ibuprofen-treated group (5/7) was greater than cimetidine-treated group (3/7) and “cimetidine + ibuprofen”-treated group

(3/7) (Figure 4).

*The spleen index in cimetidine and/or ibuprofen-treated mice*

The spleen indexes were  $0.48 \pm 0.03$  in the healthy control group,  $3.39 \pm 0.31$  in un-treated cancerous mice,  $2.15 \pm 0.36$  in the cimetidine-treated group,  $1.80 \pm 0.44$  in ibuprofen-treated mice and  $2.55 \pm 0.36$  in the “cimetidine + ibuprofen”-treated group. The spleen index in the un-treated cancer-bearing mice was larger than healthy mice ( $P < 0.0001$ ) (Figure 5). Treatment of the BC-bearing mice with cimetidine, ibuprofen or both drugs reduced the spleen index as compared with

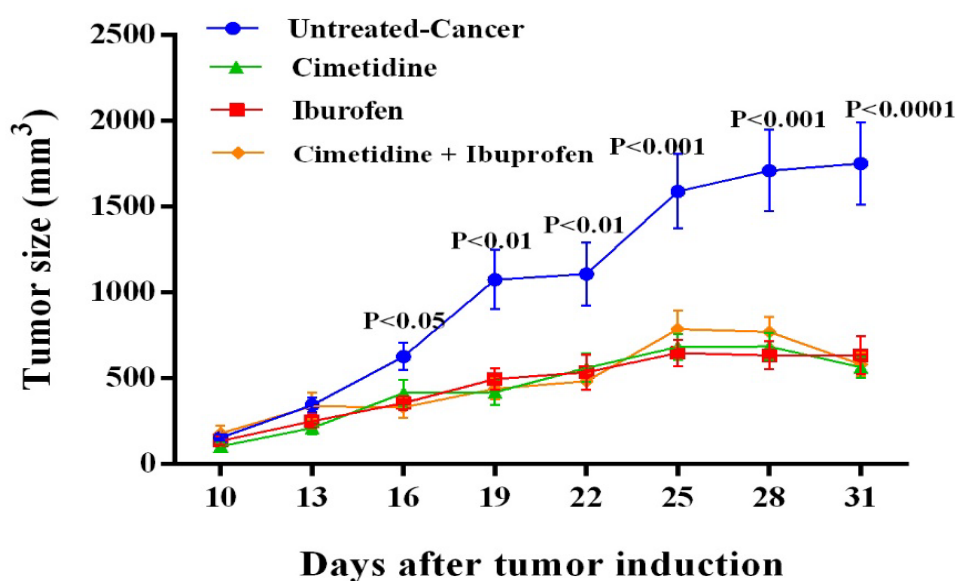


Figure 2. Effects of Treatment with Cimetidine and Ibuprofen on Tumor Sizes in Mice with Breast Cancer. The tumor sizes in cancerous mice treated with dose 20 mg/kg cimetidine and/or ibuprofen on days 16, 19, 22, 25, 28 and 31 after tumor induction were significantly lower than in non-treated cancer bearing mice.

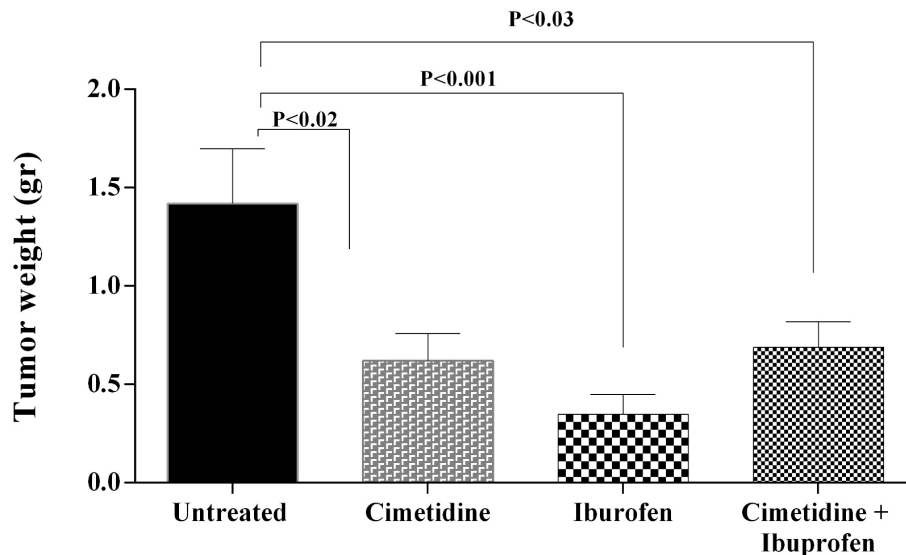


Figure 3. Effects of Treatment with Cimetidine and Ibuprofen on Tumor Weights in Mice with Breast Cancer. The tumor weights were reduced in breast cancer bearing mice treated with dose 20 mg/kg of cimetidine or/and ibuprofen as compared with un-treated cancerous mice.

un-treated cancerous mice ( $P<0.03$ ,  $P<0.02$  and  $P<0.05$ , respectively). No statistically significant differences were detected between cimetidine-and/or ibuprofen-treated mice regarding the spleen indexes.

*The splenic Th1- and Treg cells in cimetidine and/or ibuprofen-treated mice*

The percentages of the splenic Th1- and Treg cells were  $2.46 \pm 0.35$  and  $2.22 \pm 0.86$  in the healthy group,  $0.70 \pm 0.12$  and  $3.56 \pm 0.74$  in un-treated cancerous mice,  $2.11 \pm 0.58$  and  $1.84 \pm 0.97$  in cimetidine-treated group,  $1.90 \pm 0.29$  and  $2.00 \pm 1.13$  in ibuprofen-treated mice, and  $1.75 \pm 0.23$  and  $1.72 \pm 0.97$  in “cimetidine + ibuprofen”-treated group, respectively (Figure 6 and Figure 7). The

un-treated cancerous mice showed a lower percentage of the splenic Th1 cells and higher percentage of the splenic Treg cells in comparison with healthy mice ( $P<0.003$  and  $P<0.04$ , respectively). The cimetidine, ibuprofen and “cimetidine + ibuprofen”-treated BC mice exhibited higher frequencies of the splenic Th1 cells compared with un-treated cancerous mice ( $P<0.05$ ,  $P<0.007$  and  $P<0.005$ , respectively) (Figure 6). The frequencies of the splenic Treg cells in cimetidine, ibuprofen and “cimetidine + ibuprofen”-treated BC mice were reduced compared to un-treated cancerous mice ( $P<0.02$ ,  $P<0.03$  and  $P<0.01$ , respectively) (Figure 7). The differences of the percentages of the splenic Th1- and Treg cells between cimetidine and/or ibuprofen-treated groups were not significant.

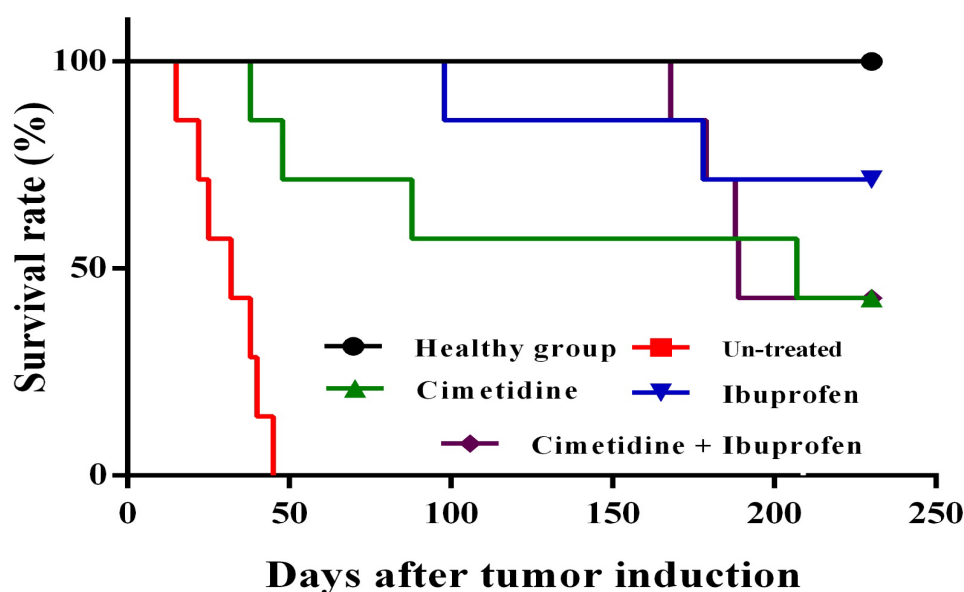


Figure 4. Effects of Treatment with Cimetidine and Ibuprofen on Survival Rate in Mice with Breast Cancer. The survival rate was enhanced in breast cancer bearing mice treated with dose 20 mg/kg of cimetidine or/and ibuprofen as compared with that in un-treated cancerous mice.

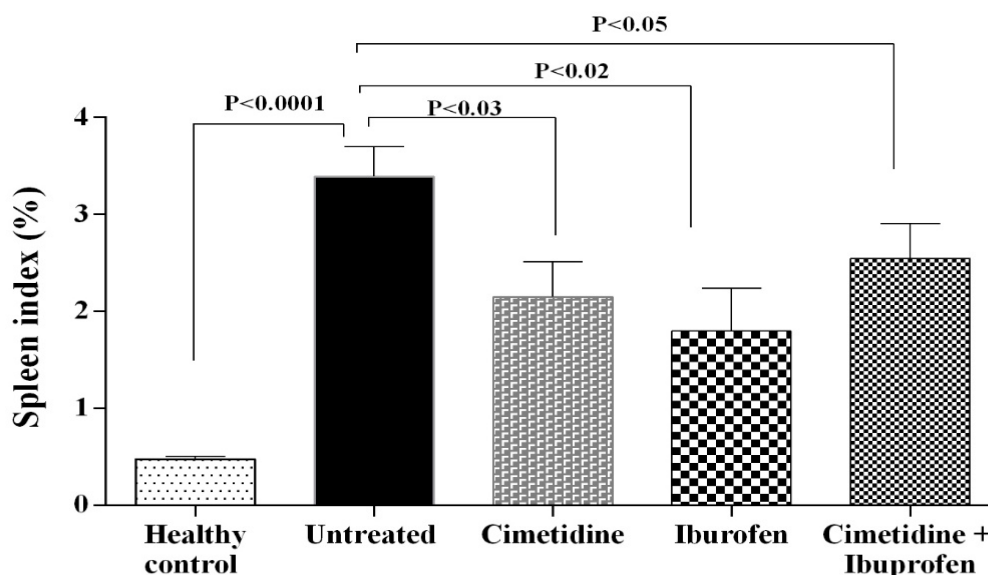


Figure 5. Effects of Treatment with Cimetidine and Ibuprofen on Spleen Index in Mice with Breast Cancer. The spleen index was greater in un-treated cancer bearing mice compared with healthy group and the treatment of breast cancer bearing mice with dose 20 mg/kg of cimetidine or/and ibuprofen reduced the spleen index compared with that in un-treated cancerous mice.

*The plasma IFN- $\gamma$  and TGF- $\beta$  levels in cimetidine and/or ibuprofen-treated mice*

The plasma IFN- $\gamma$  and TGF- $\beta$  levels were  $118.41 \pm 16.51$  Pg/ml and  $173.22 \pm 12.15$  Pg/ml in healthy group,  $58.76 \pm 12.73$  Pg/ml and  $358.51 \pm 40.90$  Pg/ml in un-treated cancerous mice,  $118.09 \pm 11.35$  Pg/ml and  $168.61 \pm 36.32$  Pg/ml in cimetidine-treated group,  $156.49 \pm 13.20$  Pg/ml and  $197.43 \pm 37.71$  Pg/ml in ibuprofen-treated mice, and  $207.86 \pm 53.03$  Pg/ml and  $151.99 \pm 27.68$  Pg/ml in “cimetidine + ibuprofen”-treated group, respectively (Figure 8).

As demonstrated in Figure 8, the plasma quantities

of the IFN- $\gamma$  in un-treated cancerous mice were significantly reduced compared with the healthy mice ( $P < 0.01$ ). Treatment of BC-bearing mice with cimetidine, ibuprofen or both drugs increased the plasma amounts of the IFN- $\gamma$  in comparison with un-treated cancerous mice ( $P < 0.004$ ,  $P < 0.0001$  and  $P < 0.03$ , respectively). Statistical analysis showed that the plasma IFN- $\gamma$  levels in ibuprofen-administrated mice were significantly higher than cimetidine-treated group ( $P < 0.05$ ). Although, the “cimetidine + ibuprofen”- administrated mice showed greater plasma quantities of IFN- $\gamma$  compared with cimetidine or ibuprofen groups, but differences were not

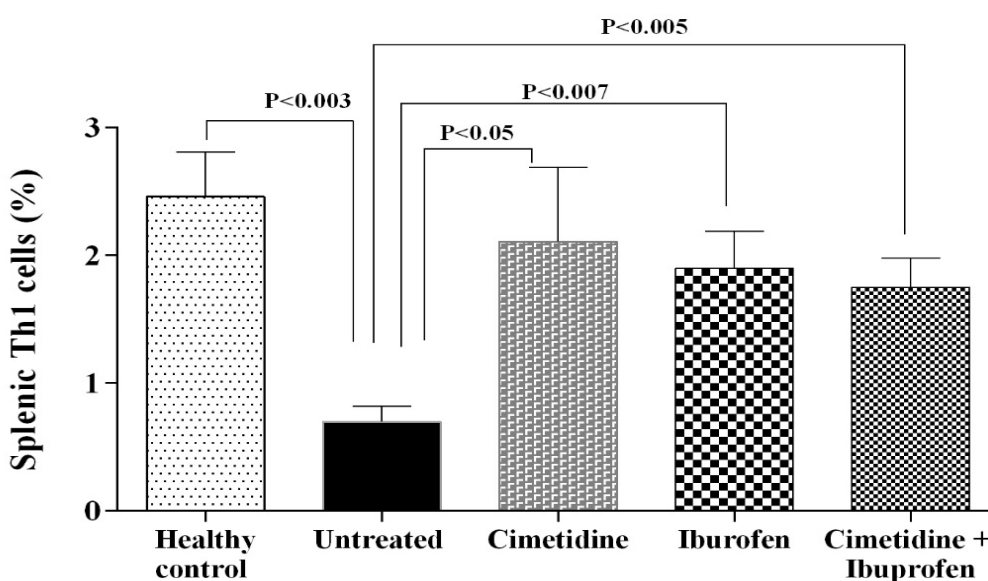


Figure 6. Effects of Treatment with Cimetidine and Ibuprofen on the Percentage of Splenic Th1 Cells in mice with Breast Cancer. The percentage of the splenic Th1 cells was reduced in untreated cancer bearing mice compared with healthy group and treatment of breast cancer bearing mice with dose 20 mg/kg of cimetidine or/and ibuprofen increased the percentage of the splenic Th1 cells compared with that in untreated cancerous mice.

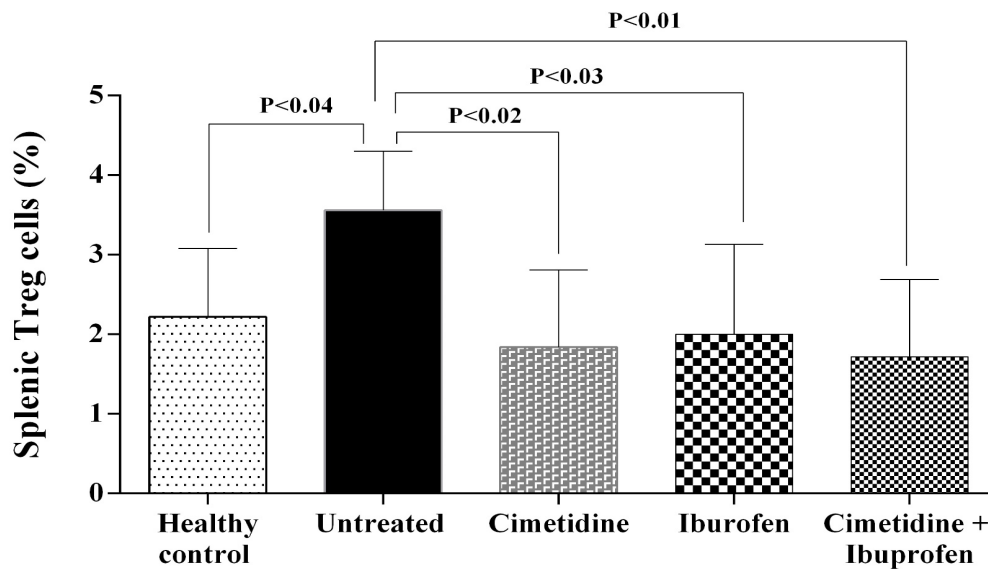


Figure 7. Effects of Treatment with Cimetidine and Ibuprofen on the Percentage of Splenic Treg Cells in Mice with Breast Cancer. The percentage of the splenic Treg cells was increased in untreated cancer bearing mice compared with healthy group. Treatment of breast cancer bearing mice with dose 20 mg/kg of cimetidine or/and ibuprofen reduced the percentage of the splenic Treg cells compared with that in untreated cancerous mice.

significant. The plasma levels of TGF- $\beta$  in cimetidine, ibuprofen and “cimetidine + ibuprofen”-treated BC mice were reduced compared to untreated cancerous mice ( $P < 0.006$ ,  $P < 0.02$  and  $P < 0.002$ , respectively) (Figure 8). The differences of the plasma levels of TGF- $\beta$  between cimetidine and/or ibuprofen-treated groups were not significant.

*The intra-tumoral T-bet, FOXP3, GATA3 and ROR $\gamma$ t expression in cimetidine and/or ibuprofen-treated mice*

The fold changes expression of T-bet and FOXP3 were  $1.00 \pm 0.07$  and  $1.00 \pm 0.33$  in un-treated cancerous mice,  $3.55 \pm 1.77$  and  $0.24 \pm 0.10$  in cimetidine-treated group,  $2.08 \pm 1.35$  and  $0.14 \pm 0.05$  in ibuprofen-treated mice, and  $6.23 \pm 1.12$  and  $0.18 \pm 0.06$  in “cimetidine + ibuprofen”-treated group. The fold changes expression of

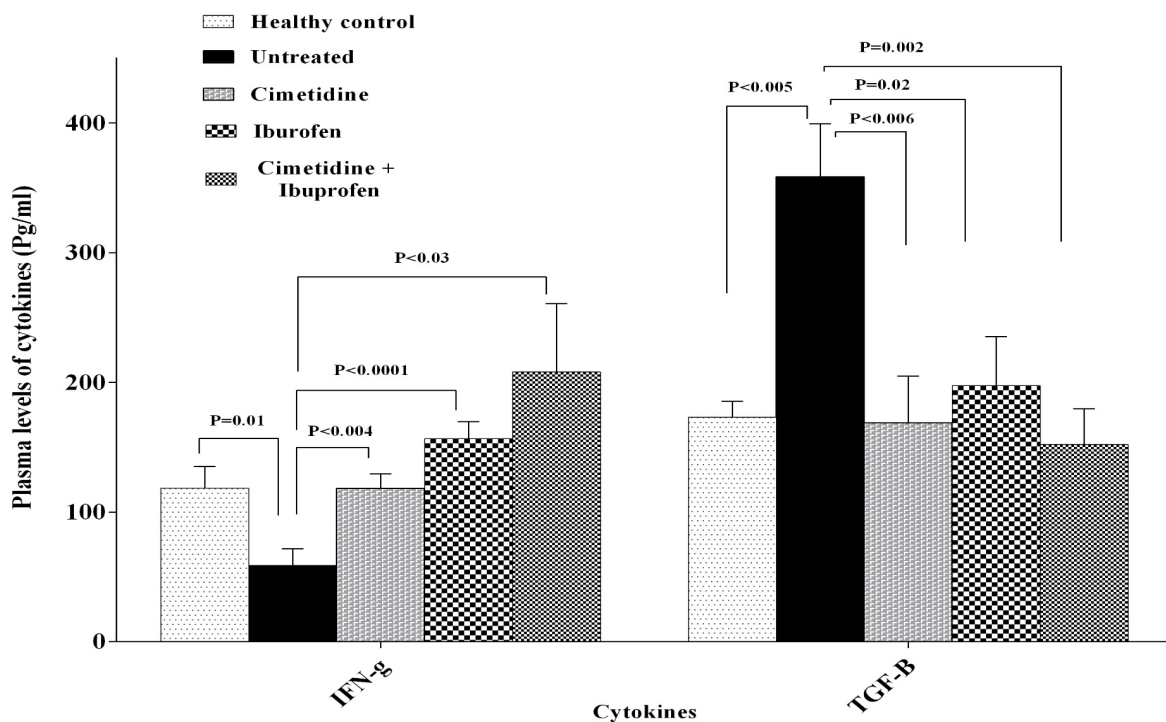


Figure 8. Effects of Treatment with Cimetidine and Ibuprofen on the Plasma Levels of IFN- $\gamma$  and TGF- $\beta$  in Mice with Breast Cancer. The plasma IFN- $\gamma$  levels were reduced, while the plasma TGF- $\beta$  levels were increased in untreated cancer bearing mice compared with the healthy group. Treatment of breast cancer bearing mice with dose 20 mg/kg of cimetidine or/and ibuprofen increased the plasma IFN- $\gamma$  levels, whereas reduced the TGF- $\beta$  levels compared with those in untreated cancerous mice.

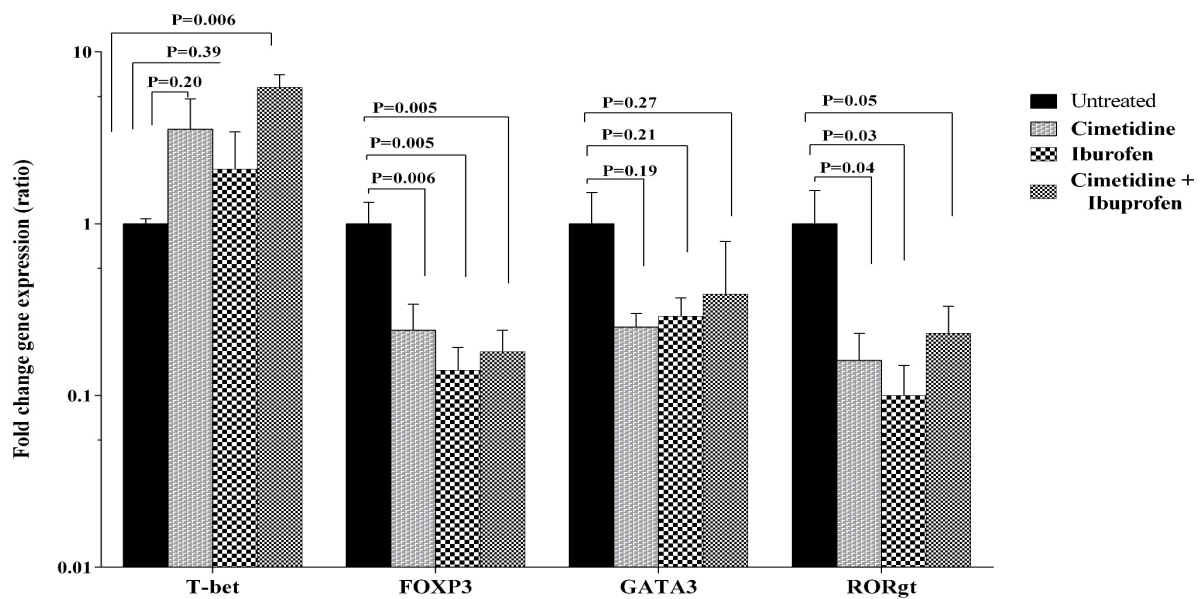


Figure 9. Effects of Treatment with Cimetidine and Ibuprofen on the Intra-Tumoral T-bet, FOXP3, GATA3 and RORgt Expression in mice with Breast Cancer. Treatment of breast cancer-bearing mice with dose 20 mg/kg of cimetidine plus ibuprofen significantly increased the T-bet expression compared with untreated cancerous mice. Treatment of cancerous mice with dose 20 mg/kg of cimetidine or/and ibuprofen significantly reduced the FOXP3 and RORgt expression compared with that in untreated cancerous mice. Treatment of cancerous mice with dose 20 mg/kg of cimetidine or/and ibuprofen also reduced the GATA3 expression compared with that in untreated cancerous mice, but differences were not significant.

T-bet was greater in “cimetidine + ibuprofen”-treated mice compared with un-treated cancerous mice ( $P < 0.006$ ). The difference of the T-bet expression between cimetidine- or ibuprofen-treated mice with un-treated cancerous mice was not significant ( $P = 0.20$  and  $P = 0.39$ , respectively) (Figure 9). The fold change expression of T-bet was also higher in “cimetidine + ibuprofen”-treated mice compared with ibuprofen-treated mice ( $P < 0.05$ ). The difference

in the expression of T-bet between cimetidine- and ibuprofen-treated mice was not significant. The expression of FOXP3 was reduced in cimetidine, ibuprofen and “cimetidine + ibuprofen”-treated BC mice compare to untreated cancerous mice ( $P < 0.006$ ,  $P < 0.005$  and  $P < 0.005$ , respectively) (Figure 9). The differences of the intra-tumoral expression of FOXP3 between cimetidine and/or ibuprofen-treated groups were not significant.

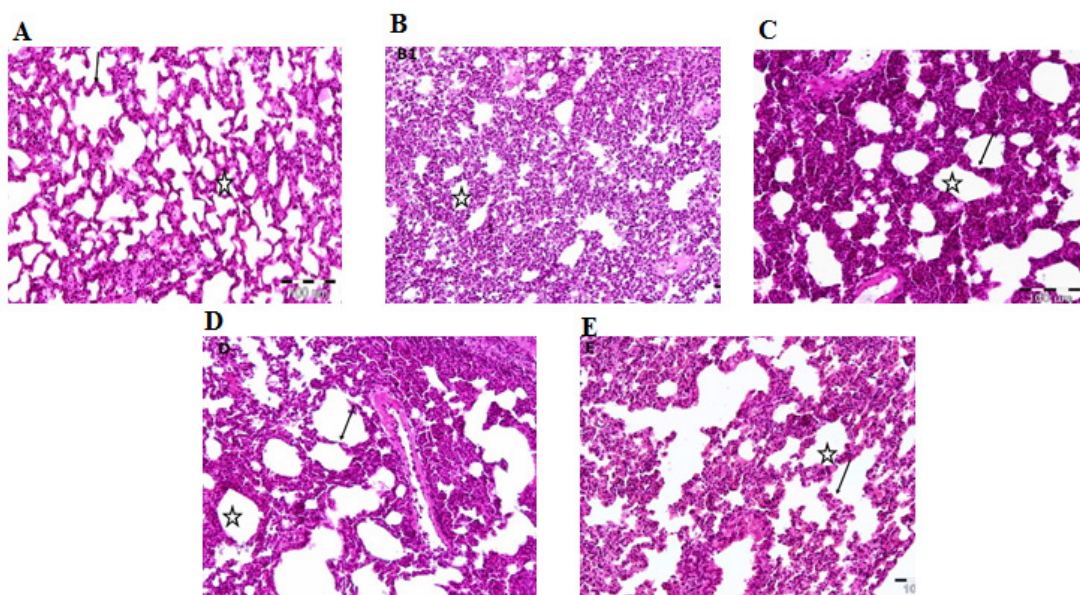


Figure 10. Hematoxylin and Eosin Staining of Lung Sections. 10A shows lung pattern from normal mice. 10B shows the lung pattern from untreated breast cancer mouse. The alveolar space is reduced, while the thickness of alveoli wall was increased due to metastases formation. 10C, 10D and 10E indicate the lung patterns form ibuprofen-, cimetidine-, and “ibuprofen + cimetidine”-breast cancer treated mice, respectively. As indicated the alveolar spaces are increased, while the thickness of alveoli walls were reduced in treated breast cancer mice compared to untreated breast cancer mouse. The star and arrow symbols indicate the alveolar space and the thickness of alveoli wall, respectively.



The fold changes expression of GATA3 and ROR $\gamma$ t were  $1.00 \pm 0.52$  and  $1.00 \pm 0.56$  in un-treated cancerous mice,  $0.25 \pm 0.05$  and  $0.16 \pm 0.07$  in cimetidine-treated group,  $0.29 \pm 0.08$  and  $0.10 \pm 0.05$  in ibuprofen-treated mice, and  $0.39 \pm 0.40$  and  $0.23 \pm 0.10$  in “cimetidine + ibuprofen”-treated group. The differences of the intra-tumoral expression of GATA3 between untreated and cimetidine and/or ibuprofen-treated groups were not significant (Figure 9). The fold changes expression of ROR $\gamma$ t was reduced in cimetidine, ibuprofen and “cimetidine + ibuprofen”-treated BC mice compare to untreated cancerous mice ( $P < 0.05$ ,  $P < 0.03$  and  $P < 0.05$ , respectively) (Figure 9). The differences of the intra-tumoral expression of ROR $\gamma$ t between cimetidine and/or ibuprofen-treated groups were not significant.

### Histopathological analysis

Hematoxylin and eosin staining, and microscopic observations showed histopathological alterations in lung tissues from untreated breast cancer mice. As the space of the pulmonary alveoli reduced, while the thickness of alveoli wall increased in untreated breast cancer mice due to pathological reactions. A few metastatic colonies were also observed in lung tissues from some untreated breast cancer mice. The alveolar spaces are increased, while the thicknesses of alveoli walls were reduced in treated breast cancer mice, especially in “ibuprofen + cimetidine”-breast cancer treated mice compared to untreated breast cancer mice (Figure 10).

## Discussion

The results of this study indicated that the treatment of BC-bearing mice with cimetidine and/or ibuprofen prevented the weight loss, reduced the tumor size and weight, and enhanced the survival rate compared with the non-treated cancer-bearing mice. In an animal model of lung carcinoma, cimetidine reduced tumor growth and improved the survival rates of tumor-bearing mice (Zheng et al., 2013). The use of cimetidine also increased the survival time in patients with colorectal cancer (Ali et al., 2018). Furthermore, in a mouse model of colorectal cancer, it was found that treatment with ibuprofen prevented the loss of body weight, reduced the tumor volume, and enhanced the survival rate (Yao et al., 2005). The use of ibuprofen also reduced the risk of mortality in patients with lung cancer (Bittoni et al., 2017).

Elevated quantities of histamine have been detected in some malignancies such as breast, prostate, ovarian, cervical- and lung cancer (Kubecova et al., 2011). Various types of malignant cells also express the H2 receptor, and histamine may directly induce the expansion of certain tumor cells and/or exerts pro-tumor activities through H2 receptor-mediated immune suppression (Falus and Gilicze, 2014; Faustino-Rocha et al., 2017; Jafarzadeh et al., 2019). The overexpression of COX-2 and PGE2 was also reported in cancerous cells, which are related to tumor progression and resistance to current therapeutic elements (Pang et al., 2016). Therefore, cimetidine as an H2 blocker agent and ibuprofen as an inhibitor of COX

can interfere with tumor development and progression. Cimetidine and ibuprofen may directly and indirectly influence tumor growth. Cimetidine induces apoptosis in the colorectal cancer cells, salivary gland tumor cells and gastric cancer cells (Fukuda et al., 2007; Kubecova et al., 2011). Cimetidine possesses anti-tumorigenic effects via triggering of apoptosis in tumor cells, suppressing the vascular endothelial growth factor (VEGF) expression, preventing angiogenesis, and reducing the adhesion of tumor cells (Kawase et al., 2009; Kubecova et al., 2011; Jafarzadeh et al., 2019). Ibuprofen may also exert anti-tumor effects by inducing of apoptosis in cancer cells, inhibiting of tumor cell expansion, inducing of the tumor suppressor proteins and preventing of angiogenesis (Akrami et al., 2015; Upadhyay et al., 2016).

The results presented here also showed that the spleen index was augmented in un-treated cancerous mice. Treatment of BC-bearing animals with cimetidine and/or ibuprofen reduced the spleen index. The beneficial impacts of cimetidine on the spleen architecture were also reported in animals with burn or sulfur mustard injuries (Ebtekar and Hassan, 1993; Gharegozloo et al., 2004; Jafarzadeh et al., 2010). In accordance with our results, it has been observed that treatment with ibuprofen decrease the spleen weight in the colon tumor-bearing mice (Norden et al., 2015).

We have observed the treatment of BC-bearing mice with cimetidine and/or ibuprofen modulated the number of the splenic Th1- and Treg cells, plasma levels of IFN- $\gamma$  (a Th1 cell-related cytokine), plasma levels of TGF- $\beta$  (a Treg cell-related cytokine), intra-tumoral expression of the T-bet (a main Th1-related transcription factor) and intra-tumoral expression of the FOXP3 (a main Treg-related transcription factor) compared with un-treated cancerous mice.

In accordance with our findings, it has been revealed that the anti-tumorigenic effects of the cimetidine may be performed via stimulating the proper immune responses, such as enhancing macrophages activation, increasing the NK cell activity, reducing the development of myeloid derived-suppressive cells (MDSCs), inhibiting Treg cell functions, up-regulating anti-tumor cytokines (including IL-2, IL-12, IL-15, IFN- $\gamma$ , TNF- $\alpha$  and TNF- $\beta$ ), improving the functions of tumor-infiltrating lymphocytes (TIL), and increasing the immunostimulatory characteristics of DCs (Li et al., 2005; Kubecova et al., 2011; Li et al., 2013; Jafarzadeh et al., 2019). Moreover, cimetidine abolishes the MDSC-induced T cell inactivation and restores the IFN- $\gamma$  production (Zheng et al., 2013; Jafarzadeh et al., 2019). The number of NK cells, total T lymphocytes, and Th cells was also enhanced in cimetidine-treated patients with gastrointestinal cancers (Li et al., 2005; Kubecova et al., 2011; Jafarzadeh et al., 2019).

In addition, cimetidine also inhibits the histamine-induced IL-10 expression, while promoting the histamine-reduced IL-12 (a key cytokine of Th1 cell differentiation) secretion in lipopolysaccharide (LPS)-stimulated DCs (Zhang et al., 2011; Jafarzadeh et al., 2019). When mixed with the hepatitis B vaccine, cimetidine enhances the IL-12 and IFN- $\gamma$  quantities, while decreasing the IL-10

concentrations in immunized mice (Shemanski, 2016). Cimetidine also has inhibitory impacts on the Treg cell functions (Wang et al., 2008). Cimetidine leads to FOXP3 degradation (a Treg cell-related major transcription factor) accompanied by the impairment of Treg cell activity (Zhang et al., 2016; Jafarzadeh et al., 2019). Hence, cimetidine has the capacity to directly or indirectly correct the Th1 cell-mediated immune responses in BC-bearing mice.

As mentioned earlier, the COX-2/PGE2 axis strongly inhibits the anti-tumor immune responses through suppressing the NK cell activities, inhibiting the DC maturation, damping the CTL responses, increasing the Treg cell activation, promoting the M2 macrophage development, enhancing the MDSC differentiation and reinforcing the Th2 cell-related responses (Liu et al., 2015). PGE2 also has repressive impacts on the Th1 cells via the preventing the IL-12 production by antigen-presenting cells (APCs), while promoting the IL-10 generation (Rodriguez et al., 2014). PGE2 also reinforces the inhibitory activity of Treg cells (Sahin et al., 2013). Therefore, ibuprofen may neutralize the effects of PGE2 on Th1- and Treg cells which lead to the potentiation of anti-tumor immunity. Ibuprofen-treated mice with postpartum BC exhibit lower involution-related tumor burden, higher numbers of T cells in the tumor border regions, and greater numbers of mature macrophages, and higher levels of intratumoral Th1/M1-derived cytokines such as TNF- $\alpha$ , IL-12, and IL-2 (Pennock et al., 2018).

Our results indicated that the plasma IFN- $\gamma$  levels in the ibuprofen-administrated mice were significantly higher than the cimetidine-treated group. These results revealed that ibuprofen may have more powerful effects than cimetidine concerning the IFN- $\gamma$  induction. It was observed that the difference in T-bet expression between the cimetidine- or ibuprofen-treated mice and the un-treated cancerous mice was not significant. The “cimetidine + ibuprofen”-treated mice exhibited significantly greater T-bet expression compared with the untreated BC mice. These results demonstrated that combination therapy using “cimetidine + ibuprofen” may be more efficient than a single treatment with cimetidine or ibuprofen regarding the T-bet expression.

The present results also indicated that the treatment of BC mice with cimetidine, ibuprofen and “cimetidine + ibuprofen” reduced the intra-tumoral expression of ROR $\gamma$ t. It has been shown that 4T1 tumor cell-secreted PGE2 promotes IL-23 and Th17 cell development during BC progression (Qian et al., 2013). Therefore, the intra-tumoral expression of Th17 cell-related elements, such as ROR $\gamma$ t can be attributed to PGE2-mediated Th17 cell development (1). Accordingly, ibuprofen as an inhibitor of PGE2 production can prevent Th17 cell development. Furthermore, the inhibitory effects of cimetidine on the Th17 cell responses were also reported (Jafarzadeh et al., 2019); however, the exact mechanism of action of cimetidine on Th17 cells remains unclear and should be further investigated in future studies.

The intra-tumoral expression of GATA3 was also lower in the cimetidine-, ibuprofen- and “cimetidine + ibuprofen”-

treated groups compared with the untreated-BC mice, but the differences were not significant. There are controversies regarding the pro- or anti-tumoral effects of Th2 cells (Chraa et al., 2019). Although, there are reports concerning the modulatory effects of cimetidine and ibuprofen on Th2 cell-related responses (Machado et al., 2010; Jafarzadeh et al., 2019), the role of Th2 cells during malignancies and their modulation need to be clarified in future studies.

Ibuprofen-treated mice displayed greater body weight and survival rate, while lower tumor and spleen index compared with cimetidine-treated mice, although differences did not reach to a meaningful value. However, the plasma IFN- $\gamma$  levels in ibuprofen-administrated mice were significantly higher than cimetidine-treated group. Thus, it seems that ibuprofen exerts better effects than cimetidine in this animal tumor model. With exception of the T-bet expression, no remarkable differences were observed between the “cimetidine + ibuprofen”-treated mice and cimetidine or ibuprofen-treated mice regarding the other investigated parameters. Thus, synergistically effects of the cimetidine and ibuprofen can be excluded concerning the investigated parameters.

In summary, cimetidine and ibuprofen modulated the major effector T cell-related parameters in a mouse model of BC. Cimetidine and ibuprofen also displayed profitable effects on the body weight, survival rate, tumor size, and spleen index in investigated BC model. The immunotherapeutic potentials of cimetidine and ibuprofen for cancer treatment need more considerations.

## Author Contribution Statement

Study conception and design: AJ; data collection: OO, FT, MTR, MN, AT; analysis and interpretation of results: AJ, ZMH; draft manuscript preparation: AJ, OO, FT; All authors reviewed the results and approved the final version of the manuscript.

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## Conflict of interest

The authors declare no conflict of interest.

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