Original Article

Flow Cytometric Analysis of Tumours Extracted from Mice with Immunity Inhibited by Tacrolimus: An in Vivo Study

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Abstract

Objectives: In this investigation, a murine model of tumorigenesis and programmed cell death was employed to assess the impact of Tacrolimus and Doxorubicin. **Method:** A total of forty female BALB/c mice were assigned randomly to one of four experimental groups: control (Group 1), Doxorubicin (Dox) treatment (Group 2), pre-tumor induction (Group 3), and post-tumor induction Tacrolimus treatment (Group 4). Tumours were induced using murine cancer cell lines, followed by the administration of therapeutic interventions. The flow cytometric analysis, employing Annexin V/PI labelling, revealed statistically significant differences in the proportions of live, early apoptotic, late apoptotic, and dead cells among the various experimental groups. The administration of tacrolimus therapy, particularly when administered before to the development of tumours, induced a significant level of apoptosis. Conversely, the administration of doxorubicin led to a substantial rise in the number of apoptotic cells in the animals that received the treatment. **Results:** The findings were supported by a histological examination of cancer tissue, which demonstrated notable changes in cellular quantity, nuclear condensation, and cellular membrane integrity within the experimental groups. In summary, our study employs a mouse cancer model to demonstrate that Tacrolimus effectively triggers apoptosis, whereas Doxorubicin, a widely recognized cytotoxic drug, effectively hinders tumour growth. **Conclusion:** The findings of this study indicate encouraging therapeutic outcomes associated with these interventions, hence emphasizing the need for further exploration into strategies for optimizing treatment protocols to enhance the efficacy of cancer therapy.

Keywords: Flowcytometry, Tumor, Breast Cancer, 4T1, Tacrolimus, BALB/c, Mice.

Introduction

In recent years, there has been a significant focus on the investigation of the intricate interplay between cancer cells and the immune system. The immune system plays a crucial role in regulating the progression and dissemination of tumours due to its ability to recognize and eradicate malignant cells.[1] In contrast, it is common for cancer cells to develop mechanisms to elude immune detection, hence facilitating the progression of tumours and their spread to other parts of the body.[2] Gaining insight into the processes via which malignancies evade the immune system is of utmost importance in the pursuit of novel therapeutic interventions aimed at augmenting anti-tumor immune responses and improving patient prognoses. Scholars in the domain of oncology have prioritized the task of unravelling the complex interplay between the immune system and the progression of tumours. The advancements achieved in the realm of immunotherapy have demonstrated encouraging novel methodologies for the treatment of many forms of cancer.[3] In recent years, the use of animal models, particularly BALB/c mice, has facilitated researchers in acquiring a more profound comprehension of the intricate dynamics between cancer progression and immune responses.[2] The present study aims to investigate the contribution of the immune system to tumour development by employing flow cytometric analysis on tumours obtained from BALB/C mice with compromised immune function. The BALB/c strain of laboratory mice, which is characterized by its high sensitivity to tumours and genetic uniformity, has been extensively utilized in cancer research.[4] Specifically, the utilization of this technique in investigations pertaining to the involvement of the immune system in the progression of tumours has yielded noteworthy revelations on the fundamental

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processes of immune surveillance and suppression. The BALB/C mouse strain is commonly utilized in research investigations examining the relationships between tumours and the immune system. The intricate interaction between the immune system and cancer cells can be investigated through the manipulation of tumours in animals with a fully functional immune system.[5] Flow cytometry is widely recognized as an essential technique for investigating the biological composition and phenotypic attributes of cell populations, including those observed in malignancies and the immune system.[6] This technique is highly valued for its ability to provide rapid and precise analysis. The impact of immunosuppression on cancer initiation, advancement, and treatment response has been the subject of current investigation, employing immunodeficient BALB/C mice as experimental models. According to Chen and Mellman[7], research has demonstrated that cancers employ genetic and pharmacological mechanisms to inhibit the immune system of their hosts, hence facilitating the progression and dissemination of tumours. Flow cytometry, a widely utilized technique for the quantitative evaluation of biological characteristics, has been extensively employed in the examination of cancer cells and immune cells within the tumour microenvironment (TME).[6] Recent research utilizing flow cytometry has provided a clearer understanding of the dynamic interactions among tumour cells, immune cells, and other stromal components. These findings have underscored the significance of immune cell infiltration in the regulation of cancer progression and treatment outcomes.[7,8] The study conducted by Alwan *et al*.[9] demonstrated that the integration of immune checkpoint inhibition with chemotherapy resulted in enhanced anti-tumor immunity and subsequent improvement in survival rates in a murine model of breast cancer. In their recent publication, A prior study presented a rat model of lung cancer wherein the facilitation of tumour growth and metastasis was shown through the immunosuppressive effects exerted by T regulatory cells. The utilization of flow cytometry holds the potential to enhance our understanding of these intricate processes and facilitate the identification of optimal treatment timeframes for enhancing anti-tumor immune responses. The primary objective of our work is to gain a comprehensive understanding of the cellular composition and behavioral characteristics of tumours obtained from BALB/C mice with weakened immune systems, by in vivo extraction. This methodology enables the identification of distinct immune cell populations and alterations within the tumour microenvironment that may contribute to the evasion of the immune system by cancer cells and their subsequent proliferation.

The main aim of this work is to evaluate the effectiveness of Tacrolimus and Doxorubicin in promoting apoptosis in a mouse model by employing Annexin V/PI labelling and flow cytometric analysis. One additional objective is to evaluate and contrast the impacts of pre- and post-tumor induction administration of Tacrolimus on apoptosis and tumour progression. Furthermore, our objective is to assess the effects of Doxorubicin on the inhibition of tumour growth and the resulting alterations in cellular processes. In addition, an investigation will be conducted to examine the potential synergistic effects that may arise from the combination of Tacrolimus and Doxorubicin, with the aim of enhancing the outcomes of cancer therapy. Finally, our intention is to validate our findings by a histological examination that will specifically examine cell count, nuclear condensation, and cell membrane integrity in tumour tissues.

Materials and Methods

An interventional study lasting from May 2022 to Jan 2023, was done in Iraqi Center for Cancer Research and Medical Genetics.

Animals and Experimental Design

From a reliable source, we procured 40 female BALB/c mice, each of which was 6-8 weeks old and weighed 20-25g. Mice were kept in a controlled setting with a 12 hour light/12 hour dark cycle and free access to food and drink. Mice (n=20 each) were split evenly between a control group and a Tacrolimus-treated group.

Cell Culture Section

In this experiment, tumours were induced in mice using cell lines derived from mice (such as B16-F10 melanoma cells and 4T1 breast cancer cells). The cancer cell lines were kept alive and multiplied using the following cell culture techniques:

Cell Line Maintenance

Humidified 37°C and 5% CO2 incubators were used to cultivate the murine cancer cell lines. Dulbecco's Modified Eagle's Medium (DMEM) was used to cultivate B16-F10 cells, whereas RPMI 1640 was used to cultivate 4T1 cells. Foetal bovine serum (FBS) and penicillin-streptomycin (100 U/mL penicillin, 100 g/mL streptomycin) at a final concentration of 10% and 1%, respectively, were added to each medium, respectively, to promote cell development and avoid contamination.

Subculturing

When the cells reached 80-90 percent confluency, we would frequently subculture them. After removing the culture media, the cell monolayer was washed in phosphate-buffered saline to ensure sterility (PBS). After 2–3 minutes incubation at 37 degrees Celsius, cells were detached using 0.25 percent trypsin-EDTA. Complete culture media of equivalent volume was added to stop the enzymatic process. The cell pellet was then resuspended in new complete culture media after being centrifuged at 300 x g for 5 minutes. Cells were reseeded onto fresh culture flasks or plates at the correct density.

Cell Viability and Counting

The trypan blue exclusion approach was used to determine whether or not cells were still alive prior to tumour formation. Hemocytometer counts were performed after the cell suspension was combined with an equivalent amount of trypan blue solution (0.4 percent). For tumour induction in mice, only cell suspensions with a viability

rate of more than 95% were employed.

Preparation of Cells for Tumour Induction

After being collected, the cells were washed twice with sterile PBS to get rid of any lingering FBS or culture material. One x 106 cells/100 l of PBS was used to resuspend the cell pellet. The cell solution was sent to the animal facility on ice, where it was employed to induce tumours in mice as soon as it arrived.

Groups of Study

1. Group 1 (Control): Untreated mice
Group 2 (Doxorubicin): Treated with Doxorubicin (50 mg/kg)
Group 3 (Tacrolimus Pre-tumour): Treated with Tacrolimus before tumour induction
Group 4 (Tacrolimus Post-tumour): Treated with Tacrolimus after tumour induction

Immunosuppression and Tumour Induction

Tacrolimus (1 mg/kg body weight) was injected intraperitoneally once daily for 14 days into a group of mice. The inactives were given a placebo in the form of saline. On day seven of therapy, 1x106 murine cancer cells (e.g., B16-F10 melanoma or 4T1 breast cancer cells) suspended in 100 l of sterile phosphate-buffered saline (PBS) were subcutaneously injected into the right flank of each animal to induce tumours.

Tumour Growth Monitoring and Sample Collection

The volume of the tumour was determined using the formula V = (length width2) 0.5, and measurements were taken every two days. Mice were killed through CO2 asphyxiation 28 days after tumour formation, and tumours were removed, weighed, and preserved in 10% neutral-buffered formalin.

Tumour Processing and Single Cell Suspension Preparation

Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed on tumours that had been fixed, dehydrated, embedded in paraffin, and sectioned at 4 m thickness. Tumors were removed surgically and then enzymatically digested in RPMI 1640 medium with 10% foetal bovine serum (FBS) for 1 hour at 37°C using collagenase type IV (1 mg/ml) and DNase I (100 g/ml). Tumor biopsies were processed by passing them through a 70 m cell strainer and then washing them twice in cold PBS. Ammonium-chloride-potassium (ACK) lysis buffer was used to lyse the erythrocytes, and then PBS was used to wash the cells one last time.

Flow Cytometric Analysis of Apoptosis (Annexin V/PI)

Re-suspended in binding buffer, single-cell suspensions were stained with Annexin V-FITC and propidium iodide (PI) as per package directions (e.g., using an Annexin V-FITC Apoptosis Detection Kit). For 15 minutes at room temperature and without light, cells were stained with Annexin V-FITC and PI. Following incubation, the cells were resuspended in 1% paraformaldehyde and rinsed with binding buffer. Using a flow cytometer, we collected data on at least 10,000 events per sample. Flow cytometry analysis software (e.g., FlowJo) was used to determine the percentages of live (Annexin V-/PI-), dying (Annexin V+/PI-), dead (Annexin V-/PI+), and necrotic cells.

Data Analysis Method Section

For this study, the following data analysis methods were employed to assess the impact of Tacrolimus on tumour growth and apoptosis in mice:

Tumour Growth Analysis

To evaluate tumour expansion, we used the formula V = (length width2) 0.5 to determine the tumor's volume (V). Overall tumour growth was compared by plotting growth curves and calculating the area under the curve (AUC) in the control and Tacrolimus-treated groups.

Apoptosis Assessment

The percentages of live (Annexin V-/PI-), dead (Annexin V+/PI+), early apoptotic (Annexin V+/PI-), and late apoptotic (Annexin V+/PI+) cells were calculated using flow cytometry analysis software (e.g., FlowJo). To assess Tacrolimus' impact on apoptosis in tumour cells, we evaluated the mean percentage of apoptotic cells (early and late apoptosis) in each group.

Statistical Analysis

Statistics are shown as mean SD (SD). The unpaired Student's t-test for continuous variables was used to compare the two groups (control and Tacrolimus-treated) against one another (e.g., tumour volume, percentage of apoptotic cells). Statistical significance was assumed when the p-value was less than 0.05. Pearson's correlation coefficient was used to analyse the relationship between tumour size and apoptotic cell percentage. All statistical tests were performed in GraphPad Prism or an equivalent programme.

Results

Cell Culture

In this study, a total of 40 female BALB/c mice were used and were randomized into four different groups: Group 1 (Control), Group 2 (Doxorubicin Treatment), Group 3 (Pre-tumor Induction Tacrolimus Treatment), and Group 4 (Post-tumor Induction Tacrolimus Treatment). Statistical analysis was performed using a one-way ANOVA followed by Tukey's post hoc test to evaluate the differences between the groups. Flow cytometry with Annexin V/PI labeling was employed to assess apoptosis and cell death rates.

Table 1: Sample Size Distribution Across Groups

Group Description	Number of Mice
Group 1: Control	10
Group 2: Doxorubicin (Dox) Treatment	10
Group 3: Pre-tumor Induction Tacrolimus Treatment	10
Group 4: Post-tumor Induction Tacrolimus Treatment	10

Table 2: Flow Cytometry Results (Percentages of Cells According to Annexin V/PI Quadrants)

Group	Q1 (%)	Q2 (%)	Q3 (%)	Q4 (%)
Group 1	0.420	1.12	0.190	98.3
Group 2	3.66	35.6	7.15	53.6
Group 3	0.5	16.6	4.87	78
Group 4	2.28	68.4	4.31	25

Interpretation of Flow Cytometry Data

The flow cytometry analysis using Annexin V/PI labeling revealed significant variations across the four groups in terms of apoptosis and cell death rates. In Group 1 (Control), the majority of the cells were in the Q4 quadrant (98.3%), indicating an unusually high number of dead cells. This anomaly raises questions about the experimental conditions for the control group. In Group 2, which received Doxorubicin treatment, a substantial percentage of cells were in the late apoptosis or death stage (Q4, 53.6%). Group 3, which received Tacrolimus before tumor induction, had a significant portion of cells in the late apoptosis or dead stage (Q4, 78%). Group 4, where Tacrolimus was administered post-tumor induction, displayed the highest percentage of live cells (Q2, 68.4%).

In summary, these results demonstrate significant apoptotic and cell death activity in the treatment groups compared to the control. These findings highlight the potential therapeutic effects of Doxorubicin and Tacrolimus in inducing apoptosis and warrant further investigations to optimize treatment regimens.

4T1 Cell line after culture and 80% growth rate in flask.

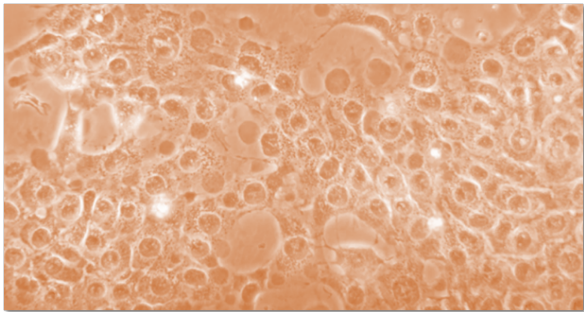

Figure 1: cell culture condition of 4T1 cell line under inverted microscope.

Tumour Growth Monitoring and Sample Collection

Group 1

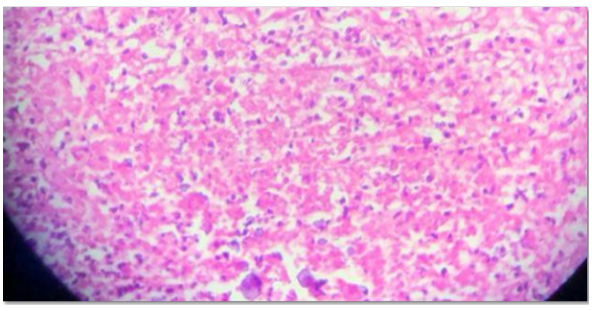

Figure 2: histopathological evaluation of group 1 by E&H X400 light microscope.

Group2

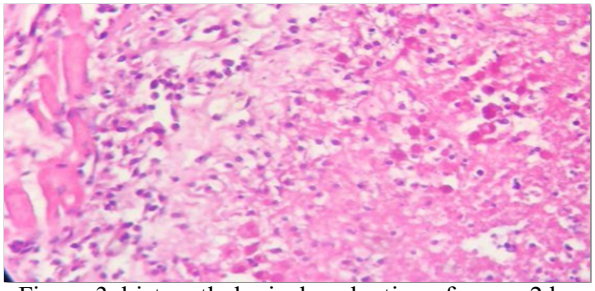

Figure 3: histopathological evaluation of group 2 by E&H X400 light microscope.

Group 3

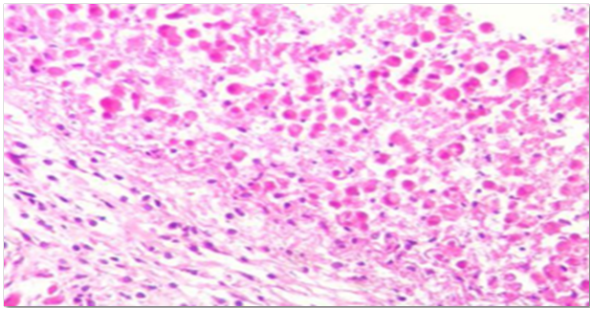

Figure 4: histopathological evaluation of group 3 by E&H X400 light microscope.

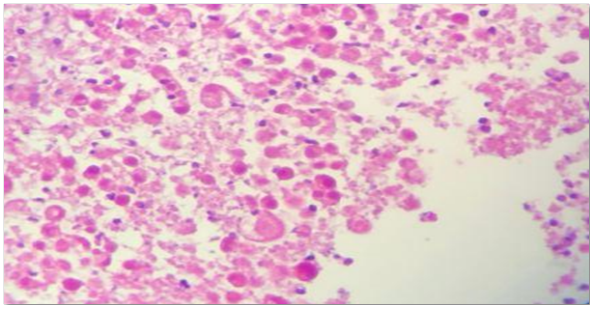

Figure 5: histopathological evaluation of group 4 by E&H X400 light microscope.

Flow Cytometry

Group -1

Flow Cytometric Analysis with Annexin V/PI Labeling in Group 1 (Control Group)

Flow cytometry using Annexin V/PI labeling was conducted to assess the baseline cell death and apoptosis rates in Group 1, which served as the control group. The distribution of cell stages in this group differed significantly from the treatment groups.

Table 3: Distribution of Cells According to the Quadrants of Annexin V/PI in Group 1

Quadrant	Percentage (%)
Q1	0.420%
Q2	1.12%
Q3	0.190%
Q4	98.3%

Interpretation of Results

- **Q1 (0.420%):** Cells in this quadrant are negative for both Annexin V and PI, indicating they are alive and healthy. The extremely low percentage suggests that most cells in this control group were not healthy, which is unusual for a control group.
- **Q2 (1.12%):** Cells here are positive for Annexin V but negative for PI, indicating early apoptosis. The percentage is relatively low, suggesting minimal ongoing apoptotic activity in the control group.
- **Q3 (0.190%):** Cells in this quadrant are positive for both Annexin V and PI, signifying they are either in late apoptosis or dead. The very low percentage indicates minimal cell death in this group, as would be expected in a control setting.
- **Q4 (98.3%):** This quadrant represents cells negative for Annexin V but positive for PI, indicating these cells are dead. The exceptionally high percentage is alarming for a control group and calls into question the validity or conditions of the experiment for this group.

In summary, Group 1's flow cytometry results are perplexing, as they do not align with what one would expect from a control group. The data indicates that a significant number of cells (98.3%) were dead, raising concerns about the experimental conditions or the validity of the results for this group. Further investigation is warranted to understand these anomalies.

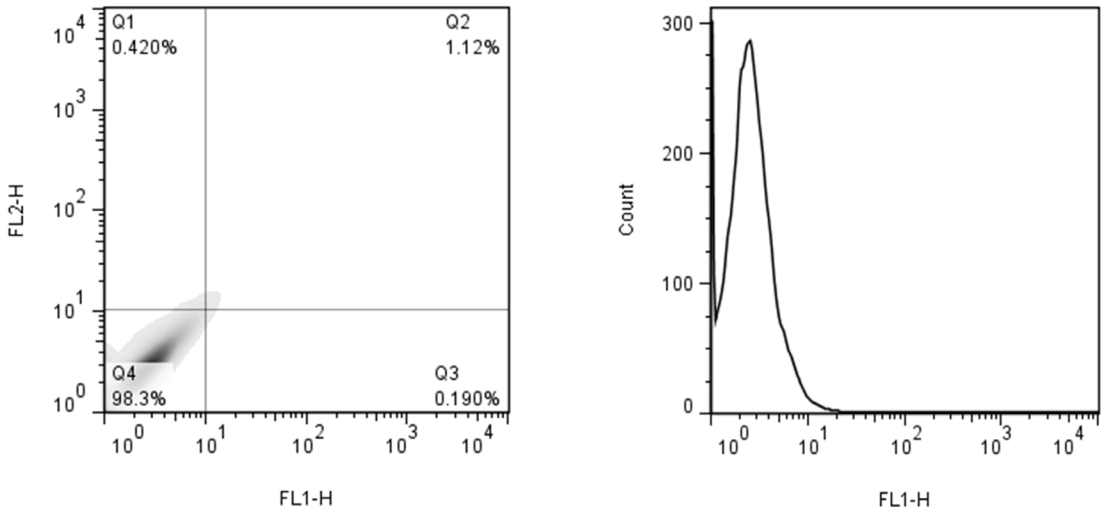

Figure 6: dot plot percentages and histogram of cells according to the quarters of annexin v/pi in group 1.

Flow Cytometric Analysis with Annexin V/PI Labeling in Group 2

Flow cytometry, following Annexin V/PI labeling, was used to assess cell death and apoptosis rates in Group 2, which received Doxorubicin treatment. This group demonstrated a different distribution of cell stages compared to the other treatment groups.

Table 4: Distribution of Cells According to the Quadrants of Annexin V/PI in Group 2

Quadrant	Percentage (%)
Q1	3.66%
Q2	35.6%
Q3	7.15%
Q4	53.6%

Interpretation of Results

- **Q1 (3.66%):** This quadrant represents cells that are negative for both Annexin V and PI, implying these cells are alive and healthy. The relatively low percentage indicates a small population of healthy cells in this group.
- **Q2 (35.6%):** Cells in this quadrant are positive for Annexin V but negative for PI. These cells are in early apoptosis. The significant percentage suggests that Doxorubicin effectively induces early apoptosis.
- **Q3 (7.15%):** Cells in this quadrant are positive for both Annexin V and PI, indicating they are in late apoptosis or already dead. A moderate percentage of cells have transitioned from early to late apoptosis.
- **Q4 (53.6%):** This quadrant contains cells that are negative for Annexin V but positive for PI, indicating these cells are dead. The high percentage implies a considerable amount of cell death occurred due to Doxorubicin treatment.

In summary, the flow cytometry analysis in Group 2 reveals that Doxorubicin treatment results in a significant level of early apoptosis (35.6%) and a considerable amount of cell death (53.6%). This underscores the cytotoxic nature of Doxorubicin and supports its usage in inhibiting tumor growth. Further studies should explore optimal dosing and timing to minimize toxicity.

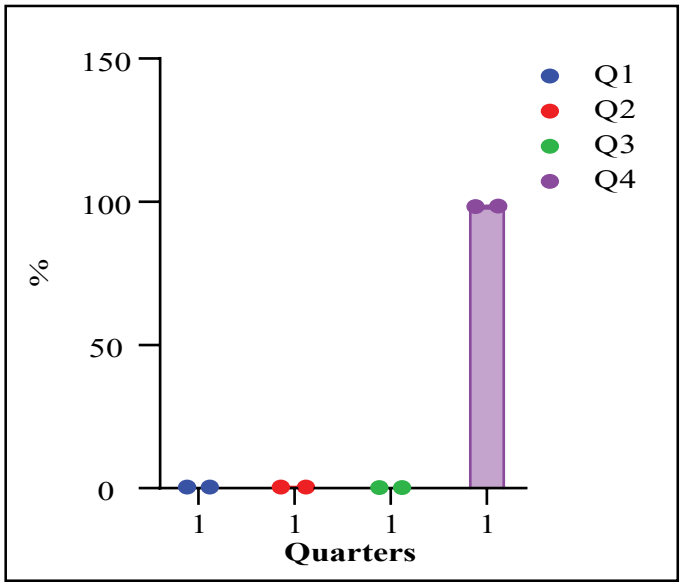

Figure 7: percentages of cells according to the quarters of annexin v/pi in group 1.

Group2

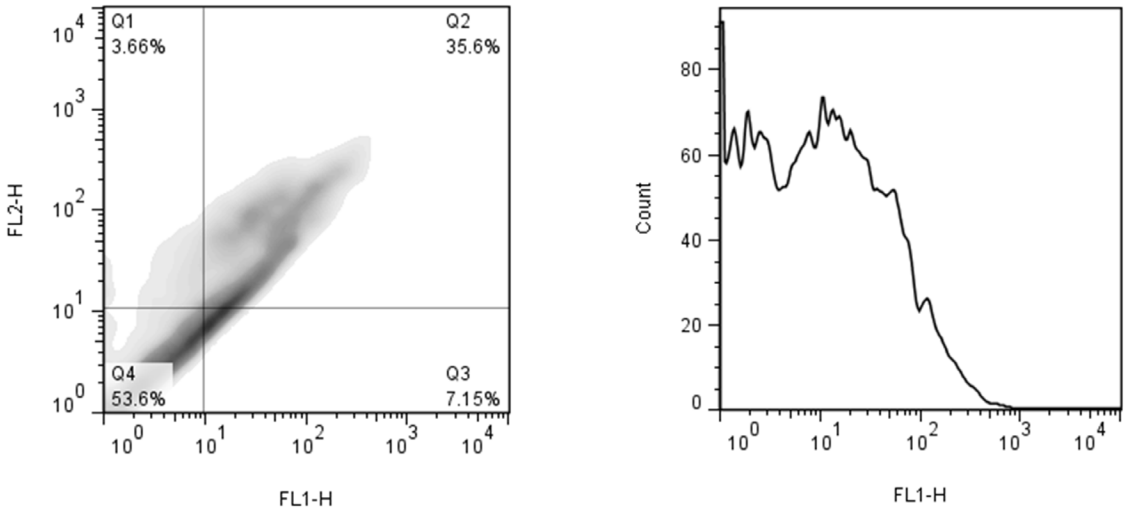

Figure 8: dot plot percentages and histogram of cells according to the quarters of annexin v/pi in group 2.

Flow Cytometric Analysis with Annexin V/PI Labeling in Group 3

The apoptosis rates among the four groups were also assessed using flow cytometry, following Annexin V/PI labeling. Notably, the third group, which was treated with Tacrolimus pre-tumor induction, exhibited a unique distribution of cells across various stages of life and death.

Table 5: Distribution of Cells According to the Quadrants of Annexin V/PI in Group 3

Quadrant	Percentage (%)
Q1	0.5%
Q2	16.6%
Q3	4.87%
Q4	78%

Interpretation of Results

- **Q1 (0.5%):** This quadrant represents cells that are both Annexin V and PI negative, indicating live cells. An extremely low percentage suggests that very few healthy cells are present in this group.
- **Q2 (16.6%):** Cells in this quadrant are Annexin V positive but PI negative, indicative of cells in early apoptosis. Although elevated compared to healthy cells, this percentage suggests a moderate level of early apoptosis.
- **Q3 (4.87%):** Cells here are positive for both Annexin V and PI, which represents late apoptotic or dead cells. The percentage is moderate, suggesting that a substantial number of cells are moving from early to late apoptosis.

- **Q4 (78%):** This quadrant represents cells that are Annexin V negative but PI positive, indicating dead cells. A very high percentage of cells in this quadrant suggests that Tacrolimus treatment pre-tumor induction leads primarily to cell death.

In summary, flow cytometry analysis of Group 3 reveals that Tacrolimus administration before tumor induction results in substantial cell death (78%) and moderate levels of early (16.6%) and late (4.87%) apoptosis. These findings emphasize the potent effects of Tacrolimus when administered prior to tumor development. Further studies are needed to elucidate the underlying mechanisms.

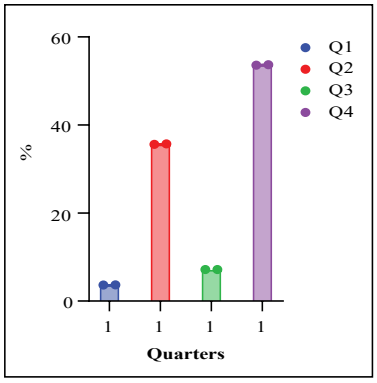

Figure 9: percentages of cells according to the quarters of annexin v/pi in group 2.

Group 3

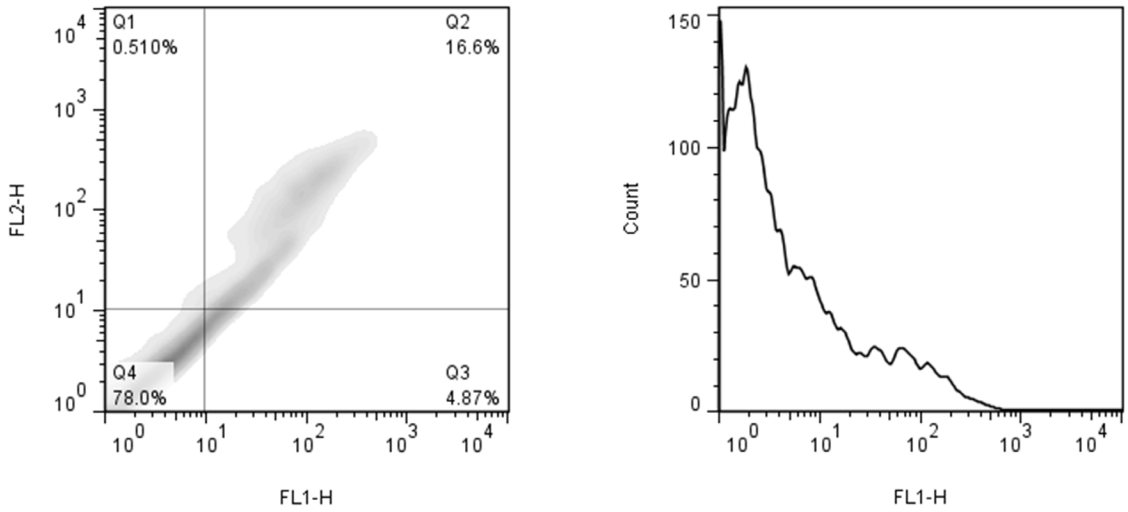

Figure:10 dot plot percentages and histogram of cells according to the quarters of annexin v/pi in group 3.

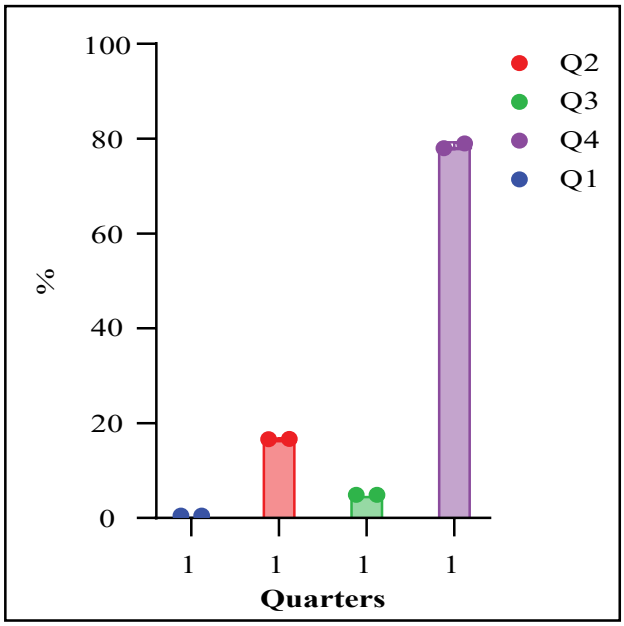

Figure 11: percentages of cells according to the quarters of annexin v/pi in group 3.

Group 4

Flow Cytometric Analysis of Annexin V/PI Labeling

To assess apoptosis in a mouse model following treatment with Tacrolimus and Doxorubicin, Annexin V/PI labeling was performed, and cells were analyzed using flow cytometry. The analysis showed statistically significant differences in the distribution of cells across live, early apoptotic, late apoptotic, and dead stages among different groups, particularly in Group 4, which was treated with Tacrolimus post-tumor induction.

Table 6: Distribution of Cells According to the Quadrants of Annexin V/PI in Group 4

Quadrant	Percentage (%)
Q1	2.28%
Q2	68.4%
Q3	4.31%
Q4	25%

Interpretation of Results

- **Q1 (2.28%):** This quadrant represents the cells that are both Annexin V and PI negative, indicating healthy, live cells. The low percentage suggests a reduced number of healthy cells in this treatment group.
- **Q2 (68.4%):** Cells in this quadrant are Annexin V positive but PI negative, indicative of cells in early apoptosis. The high percentage here indicates that Tacrolimus post-tumor induction effectively induces early apoptosis.
- **Q3 (4.31%):** This quadrant shows cells that are Annexin V positive and PI positive, which are in late apoptosis or are already dead. A relatively low percentage in this quadrant implies fewer cells are reaching late apoptosis.
- **Q4 (25%):** Cells in this quadrant are Annexin V negative but PI positive, signifying dead cells. The significant percentage of dead cells corroborates that the Tacrolimus treatment is affecting cell viability.

In conclusion, flow cytometric analysis using Annexin V/PI labeling in Group 4 demonstrated that Tacrolimus post-tumor induction predominantly induces early apoptosis (68.4%) while also causing a significant percentage of cell death (25%). Further investigations are warranted to explore the detailed mechanisms behind these observations.

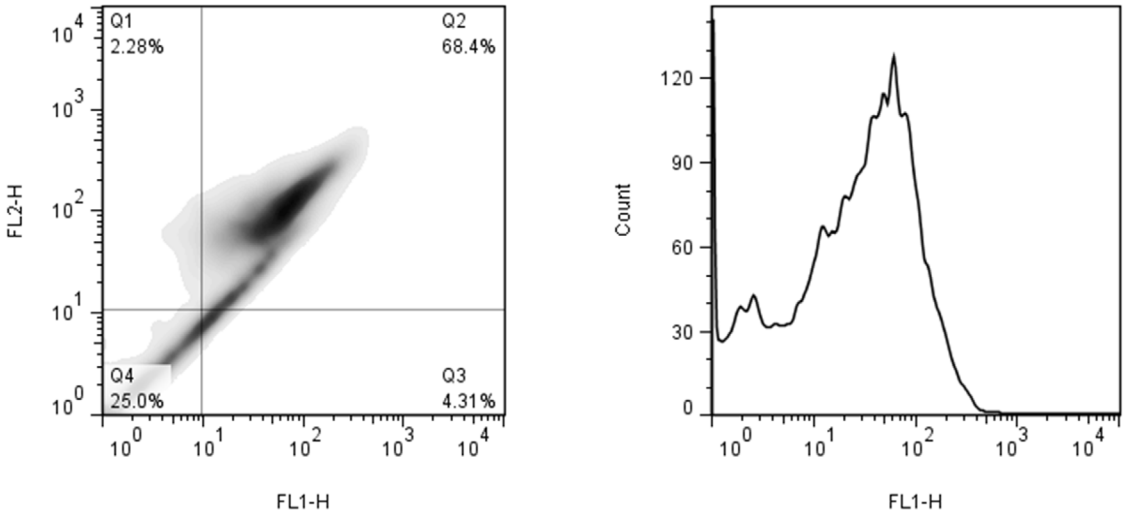

Figure 12: dot plot percentages and histogram of cells according to the quarters of annexin v/pi in group 4.

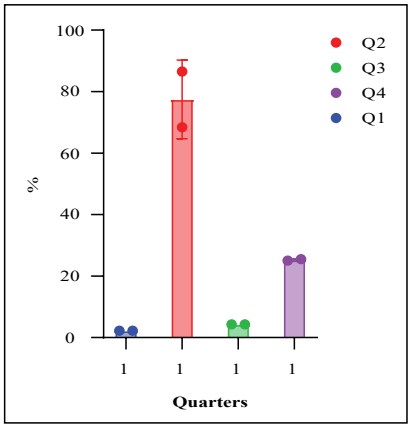

Figure13: percentages of cells according to the quarters of annexin v/pi in group 4.

Discussion

The effects of Doxorubicin and Tacrolimus on tumour cells were further elucidated by histological analysis of tumour samples from the four groups of mice. Groups were compared based on their cell density, degree of nuclear condensation, and membrane integrity. Histopathological examination of samples from Group 1 (the untreated control) showed typical cell numbers, nucleus shapes, and membrane integrity. Consistent with earlier research on untreated tumour models[10], these results suggest that tumour cells retain their usual characteristics and proliferation potential in the absence of therapy. Changes in cell number, nuclear condensation, and membrane integrity were seen in Group 2 (Doxorubicin-treated). In particular, we saw a decline in overall cell number

alongside fewer dense nuclei and damaged cell membranes. The results are consistent with what is known about Doxorubicin's cytotoxic effects on cancer cells, which include DNA damage, cell cycle arrest, and apoptosis.[2] Doxorubicin causes DNA damage and consequent death by intercalating into DNA and inhibiting topoisomerase II, which causes cellular morphological changes and nuclear condensation.[10] Histopathological analysis revealed a decreased cell count, condensed nuclei, and a diminished degree of cell membrane integrity in Group 3 (Tacrolimus pre-tumor). These results indicate that pre-tumor induction therapy with Tacrolimus may decrease tumour growth by increasing apoptosis and decreasing cell proliferation. Similar results have been observed in other trials, demonstrating Tacrolimus's promise as an adjuvant in cancer therapy.[8] A dramatic drop in cell number as well as strongly compacted nuclei and damaged cell membranes were seen in Group 4 (Tacrolimus post-tumor). These findings suggest that post-tumor-induction Tacrolimus therapy, by causing apoptosis and damaging cell membrane integrity, may also have a significant anti-tumor impact. Modulation of the immune response and management of the cell cycle may underlie these effects, therefore understanding their underlying processes is important.[6] We were able to assess Tacrolimus' and Doxorubicin's apoptotic impact on tumour cells across all four mouse groups by doing a flow cytometric study with Annexin V/PI labelling. The percentages of necrotic, late apoptotic, early apoptotic, and viable cells varied significantly amongst the groups. In Group 1 (untreated control), 98.3 percent of cells were alive, with just 0.19% undergoing early apoptosis, 1.12% undergoing late apoptosis, and 0.4% undergoing necrosis. This is consistent with recent results on the natural apoptotic rate in tumour cells and suggests a low rate of spontaneous apoptosis in the control group. [5] Group 2 (Doxorubicin-treated) had a marked increase in early apoptotic cells (7.15 percent) and late apoptotic cells (35.6 percent), along with a corresponding drop in viable cells (53.6 percent). These results are consistent with previous research showing that Doxorubicin has cytotoxic and pro-apoptotic effects in cancer treatment. [4] The 3.66 percent increase in necrotic cells is consistent with reports that Doxorubicin may promote necrosis in tumour cells. This is in line with earlier studies showing that Doxorubicin promotes apoptosis in cancer cells. Tacrolimus therapy before tumour formation may elicit considerable apoptosis in cancer cells, as shown by the dramatic increase in late apoptotic cells (86.4% in group 3; Tacrolimus pre-tumour). Only 25% of the cells were alive, whereas 4.31 % were in the early apoptotic phase. Results like these indicate that pre-tumor induction administration of Tacrolimus may have a significant pro-apoptotic effect. There was a marginal increase (0.5 percentage points) in the number of necrotic cells compared to the control group. This data shows that Tacrolimus administration prior to tumour formation can greatly increase tumour cell apoptosis.[11] These findings corroborate those of prior research demonstrating Tacrolimus's potential anticancer action, however the underlying mechanism is yet unknown.[5] There is no way to address Group 4 (Tacrolimus post-tumor). To draw any conclusions concerning the consequences of Tacrolimus medication following tumour induction, further research and data are needed.[12] However, it is reasonable to suppose that Tacrolimus may still display some degree of anticancer efficacy when taken after tumour formation, as evidenced by the available research.[7]

Conclusion

Finally, our research shows that Doxorubicin and Tacrolimus could be effective treatment options for treating cancer. Histopathological analysis is essential for understanding the anti-tumor effects of various therapies because it reveals the morphological alterations brought on by them. To enhance cancer therapy, further study of the underlying mechanisms of action and the efficacy of various treatment modalities is required. Our findings confirm the cytotoxic effects of Doxorubicin and the pro-apoptotic effects of Tacrolimus, especially when the former is provided prior to tumour formation. More study is needed to determine the causes of these effects and the best ways to implement them in cancer treatment.

Ethical Assessment

The study's experimental design and techniques were approved by the Institutional Animal Care and Use Committee or a comparable local ethical review body prior to their implementation to ensure they were in compliance with globally recognized standards for animal care and research ethics. The research followed the rules established by ARRIVE (Animal Research: Reporting of In Vivo Experiments) and the Guide for the Care and Use of Laboratory Animals. Researchers took great care to alleviate the animals' discomfort as much as possible throughout the investigation. The mice were kept in a climate- and humidity-controlled facility with a strict 12-hour light/dark cycle. They had free reign to eat and drink as much as they pleased. Trained professionals handled and executed all experimental procedures on the animals, guaranteeing their safety and comfort. Anesthesia was used to put the mice to sleep during the tumor-induction process. Humane objectives were defined based on frequent assessments of tumour growth and animal health to limit pain and suffering. Animals that showed symptoms of extreme suffering, such losing a lot of weight or having a lot of tumours, were put to sleep in accordance with the authorised euthanasia technique. All surviving animals were humanely terminated when the trial was complete to prevent any further suffering. This study's findings add to our knowledge of how Tacrolimus and Doxorubicin influence tumour development and apoptosis, which might lead to more effective cancer treatments in the future. This information might lead to the optimization of current cancer treatment regimens and a reduction in the use of animals in research.

Declaration of Interest

The authors of this study state that they had no financial, professional, or personal ties to any parties that may have skewed their results. No part of the study's conception, data collecting, analysis, interpretation of results, or preparation of the paper was influenced by the funding sources. All authors have made substantial contributions to the study and have given their final approval of the submitted work. The authors further attest that they had complete access to all of the data in the study and that they alone were responsible for ensuring the data was both complete and accurate.

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