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Original Article

To Evaluate the Safety and Efficacy of Autologous Non-specific Activated T-Cells Therapy For 7,12-Dimethylbenz[A]Anthracene (DMBA) Induced Breast Cancer in Female Rats: A Supportive Care

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ABSTRACT

Background and Objectives: Breast cancer persist a core cause of mortality in women, and current treatments like Tamoxifen often fail to address immune suppression within the tumor microenvironment. Autologous nonspecific activated T-cell therapy, either alone or combined with Tamoxifen, has shown promise in enhancing immune responses and overcoming these barriers in treating breast cancer induced by DMBA in female rats. **Materials and Methods:** The study involved administering DMBA to induce breast cancer in female rats, grouped into following groups; normal control, disease control, Tamoxifen alone, and three T-cell therapy groups; one combination with tamoxifen. T-cell therapy involved isolation, culture, and activation of MNCs from rats, administered intravenously and intra-tumor in combination with Tamoxifen. Tumor growth, immune markers, cytokines, and inflammatory markers were monitored. **Key Observations:** T-cell therapy showed promising tolerance and efficacy in treating aggressive tumor progression. While tamoxifen partially reduced tumor size and inflammation, its immune impact was minimal. Intravenous T-cell therapy yielded limited results, whereas intratumor T-cell administration led to significant tumor regression and immune enhancement. Combined intratumor T-cell therapy with tamoxifen achieved near-complete tumor regression, increased CD3, CD4, CD8, lymphocyte counts, elevated INF-γ, and reduced IL-6. Histology confirmed improved tissue regeneration, supporting the potential of combined immune and conventional therapies. **Conclusion:** The study found that the combination of intratumor T-cell administration and tamoxifen effectively treated DMBA-induced breast cancer, leading to near-complete tumor regression, elevated interferon-gamma levels, and reduced IL-6. The therapy also promoted tumor suppression and immune system recovery, resulting in increased lymphocyte counts and CD4+ T-Cell Subsets.

Keywords: Breast Cancer, Cytokine, DMBA, Mononucleated cells, T cell therapy, Tamoxifen, Tumor Regression.

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INTRODUCTION

Cancer is a complex disease defined by the abnormal growth of cells, resulting from numerous alterations in genes that disrupt the interplay between cellular division and apoptosis.[1] Globally, breast cancer persists a major cause of cancer-related deaths in women, with challenges in early detection and treatment due to the aggressive nature of certain subtypes.[2] Breast cancer persists to be one of the foremost prevalent and life-threatening malignancies in women worldwide, accounting for significant morbidity and mortality. WHO reports that around 2.3M women are confirmed with breast cancer annually, with over 685k deaths globally in 2020 alone.[3] Despite improvements in therapies and diagnostic tools, the heterogeneity and complexity of breast cancer necessitate the continuous exploration of novel therapeutic approaches. Immunotherapy has come forward as a promising method, with the potential to strengthen the body's immune defenses against cancer cells. Specifically, the use of non-specific activated T-cells offers a novel approach that may overcome some limitations of current treatments by leveraging the body's defense mechanism to target and eradicate malignant cells.[4] Mononuclear Cells (MNCs) are a category of white blood cells distinguished by having a single, round nucleus, which includes lymphocytes, monocytes, and stem cells. They are core components of the immune system and are involved in a range of physiological functions.[5] T cells, particularly cytotoxic T lymphocytes, play an

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important role in immuno surveillance and cancer defense by scanning cancer cells for specific antigens and inducing apoptosis upon detection. This precise mechanism underscores their significance in distinguishing and eliminating malignant cells. In T cell therapy, tumor-specific T cells are introduced to boost the immune system's response to cancer, offering a hopeful strategy in cancer therapy. Such immunotherapeutic strategies align with the concept of autologous T cell therapy, as explored in our research on the efficacy of non-specific T cell therapy for DMBA-triggered breast cancer in rats.[1] The DMBA-induced breast cancer model in rats is a widely used preclinical model for studying breast cancer progression and evaluating new therapeutic approaches. DMBA, a potent polycyclic aromatic hydrocarbon, initiates tumorigenesis by forming DNA adducts, which cause genetic mutations and uncontrolled cell growth. This model is highly valued because it mimics hormone-responsive breast cancers in humans, primarily estrogen receptor-positive tumors, which represent the most common subtype of human breast cancer.[6] Current breast cancer treatments, including chemotherapy, radiotherapy, and hormone therapy, face several challenges, especially in aggressive and resistant cases like those observed in DMBA-induced models. Chemotherapy lacks specificity, leading to systemic toxicity and off-target effects such as immunosuppression and organ damage.[7] Hormone therapies, while effective in estrogen receptor-positive tumors, often encounter resistance due to mutations in estrogen receptor pathways, limiting their long-term efficacy.[8] Additionally, radiotherapy, though effective for localized tumors, does not fully prevent recurrence or metastasis and can cause damage to surrounding healthy tissues.[9] Traditional treatments also fail to adequately stimulate the immune system, allowing tumors to evade immune surveillance. While T-cell therapies, particularly autologous approaches, have achieved success in treating hematologic cancers, their efficacy in solid tumors, including breast cancer, remains limited.[10] This study focuses on exploring the safety and efficacy of non-specific activated T-cells in treating DMBA-induced breast cancer in rats. Non-specific activation of T-cells involves stimulating these immune cells without targeting a specific antigen, potentially enhancing their capability to recognize and destroy a broad range of cancer cells.[11] Unlike antigen-specific therapies, which may be limited by tumour antigen heterogeneity and the potential for immune escape mechanisms, non-specific activation aims to broadly stimulate the immune system, providing a more versatile approach to targeting cancer cells.[12]

Research hypothesis

We hypothesized that autologous nonspecific activated T-cell therapy will demonstrate both safety and efficacy in reducing tumor growth and improving survival rates in DMBA-triggered breast cancer in female rats, without causing significant side effects. Furthermore, we expect this approach to heighten the immune activity against tumor cells, contributing to more effective and sustainable cancer treatment strategies.

MATERIALS AND METHODS

Collection of chemicals and materials

All the reagents and materials which were used in this study were sourced from Gibco, Sigma and Hi-Media, purchased from authenticated vendors in Bangalore, India. The equipment and facilities utilized for this research are in the tissue culture laboratory within the Department of Pharmacology at KCP, Bangalore.

Experimental animals

Wistar female rats of weight 150-200 g were used for the experiment. Prior to the experiment animals were kept for acclimatization for one week. These animals were kept in well-ventilated rooms with a 12-hr light-dark cycle and maintained at a temperature of 25±2°C. The animals were given free access to a standard diet and water. The experimental protocol was approved by the IAEC (Institutional Animal Ethical Committee) of Karnataka College of pharmacy, Bangalore and the animal experiments were conducted according to the CPCSEA.

Experimental design

Female adult wistar rats were weighed (150 to 200 g) were used for the experiment and all the animals were grouped into six groups; each group contained 10 animals (n=10), except the normal group which comprised 6 animals. Group I served as Normal control were given drinking water *ad libitum*. The Group II served as Disease control, were divided into two models: Model-1 animals were given single dose of DMBA (25 mg/rat in 0.5 mL of sunflower oil) subcutaneous injection into the mammary glands and Model-2 animals were given single dose of DMBA (5 mg/kg bodyweight in 0.5 mL of almond oil) through intragastric route by using the oral gavage. Group III served as standard group were given oral Tamoxifen 40 mg/kg bodyweight/day for 10 days,[13] after confirmation of the breast tumor (Model-1). Group IV served as Cellular therapy-1, given single administration of Nonspecific Activated T cells through Intravenous route, after confirmation of the breast tumor (Model-1). Group V served as Cellular therapy-2, given single administration of Nonspecific Activated T cells (1.5 mL blood; 4-8 million MNCs) through Intra-tumor injection locally, after confirmation of the breast tumor (Model-1). Group VI served as combination therapy, given single administration of Nonspecific Activated T cells through intra-tumor injection locally+oral Tamoxifen 40 mg/kg bodyweight/day, after confirmation of the breast tumor (Model-1).

Induction of breast tumor

Method 1: Animals were given single dose of DMBA (25 mg/ kg in 0.5 mL of sunflower oil) per rat through subcutaneous route directly to the mammary glands of the rat.[14]

Method 2: Animals were given single dose of DMBA (5 mg/ kg bodyweight in 0.5 mL of almond oil) through intragastric route by using the oral gavage.[14]

Preparation of T cells

Isolation of MNCs

As per the modified protocol mentioned by Lefort CT *et al.*, (2010)[15] under anesthesia, 1.5 mL of blood was withdrawn from the experimental rat by cardiac puncture technique and transferred to an EDTA tube. The blood sample was diluted with an equal volume of normal saline. A 15 mL centrifugation tube was taken. In this tube Density gradient media (HiSep, Hi-Media) was placed at the bottom. The diluted blood was then slowly added on top of the gradient using a sterile pipette, taking care to avoid mixing, maintaining a HiSep to blood ratio of 1:3. The tubes were kept for centrifugation at 1000 rpm for 20 min. Afterward, using a sterile pipette the upper portion which contained platelets and plasma was removed carefully, leaving behind the mononuclear cell layer (buffy coat) at the interface. This MNCs layer was gently aspirated and transferred to a new tube. The harvested cells were washed with normal saline twice by centrifuging at 1500 rpm for 10 min. After the final centrifugation, the supernatant was discarded, and the cells were then resuspended in the culture media; Dulbecco's Modified Eagle's Medium (DMEM).

Cell counting calculation

In a separate tube, 10 µL of MNC suspension was combined with 90 µL of crystal violet, creating a 10-fold dilution factor (df). From this mixture, 10 µL of the stained cell suspension was then further diluted with 90 µL of crystal violet, resulting in an additional 10-fold dilution factor. This brings the total dilution factor to 100-fold. Finally, 10 µL of this 100-fold diluted stained cell suspension was loaded onto a hemocytometer for cell counting. The cells were observed under upright microscope and calculated total cell count using the following formula: Total Cell Count (TCC) in million cells per mL=average number of cells counted using hemocytometer x cell suspension total volume (a) in mL x dilution factor (df)x10^4.

Culture of T Lymphocytes

Culture of T Lymphocytes were as per the modified protocol mentioned by Lefort CT *et al.*, (2010).[15] 24-Well plates were used for the culture of T Lymphocytes (1.5 mL blood; 4-8 million MNCs). By using a sterile pipette, the MNCs were transferred to the each well in 1 mL of DMEM; containing 10% FBS, 1% antibiotic (50-100 I.U./mL of penicillin-streptomycin solution). Based on the number of MNCs PHA-M (10 µL PHA-M for each 1 million cells) was added to each well plate; containing MNCs. Then the plates were kept for incubation in a CO_2 incubator at 37°C for 48 hr.

Treatment protocol

Following the confirmation of breast tumors (approximately 2 weeks post-DMBA in Model-1), treatments were given to all the treatment groups. In Group IV (Cellular Therapy-1), a single dose of nonspecific activated T-cells was administered intravenously to assess the systemic effects of immune cell activation on tumor suppression. In Group V (Cellular Therapy-2), a single dose of s intra-tumor injection of nonspecific activated T-cells was delivered directly into the tumor to evaluate the localized immune response. In Group VI (Combination Therapy), nonspecific activated T-cells were injected into the tumor, alongside oral administration of Tamoxifen (40 mg/kg body weight/day), to investigate the potential synergistic effects of combining immune cell therapy with anti-estrogen treatment for enhanced tumor regression.

Safety was continuously monitored throughout the study using various parameters, including behavioral observations, water intake, food consumption, urination patterns, and overall mortality rates. To assess the efficacy of the treatments, mammary lesion growths were tracked through pictographic imaging. Blood samples were used for the Immunological and hematological evaluations; were conducted by measuring Total Leukocyte Counts (TLC), lymphocyte counts, and calculating the LMR and NLR. Plasma immunological profiling was performed to assess levels of IL-6 and IFN-γ[16] (briefly, the sandwich ELISA method detects IL-6 and IFN-γ using a capture antibody coated on a plate, followed by sample addition, a detection antibody, and an enzyme-linked secondary antibody. A substrate reaction produces a color change, measured spectrophotometrically), while lymphocyte subsets, including CD3, CD4, CD8, and absolute lymphocyte counts, were analyzed (Lymphocyte subsets, including CD3 (T cells), CD4 (T helper cells), CD8 (Cytotoxic T cells), and Absolute Lymphocyte count (ALC), are analyzed using flow cytometry. Blood samples are stained with fluorescently labeled monoclonal antibodies specific to CD3, CD4, and CD8. After staining, the sample is run through a flow cytometer, which detects and quantifies each subset. The absolute counts are calculated by multiplying the total lymphocyte count (from a complete blood count) by the percentage of each subset).[17,18] Additionally, the Systemic Immune Inflammation index (SII) was calculated (Platelet×NLR (Neutrophil-to-Lymphocyte Ratio), and a detailed histopathological evaluation of tissue samples was performed as previously established method[19] (Briefly, the histopathology procedure for a tumor biopsy involves fixation in formalin, gross examination, and tissue processing (dehydration, clearing, and paraffin embedding). The tissue is then sectioned (3-5 µm), stained with H&E) to further assess the therapeutic impact.

Statistical analysis

Results are shown as Mean±SEM, (n=10 rats in each group). GraphPad prism 10.3.1 statistical software was used to evaluate the data. One-way Analysis of Variance (ANOVA) followed by Tukey's test and t-test were used to determine the significance of difference between all the groups. The $p<0.05$ were considered significant.

RESULTS

This study investigated the safety and efficacy of various treatments, including standard therapy (Tamoxifen), Intravenous (IV) and Intra-Tumor (IT) T-cell therapies, and a combination of T-cells with Tamoxifen, in a DMBA-induced breast cancer model. Key immunological, inflammatory, hematological parameters and histopathological studies were analyzed to assess the therapeutic outcomes.

Cancer induction

Cancer induction was carried out using two models. Model 1 involved a single subcutaneous dose of DMBA (25 mg/rat in 0.5 mL of sunflower oil), leading to breast tumor development within two weeks. Model 2, using an intragastric DMBA dose (5 mg/kg, b.w in 0.5 mL of almond oil), showed no tumor formation even after 60 days. Significant immunological and inflammatory changes were observed in Model 1, indicating successful tumor induction, while Model 2 showed no significant deviations from the control (Figure 1). Based on these results, Model 1 was selected for further experiments due to its effectiveness in inducing breast tumors.

Safety assessment

The safety assessment was done by comparing behavioral and physiological changes across experimental groups following DMBA-induced mammary tumors and subsequent treatments. The NC (normal control) group exhibited normal behavior, water intake, food consumption, and urination, indicating stable health. In contrast, the DC (disease control) group, which received DMBA (25 mg/rat subcutaneously), showed signs of systemic distress, including abnormal behavior, reduced water and food intake, and increased urination, highlighting the impact of tumor induction. Post-treatment, the STD (Tamoxifen 40 mg/kg orally), Cellular Therapy-1 (IV inj. of T-cells derived from 1.5 mL blood; 4-8 million MNCs), Cellular Therapy-2 (intra-tumor inj. of T-cells derived from 1.5 mL blood; 4-8 million MNCs), and Combination Therapy (T-cells+Tamoxifen 40 mg/kg, p.o.) groups all demonstrated a return to normal behavior, physiological functions, and no observed mortality, suggesting these treatments are well-tolerated and effective in restoring health.

Efficacy assessment

Mammary lesions growth by pictograph image

The pictograph (Figure 2) shows mammary tumor progression after DMBA (25 mg/rat, subcutaneous) across treatment groups. The Disease Control group displayed significant tumor growth. The Standard Tamoxifen group (40 mg/kg) showed partial tumor reduction or stabilization was observed. The Cellular Therapy-1 group (IV T-cells) exhibited minimal tumor change, while the Cellular Therapy-2 group (intra-tumor T-cells) showed substantial tumor shrinkage. The Combination Therapy group (intra-tumor T-cells+Tamoxifen) demonstrated near-complete tumor regression, highlighting the superior efficacy of combination and localized T-cell treatments.

Leukocyte and Lymphocyte Counts

In the Disease Control (DC) group, leukocyte counts significantly increased compared to the Normal Control (NC) group,

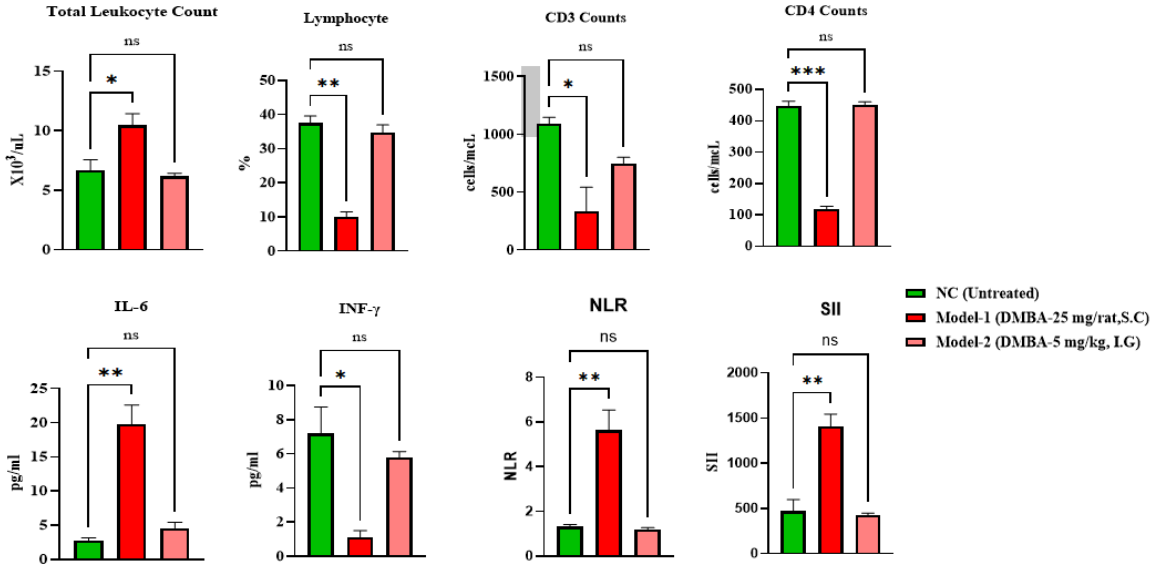

Figure 1: Comparative Analysis of Immunological and Inflammatory Parameters Across Two DMBA-Induced Models in Rats.

reflecting a heightened systemic inflammatory response triggered by DMBA-induced carcinogenesis (Table 1). Treatment with Tamoxifen significantly decreased leukocyte counts ($p<0.05$), while Cellular Therapy-1 led to a further reduction ($p<0.01$). Cellular Therapy-2 and the combination therapy maintained moderate leukocyte levels, with the combination therapy showing a significant reduction when compared with the DC group ($p<0.05$). Lymphocyte counts were significantly reduced in the DC group, indicating pronounced immunosuppression. Both Cellular Therapy-2 and combination therapy led to a significant increase in lymphocyte counts ($p<0.05$), with the combination therapy group displaying the most notable immune recovery (Table 1).

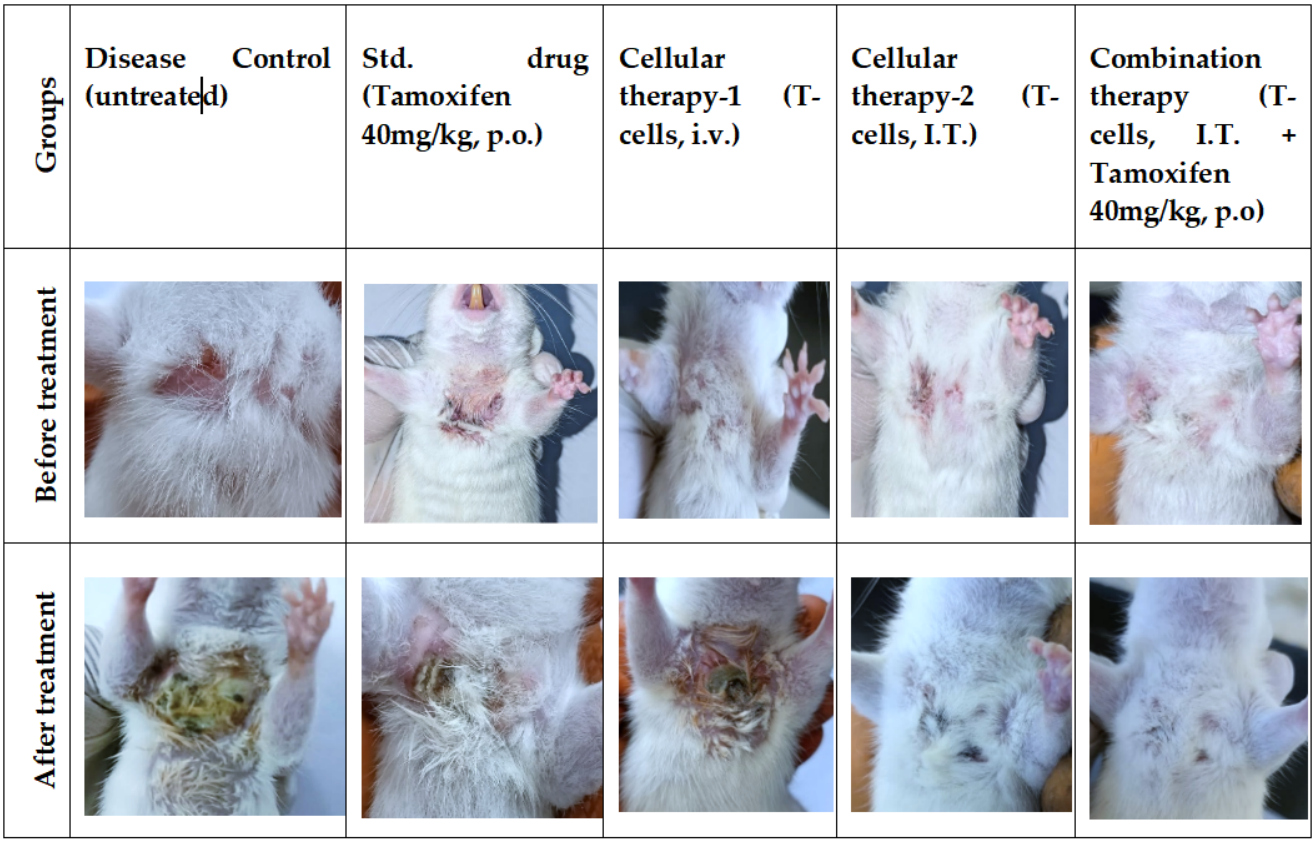

Figure 2: Pictograph image before and after treatment of the breast tumour female rats.

The image compares different treatments for a disease in an animal model. It includes 5 groups: untreated control, Tamoxifen 40 mg/kg, p.o., T cells (T-cells derived from 1.5 mL blood; 4-8 million MNCs, i.v. and i.t. injection), and a combination of T cells (with i.t. injection) with Tamoxifen. The untreated group worsens, while Tamoxifen shows partial improvement. T cell therapies have mixed effects, with IT performing better than IV. The combination therapy shows the best healing, suggesting a synergistic effect. This study highlights the potential of immune cell and hormone-based therapies for disease treatment.

Table 1: Comparison of TLC, Lymphocytes, NLR, LMR and SI-index values in NC, DC and treatment groups.

Groups	NC (Untreated)	DC (DMBA-25 mg/rat, S.C)	STD (Tamoxifen, 40mg/kg P.O)	Cellular therapy-1 (T cells, I.V.)	Cellular therapy-2 (T cells, I.T.)	Combination therapy (T-cells, intra-tumour + Tamoxifen 40 mg/kg, P.O)
TLC values	6.7±0.61	10.47±0.68$^{\#}$	6.4±0.86*	4.3±0.23**	6.8±0.87*	5.9±0.15*
Lymphocytes	33.60±5.4	10.40±0.7$^{\#\#}$	21.40±2.0	19.50±1.20	26.30±0.9*	26.45±0.75*
NL-Ratio	1.8±0.44	6.9±0.65$^{\#\#}$	3.1±0.24*	3.9±0.805*	2.34±0.23**	2.5±0.1**
LM-Ratio	6.8±0.54	3.07±0.66$^{\#\#}$	3.3±0.18	2.5±0.33	3.9±0.37	4.3±0.25
SII values	470.1±90.77	1408±95.82$^{\#\#}$	538.0±36.73**	968.3±222.8	832.8±38.71* (*t*-test)	491.9±10.35**

Values are expressed as Mean±SEM, (n=10), $^{\#}$ $p<0.05$, $^{\#\#}p<0.01$ when compared with Normal control, * $p<0.05$, ** $p<0.01$ when compared to Disease control. using one-way ANOVA, *t*-test and followed by Tukey's multiple comparison test.

Neutrophil-to-Lymphocyte Ratio (NLR) and Lymphocyte-to-Monocyte Ratio (LMR)

The NLR, a key marker of systemic inflammation and immune dysregulation, was significantly elevated in the DC group, demonstrating the role of NLR in cancer-related inflammation. All treatment groups, including Tamoxifen, Cellular Therapy-1, Cellular Therapy-2, and combination therapy, significantly reduced NLR values compared with the DC group ($p<0.05$), with Cellular Therapy-2 and combination therapy being the most effective (Table 1). Similarly, the LMR was decreased significantly in the DC group, indicating immune suppression. Though there was no statistically significant difference in LMR between the treatment's groups, combination therapy showed the highest trend toward normalization of LMR (Table 1).

Inflammatory Markers (IL-6 and INF-γ)

IL-6 levels were elevated in the DC group. Tamoxifen and Cellular Therapy-1 modestly reduced IL-6 levels, though not significantly, while both Cellular Therapy-2 and combination therapy significantly lowered the IL-6 levels ($p<0.05$) (Figure 3). INF-γ levels were reduced in the DC group but significantly increased by combination therapy ($p<0.001$) and Cellular Therapy-2 ($p<0.05$) (Figure 4).

T-Cell Subsets (CD3, CD4, CD8)

In line with the immunosuppressive effects of DMBA, the DC group exhibited a significant reduction in CD3, CD4, and CD8 counts, markers of immune competence (Table 2). Tamoxifen and Cellular Therapy-1 produced slight, non-significant increases in these immune cell populations. However, Cellular Therapy-2 and combination therapy both resulted in significant increases in CD3, CD4, and CD8 counts, with combination therapy showing

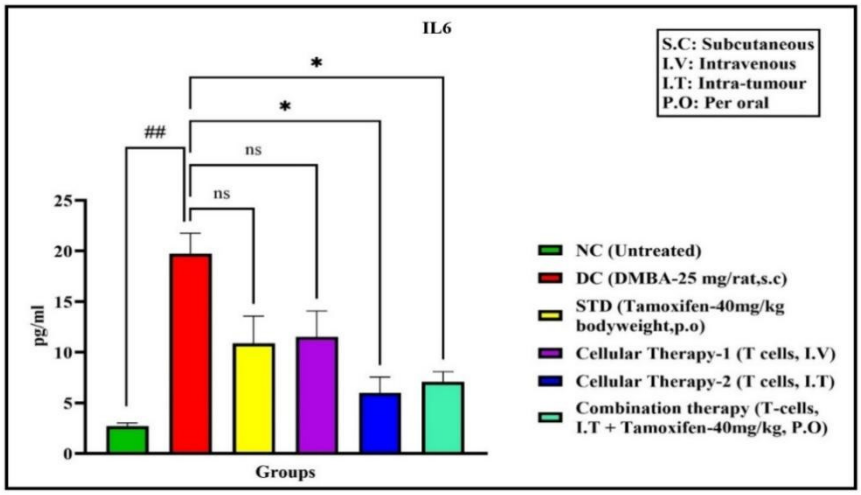

Figure 3: Comparison of IL- 6 values in NC, DC and treatment groups. Values are expressed as Mean±SEM, (n=10), ## $p<0.01$ compared with Normal control, ns $p>0.05$, *$p<0.01$ compared with Disease control.

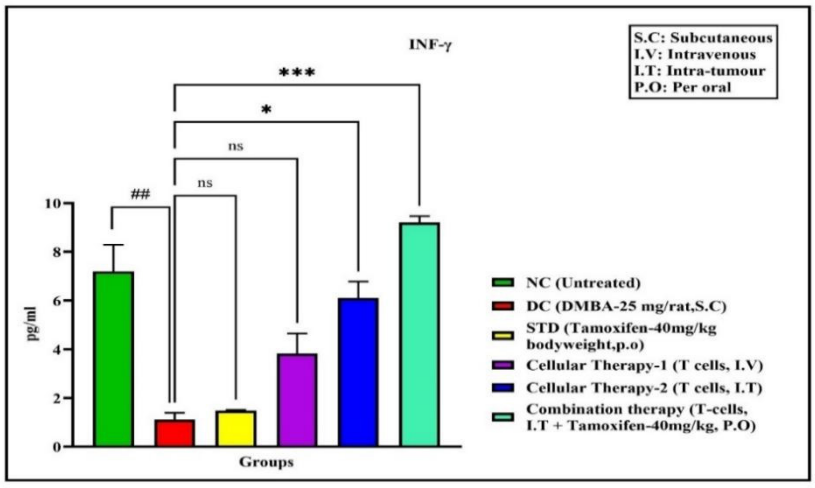

Figure 4: Comparison of INF- γ values in NC, DC and treatment groups. Values are expressed as Mean±SEM, (n=10), ##$p<0.01$ compared with Normal control, ns $p>0.05$, * $p<0.01$, ***$p<0.001$ when compared with Disease control.

the most substantial recovery ($p<0.05$ in CD3, $p<0.01$ in CD4, and $p<0.05$ in CD8) (Table 2).

Absolute Lymphocyte Counts and Systemic Immune Inflammation Index (SII)

Absolute lymphocyte counts were significantly reduced in the DC group, indicative of immune suppression. While Tamoxifen and Cellular Therapy-1 produced modest, non-significant increases, both Cellular Therapy-2 and combination therapy significantly increased absolute lymphocyte counts ($p<0.05$ and $p<0.01$, respectively), suggesting their efficacy in immune restoration (Table 2). The SII, a marker of systemic inflammation and immune status, was significantly elevated in the DC group. Tamoxifen significantly reduced SII ($p<0.01$), while Cellular Therapy-2 and combination therapy further decreased SII, with combination therapy showing the most significant reduction ($p<0.05$ and $p<0.01$) (Table 1).

Histopathological analysis

The histopathological analysis across treatment groups showed distinct therapeutic effects on breast tumor tissue (Figure 5). In the DC group (A), tumor invasion disrupted the dermis with clear adipose vacuoles and extensive fibrosis, indicating a desmoplastic response. Thickened stroma and increased vascularization reflected tumor progression and reliance on blood supply. In the STD group (B), the tumor mass was partially controlled, with defined boundaries and an organized stroma, suggesting reduced aggressiveness. Surrounding tissues were better preserved, indicating tamoxifen's anti-estrogen effect in slowing tumor growth.

In the Cellular Therapy-1 (C), tissue architecture was preserved, with an organized adipose layer and reduced fibrosis, suggesting decreased tumor activity. Structured stroma and T-cell infiltration highlighted an immune response, while vascularization pointed to normal perfusion. In the second Cellular Therapy-2 (D), treated tissue appeared more organized, with intact epidermal and dermal layers and suppressed tumor cells. Structured stroma, organized blood vessels, and reduced fibrosis suggested healing. The Combination Therapy (E) showed significant tumor reduction, preserved adipose tissue, and low cellularity, indicating targeted tumor suppression. Minimal inflammation, reduced vascularization, and compromised blood supply reflected tamoxifen's anti-angiogenic effects alongside T-cell-mediated tumor destruction.

DISCUSSION

This study presents compelling evidence for the combined efficacy of Tamoxifen and intra-tumor T-cell therapy in the treatment of DMBA-induced breast cancer. The disease control group showed pronounced tumor progression, inflammation, and immune suppression, indicated by elevated leukocyte counts, increased Neutrophil-to-Lymphocyte Ratio (NLR), and reduced CD3, CD4, and CD8 counts, along with high IL-6 and low Interferon-gamma (INF-γ) levels. These markers align with previous findings on the pro-inflammatory and immunosuppressive effects of DMBA-induced carcinogenesis (DMBA-induced carcinogenesis promotes inflammation-driven tumorigenesis while simultaneously suppressing anti-tumor immunity, creating a microenvironment that favors tumor growth and progression).[20-22] While Tamoxifen alone offered partial tumor reduction and stabilized some immune parameters, its effects on immune cell counts were modest, suggesting its limited scope in immune modulation and the need for complementary therapies.[23] Intravenous T-cell therapy (Cellular Therapy-1) showed limited tumor regression and minor immune recovery, likely due to challenges in tumor site localization and effective activation of immune responses.[24] However, intra-tumor T-cell therapy (Cellular Therapy-2) demonstrated significant tumor shrinkage, fibrosis reduction, and robust immune activation, evidenced by improved lymphocyte counts, and substantial decreases in IL-6 and increases in INF-γ, confirming the efficacy of localized immune response.[25]

Table 2: Comparison of CD3, CD4, CD8, and Absolute lymphocyte counts in NC, DC and treatment groups.

Groups	NC (Untreated)	DC (DMBA-25 mg/rat, S.C)	STD (Tamoxifen, 40mg/kg P.O)	Cellular therapy-1 (T cells, I.V.)	Cellular therapy-2 (T cells, I.T.)	Combination therapy (T-cells, intra-tumour + Tamoxifen 40 mg/kg, P.O)
CD3 Counts	1093±38.0	335.5±145.5$^{\#\#}$	583.0±37.0	505.0±128.0	1243±598.5	1059±51.0*
CD4 Counts	427.0±31.0	115.0±5.0$^{\#}$	268.0±90.0	222.5±84.50	452.0±7.0*** (*t*-test)	349.0±7.0** (*t*-test)
CD8 Counts	664.5±48.50	196.0±28.0$^{\#\#}$	280.0±52.0	245.5±41.50	316.0±81.0	548.5±48.50*
Absolute lymphocyte counts	1450±11.55	555.0±54.85$^{\#}$	860.0±80.83	875.0±101.0	1570±433.0*	1997±73.33**

Values are expressed as Mean±SEM, (n=10), $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with Normal control and, * $p<0.05$, **$p<0.01$, and ***$p<0.001$ when compared with Disease control using one-way ANOVA and unpaired *t*-test followed by Tukey's multiple comparison test.

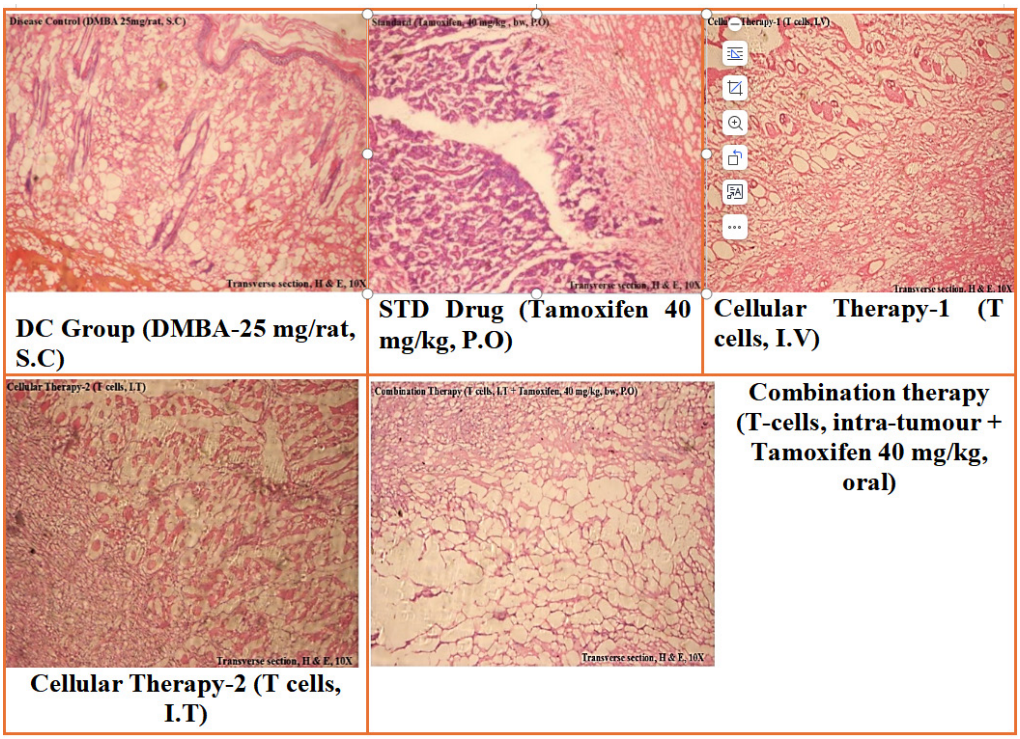

Figure 5: Histopathological evaluation of breast tumour tissues of DC and all the treatment groups. A. DC group (a-fibrotic changes, b-clear vacuoles, c-fibrous tissue), B. STD group (a-tumour mass, b-stromal response, c-apoptosis), C. Cellular therapy-1 (a-structured stroma, b-adipose layer, c-reduced fibrosis), D. Cellular therapy-2 (a-intact epidermal and dermal layer, b-absence of tumour cells, c-structured stroma and organized blood vessels, d-reduced fibrosis), E. Combination therapy (a-reduced tumour mass, b-preserved adipose tissue, c-less reactive stroma, d-reduced vascularization).

The most promising results emerged from the combination of intra-tumor T-cell therapy with Tamoxifen, leading to near-complete tumor regression, marked improvements in immune markers, and significant increases in CD3, CD4, CD8, and lymphocyte counts, along with elevated INF-γ and reduced IL-6 levels. Histopathological analysis revealed distinct therapeutic effects across groups: the disease control group showed extensive fibrosis and vascularization indicative of aggressive tumor growth; the Tamoxifen group exhibited reduced tumor mass with preserved tissue; Cellular Therapy-1 (T cells, I.V) demonstrated preserved or regenerated skin tissue architecture, organized the adipose layer suggesting restored breast tissue; intra-tumor T-cell therapy showed organized tissue structures and suppressed tumor cells; and the combination therapy group presented significant tumor regression, preserved adipose tissue, and minimal inflammation, highlighting tamoxifen's anti-angiogenic effect alongside T-cell-driven tumor destruction. These findings underline the potential of combining T-cell immunotherapy with standard treatments to achieve superior cancer control, aligning with current research supporting combinatory approaches for enhanced tumor suppression.[20-26]

CONCLUSION

This study demonstrated the safety and efficacy of autologous nonspecific activated T-cell therapy in a DMBA-triggered breast cancer model in female rats. The outcomes demonstrate that intra-tumor administration of T-cells, particularly in combination with Tamoxifen, effectively reduced tumor growth, restored immune function, and decreased systemic inflammation. Combination therapy consistently showed superior outcomes in key parameters, including leukocyte and lymphocyte counts, T-cell subsets, and inflammatory markers like IL-6 and INF-γ, indicating a synergistic effect. Safety assessments revealed that all treatments were well-tolerated, with no adverse behavioral or physiological changes post-therapy initiation. The absence of mortality further affirms the therapeutic safety of these interventions. Overall, the combination of T-cell therapy and Tamoxifen offers a promising and safe strategy for improving immune response and tumor suppression in breast cancer, warranting further investigation in clinical settings.

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ABBREVIATIONS

DMBA: 7,12-Dimethylbenz[A]Anthracene; **MNCs:** Mononuclear cells; **DMEM:** Dulbecco's Modified Eagle's Medium; **PHA-M:** Phytohemagglutinin M; **FBS:** Fetal bovine serum; **EDTA:** Ethylenediamine tetraacetic acid.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The protocol was approved by IAEC, Karnataka College of Pharmacy, Bengaluru - 560064 and Sl. No. KCP-IAEC/14/23-24/10/28/03/24.

SUMMARY

This study explores the efficacy of combining Tamoxifen with intra-tumor T-cell therapy in treating DMBA-triggered breast cancer in female rats. Results indicate that the combination therapy offers superior tumor regression, immune restoration, and reduced inflammation compared to Tamoxifen or T-cell therapy alone. Disease control rats showed high levels of inflammation and immune suppression, while Tamoxifen provided partial tumor stabilization. Intravenous T-cell therapy yielded limited immune effects, but intra-tumor T-cell therapy significantly improved localized immune response. Notably, the combination therapy achieved near-complete tumor regression and the most robust immune activation, as confirmed by histopathological analysis. This study highlights the synergistic potential of combining T-cell immunotherapy with standard treatments to enhance cancer control, paving the way for further research on optimizing combination therapies for clinical applications.

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