Electrochemotherapy Modulates Mammary Tumor Growth in Rats on a Western Diet Supplemented with Curcumin

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Received: 25 September 2020; Accepted: 10 November 2020; Published: 13 November 2020

Abstract: In the US, every 12 min, six women are diagnosed with breast cancer and one dies. This highlights a critical need for developing alternate therapies using natural compounds, which are cost effective and with less side effects. Curcumin, the yellow pigment of turmeric has been found to suppress initiation, progression, and metastasis of a variety of tumors. Multiple clinical trials highlight the efficacy of curcumin in treating breast cancer and other diseases. Our in vitro studies have demonstrated that the electrical pulse (EP) application can further enhance the effectiveness of curcumin against breast cancer cells in a therapy called electrochemotherapy (ECT). In a direct extension of these results, we studied the effect of ECT coupled with intratumoral curcumin administration (EP+Cur) on N-methyl-N-nitrosourea (MNU) induced mammary tumors in female Sprague Dawley rats. Beginning at the weaning and throughout the study, rats were fed either western diet (West) or western diet, supplemented with 1% curcumin (W+Cur). Our results showed that EP+Cur treatment led to a reduced growth rate in rats fed with W+Cur diet compared to West diet (57.14% vs. 16.67% in West diet). These results provide a foundation for further studies towards utilizing it in clinical practice.

Keywords: curcumin; western diet; breast cancer; rat mammary tumor; N-methyl-N-nitrosourea; electrochemotherapy

1. Introduction

Breast cancer is the most common cancer of women all over the world. In the US, every 12 min, six women are diagnosed with breast cancer and one dies [1]. Despite several breakthroughs in prevention, control, and the treatment of cancer, millions of patients are afflicted and incurable because of the poor response rate, high cost, and side effects of the commonly administered approved drugs. Thus, there is a critical need for effective alternate therapies using natural compounds, which are not only cost effective, but also have less side effects compared to conventional therapeutics.

Studied in this research is curcumin, the yellow pigment component of natural herb turmeric (constitutes 2–5%), that has been used for over 4000 years in the Indian subcontinent and other Asian countries as a dietary supplement, food preservative, coloring agent, and medicine [2]. In Ayurveda (science of life), turmeric is recommended for various diseases and conditions, such as skin, pulmonary, and gastrointestinal systems, aches, pains, wounds, sprains, and liver disorders [2]. The majority of the bioactivities of turmeric are attributed to curcumin, and it remains the most extensively studied compound of turmeric. The first study on curcumin’s biological activity as an antibacterial agent was reported in 1949 in Nature against staphylococcus aureus, salmonella paratyphi, trichophyton gypseum, and mycobacterium tuberculosis strains [3].
Curcumin has been found to suppress initiation, progression, and metastasis of a variety of tumors, such as breast, cervical, colorectal, bladder, lung, skin, stomach, and oral cancers, by modulating multiple receptors, growth factors, inflammatory cytokines, kinases, enzymes, and transcription factors [2,4]. Curcumin targets several genes and proteins to modulate multiple pathways (NF-κB, PI3K/Akt/mTOR, MAPK, Wnt/β-catenin, Notch, and Hh pathways) and exert its anticancer action [5,6].

The first curcumin clinical trial was published in 1937 in Lancet on human biliary diseases [7]. Due to its excellent characteristics, the research on curcumin has increased significantly over the years, as demonstrated by more than 15,000 research articles (in PubMed) and 236 clinical trials (in ClinicalTrials.gov) with keyword curcumin as of September 2020 [8,9]. Among 49 double-blind placebo-controlled clinical trials of curcumin, 17 recent trials showed efficacy [10].

In cancer patients, most trials of curcumin involve therapy of colorectal and pancreatic cancers. Currently, 6 studies using curcumin as drug or dietary supplement for breast cancer treatment are registered with ClinicalTrials.gov [11]. Oral curcumin reduced the severity of radiation dermatitis in breast cancer patients in a randomized, double-blind, placebo-controlled clinical trial [12]. In a dose escalation trial of docetaxel with curcumin in patients with advanced and metastatic breast cancer, antitumor activity of curcumin and docetaxel combination was demonstrated [13]. A daily intake of curcumin, up to 8 g was found to be safe [13]. In another comparative, randomized, double-blind, placebo-controlled clinical trial, intravenous infusion of curcumin with paclitaxel was superior to paclitaxel with placebo in the treatment of advanced and metastatic breast cancer [14]. These studies highlight the efficacy of curcumin in breast cancer treatment.

To further enhance the effectiveness of curcumin, several approaches for increasing delivery of curcumin have been explored [15]. However, the clinical potential of these formulations is yet to be established. Towards this, electrical pulse (EP) application can be effective to increase the delivery of curcumin in cells by opening cell membrane pores, since an enhanced cytotoxicity (up to 1000-times) can be obtained at lower concentrations of curcumin [16], using this non-surgical procedure, called electrochemotherapy (ECT).

ECT is an efficient modality to treat local tumors, and it has shown promising success in the clinics for the treatment of various cancers, as it is applicable to all histology of cancers [16]. Several clinical studies have demonstrated the efficacy of ECT in treating advanced subcutaneous and cutaneous tumor nodules, including malignant melanoma, squamous cell carcinoma, basal cell carcinoma, keratoacanthoma, and breast cancer and its metastases [16–23].

ECT is effective against mammary tumors in animal models. In a spontaneous mammary tumor mouse model, weekly ECT treatment resulted in partial regression in all 38 tumors, complete remission in 23 tumors, and cure in 3 tumors [24]. In another study, two sessions of ECT treatment of incompletely excised mammary carcinoma in two male pet rats led to complete remission of tumors after 10 and 14 months [25]. Tozon et al. also showed a significant reduction in the volumes of tumor nodules treated with ECT in 2 cats and 1 dog with mammary adenocarcinoma, who were previously treated with surgery [26]. In these studies, the ECT treatment was well tolerated and no major local or systemic side effects were observed after the treatment.

In clinical studies, Campana and colleagues used ECT with bleomycin to treat chest wall recurrence (CWR) of breast cancer after mastectomy in 51 patients of median age 70 year (range 38–88), who experienced tumor progression despite re-irradiation and were refractive to several systemic treatments [27–29]. In this study, a median of two ECT courses (range 1–5) were performed to obtain a complete 2-month objective response (OR) in 43.2% of patients (22/51) and a partial response (PR) in 47.5% of patients (24/51) [27]. Rebersek et al. used ECT with cisplatin to treat 12 lesions in six metastatic breast cancer patients, showing OR in all 12 lesions (100%), CR in 4 lesions (33%), and PR in 8 lesions (67%), with more than 50% decrease in the lesion [22]. In another study of ECT to treat breast cancer skin metastasis in 7 patients, an OR of 86% was obtained, with 43% of CR and 43% of PR [30]. In the European Standard Operating Procedures for ECT (ESOPE) project, among 58 breast cancer nodules treated with ECT, 52 (~90%) showed CR and 6 (5%) showed PR [16]. In a large cohort of
119 patients with breast cancer cutaneous metastases from International Network for Sharing Practice on Electrochemotherapy (INSPECT) database from 10 European clinics, the evaluation of response in 90 patients at 2-months following ECT resulted into a CR in 45 patients (50%), PR in 19 (21%), stable disease in 16 (18%), and disease progression in 7 (8%) [31]. These and other studies [32–37] demonstrate the clinical efficacy of ECT in treating advanced, recurrent, and metastatic breast cancers, which are refractory to multiple modalities, such as surgery, chemotherapy, and radiation.

ECT is simple, repeatable, highly effective, and safe in various subsets of patients with advanced, recurring, radio- and chemo-resistant tumors. Considering its excellent outcomes, its safety, and affordability, ECT is now recommended for primary skin cancer and cutaneous metastases in national and international guidelines [38,39]. Typically, ECT is used with conventional drugs, such as bleomycin and cisplatin, which come with high cost and severe side-effects on their own. Considering this, our group specializes in ECT for breast cancer treatment using natural compounds and extracts, such as curcumin, and have shown excellent increase in their cytotoxicity against breast cancer cells [40–46]. We have shown that ECT with curcumin enhances the cytotoxicity of curcumin against breast cancer cells, while causing limited cytotoxicity in non-cancerous epithelial cells [41,42,46].

In a direct extension of these in vitro results, we studied the effect of ECT with intratumoral curcumin (EP+Cur) on N-methyl-N-nitrosourea (MNU) induced mammary tumors in Sprague Dawley (SD) rats. The SD rat model is a widely recognized model due to its strong histological and genetic resemblance with human breast tumors [47–49]. Moreover, due to the beneficial effect of dietary curcumin, we studied if western diet (West) supplemented with curcumin (W+Cur) had any correlation with the outcome of the ECT treatment with curcumin. This study is the first of its kind to use ECT with curcumin on a rodent model to determine the influence of curcumin supplement diet on the efficacy of ECT to treat tumors.

2. Materials and Methods

2.1. Animals

Forty virgin female Sprague Dawley (SD) rats (Envigo, Indianapolis, IN, USA) were utilized. Upon arrival, all rats were housed in pair with continuous access to water, food, and environmental enrichment. All animal experiments were approved by the Purdue Animal Care and Use Committee. (Protocol: 111000342).

2.2. Diets

The SD 21-day weaning rats were randomly divided into 2 groups, Group 1, and Group 2. Group 1 was fed with western (West) diet (D12079B; Research Diets, NJ, USA). The West diet (D12079B, Research Diets, Inc.) contained high fat (40% Kcal) and high carbohydrate (43% Kcal).

Group 2 was fed with western diet supplemented with curcumin (1% by weight) (W+Cur). Here, only curcumin, not turmeric was used.

The W+Cur diet (D17061304) was also prepared by Research Diets, Inc. The curcumin compound (Powder City curcumin 95%) was first weighed to add to the West diet mix at the necessary amount (10 g curcumin per 1 kg West diet) to provide the final diet mixture with 1% w/w curcumin and then this was incorporated into a premix including all dry ingredients and the color dyes, which allowed to observe the homogeneity of the mixture once the color was evenly distributed. Next, a premix of corn oil and ethoxyquin (antioxidant) was added and then the appropriate amount of butter was added to the entire mixture at the correct level. First, each ingredient was homogeneously mixed and then a small amount of water was added for the purpose of pelleting (around 5–10% of the weight of the diet). Both the diets were forced through a dye and pelleted with a “cold” extrusion process. No heat was intentionally added, but the friction of moving parts during the pelleting process might expose the diet to temperatures around 32.2 °C for less than a minute. After pelleting, the diets were dried for 2 days to remove the water added prior to pelleting. The diets were then irradiated at 37–43 °C for 40–45 min. Table 1 shows the ingredients of two diets. Figure 1a shows the experimental design of the study.
Table 1. The ingredients of western (West) diet and western diet supplemented with 1% w/w curcumin (W+Cur).

| Ingredient                  | West Diet | W+Cur Diet |
|-----------------------------|-----------|------------|
| g  | kcal | g  | kcal |
| Casein                      | 195       | 780        | 195       | 780        |
| DL-Methionine               | 3         | 12         | 3         | 12         |
| Corn Starch                 | 50        | 200        | 50        | 200        |
| Maltodextrin 10             | 100       | 400        | 100       | 400        |
| Sucrose                     | 341       | 1364       | 341       | 1364       |
| Cellulose, BW200            | 50        | 0          | 50        | 0          |
| Milk Fat, Anhydrous         | 200       | 1800       | 200       | 1800       |
| Corn Oil                    | 10        | 90         | 10        | 90         |
| Soybean Oil                 | 0         | 0          | 0         | 0          |
| Ethoxyquin                  | 0.04      | 0          | 0.04      | 0          |
| Mineral Mix S10001          | 35        | 0          | 35        | 0          |
| Calcium Carbonate           | 4         | 0          | 4         | 0          |
| Vitamin Mix V10001          | 10        | 40         | 10        | 40         |
| Choline Bitartrate          | 2         | 0          | 2         | 0          |
| Cholesterol                 | 1.5       | 1.5        | 1.5       | 0          |
| Curcumin, 95%               | 0         | 0          | 10.65     | 0          |
| FD&C Red Dye #40            | 0         | 0          | 0.025     | 0          |
| FD&C Yellow Dye #5          | 0         | 0          | 0.025     | 0          |
| Total                       | 1001.5    | 4686       | 1012.2    | 4686       |
| Curcumin (g/kg)             | 0         | 10         |           |            |

Figure 1. (a) Experimental design of the study. (b) The electrochemotherapy (ECT) with curcumin treatment: (i) BTX ECM 830 electroporator for applying square wave electrical pulses (EP) (1000 V/cm, 100 µs, 8 pulses at 100 ms interval between pulses). (ii) Needle array electrodes composed of two rows of 4 needles, used for EP application to tumor. (iii) Intratumoral administration of curcumin. (iv) EP application after 1 min of curcumin injection. The zoomed-up Inset shows a typical electrode placement on tumor for EP application.

The rats were maintained on their respective diets throughout the study.
2.3. Mammary Tumor Induction and Assessment

At 70 days age, all rats were given single injection of N-Methyl-N-nitrosourea (MNU, NG-17031; Chem Service, Inc., PA, USA) intraperitoneally (i.p.) at 50 mg/kg to induce tumors in the mammary glands, and maintained on their respective diets (Figure 1a). The MNU was delivered via i.p. injection, as this route is the most reliable, rapid, and reproducible for MNU-induced mammary tumorigenesis [50]. MNU was immediately dissolved before treatment and was protected from light. The rats were inspected weekly for mammary tumor incidence, which were palpated by hand and measured with callipers on three axes. When a tumour was first palpated, the date and its location were recorded. The tumor volumes were calculated using Length × Width × Height × π/6 in mm³.

2.4. Electrochemotherapy Treatment

When the tumors were about 1 cm, rats were treated with curcumin (C1386; Sigma Aldrich, MO, USA) and electrical pulses (EP). Figure 1b shows the equipment and procedure used for the treatment. Curcumin was first dissolved into DMSO (BP231-100; Fisher scientific, MA, USA) and was diluted further in saline to make 200 µM concentration for injections. First, curcumin injections (100 µL) were administered intratumorally. After 1 min of curcumin injection, multiple EP (1000 V/cm, 100 µs, 8 pulses at 100 ms interval between pulses) were administered using needle array electrodes (Leroy Biotech, France) composed of two rows of 4 needles (15 mm long, 2.9 mm gap between needle tips and 5.9 mm gap between rows) to cover the entire tumor area. The BTX ECM 830 electroporator (BTX, MA, USA) was used for generating EP. The rats were anesthetised during the procedure. When the mammary tumors reached 2.5 cm in any one dimension, rats were euthanized.

2.5. Quantitative Characterization of Mammary Tumor Growth

Tumor volumes post treatments were quantitatively compared to the tumor volume before start of the treatment (at day 0), using relative tumor volumes and specific growth rate (SGR). The relative tumor volumes were calculated using Equation (1).

\[ \text{Relative tumor volume} = \frac{V_1}{V_0}. \]  

(1)

The SGR calculations were done using Equation (2), as described previously [51].

\[ SGR = \ln\left(\frac{V_1}{V_0}\right) / (t_1 - t_0), \]  

(2)

where \( V_0 \) and \( V_1 \) are the tumor volumes before treatment (\( t_0 \)) and tumor volume at a particular day (day 7 or 14) after the treatment (\( t_1 \)), respectively. For quantitative characterization of tumor growth in rats with missing tumor volume measurements at days 0, 7, or 14, the tumor volumes at these time points were estimated between two known values manually in GraphPad Prism.

2.6. Statistical Analysis

Student’s t-test was used to compare groups. Tumor onset data was analyzed using the Kaplan–Meier method, followed by log rank test using chi square statistics in STATA. Cox proportional hazard regression model was also run on the tumor onset survival data to estimate the ratio of hazard rate in two diet groups. Correlation between tumor size at treatment and tumor responses was assessed using Fisher’s exact test (two tail) in JMP software.

A repeated measure ANOVA analysis was performed in JMP to compare the growth rate of all the tumors (including second and third tumors) before treatment (before day 0) for the rats who responded to the treatment to the ones who did not show a response in Cur diet with EP+Cur treatment group.

For all tests, values were considered significant at \( p < 0.05 \). Results are expressed as mean ± standard error (SE).
3. Results

3.1. Rat Postnatal Development

Administration of MNU did not induce any acute toxicity in treated rats. Figure 2a shows the average body weight of rats for West diet and W+Cur diet groups over days. No significant difference was observed in the body weight between two groups at any time point.

Figure 2. (a) The body weights of rats in both western (West) and western supplemented with 1% curcumin (W+Cur) diet groups. (b) Tumor onset analysis using Kaplan–Meier method. Y-axis shows the probability of animals without tumor incidence. (c) Volume of the first tumor at inception (earliest palpable tumor) for both the groups. (d) Number of tumors per tumor bearing animal.

3.2. Mammary Tumor Incidence and Multiplicity

The first palpable mammary tumors appeared during the 9th week (57 days) of the MNU application in both diet groups (rat age 127 days). At this time only one rat from each group developed a palpable tumor. As time progressed, the incidence of mammary tumor increased. At the end of the study, the overall incidence of palpable mammary tumors was 60% (12/20 rats) in West diet, while it was 70% (14/20 rats) in W+Cur diet, indicating similar tumor incidence in both groups. The median time to tumor onset were 182 days of age in West diet, while it was 164.5 days in W+Cur diet.

The Kaplan–Meier method was used to study the mammary tumor latency in these two diet groups. Figure 2b shows the probability of animals without tumor incidence from Kaplan–Meier method. To test if the rats in one diet group have a longer time to first occurrence of tumor than those in the other, we conducted a log rank test using chi square statistics. The $p$-value from chi square test was 0.2748, indicating that the survival curves for two groups are not significantly different from each other. Cox proportional hazard regression model was also run on the tumor onset survival data to estimate the ratio of hazard rate in two diet groups. The hazard ratio was 1.51, with a 95% confidence interval of (0.69–3.28) and $p$-value of 0.2927, indicating that the tumor onset for the rats in two diet groups are not significantly different from each other.
We also compared the volume of the first detectable tumor at palpation for both the groups. These results are shown in Figure 2c. Mean volume at tumor onset was $734 \text{ mm}^3$ for West diet, while it was $507 \text{ mm}^3$ for W+Cur diet. Even though the mean tumor volume at first palpation for W+Cur diet group was lower, it was not significantly different from West diet group. Figure 2d shows the tumor multiplicity per tumor bearing animal, which was 1.67 for West diet group and 1.86 W+Cur diet, not significantly different from each other.

### 3.3. Mammary Tumor Growth before Treatment

Further, we studied the relative change in the volume of the first tumor post appearance for both diet groups, but before the start of treatment. Figure 3a,b show the absolute tumor volumes for individual rats in West and W+Cur diet, respectively. The tumor volumes were also normalized with the tumor volume at inception to calculate the relative tumor volume, as shown in Figure 3c,d. A considerable variability was observed in the tumor growth of individual rats within both diet groups. Several rats in both groups showed exponential tumor growth. The tumor growth was similar for both diet groups.

![Figure 3](image)

**Figure 3.** Individual tumor growth curves showing absolute (a,b) and relative (c,d) tumor volumes over days post tumor appearance but before treatment start: (a,c) West diet, (b,d) W+Cur diet. The tumor volumes were normalized with the tumor volume at inception to calculate the relative tumor volume.

Together these results highlight that the W+Cur diet did not impact postnatal development, tumor incidence, latency, multiplicity, and growth rates.

### 3.4. Mammary Tumor Growth after Treatment

Tumor volumes were measured after treatment with EP+Cur for rats in West and W+Cur diet (Figure 4a,b). To quantitively characterize the tumor growth after treatment in both diet groups, relative tumor volumes and SGR were calculated at days 7 and 14 following treatment, with respect to pre-treatment volumes (on day 0). These results are shown in Tables 2 and 3 for West and W+Cur diet, respectively. Relative tumors and SGR were also measured in second and third tumors appeared in the
treated rats (Table 4). A negative SGR (i.e., reduction in tumor volume) at day 14 compared to day 0 was considered to be a tumor response. When rats on West diet were treated with EP+Cur, only 16.67% (1/6) showed a tumor response in at least one tumor nodule. On the other hand, 57.14% (4/7) rats on W+Cur diet showed tumor response in at least one nodule upon EP+Cur treatment.

Figure 4. Individual tumor growth curves showing absolute tumor volumes over days after treatment: (a) West diet with ECT with intratumoral curcumin (EP+Cur) treatment, (b) W+Cur diet with EP+Cur treatment.

Table 2. Quantitative characterization of tumor growth for rats on West diet with EP+Cur treatment. The relative tumor volumes and specific growth rate (SGR) were calculated for days 7 and 14 after treatment, compared to the tumor volume before start of the treatment (at day 0).

|                  | Day | Rat | 6   | 4   | 3   | 11  | 15  | 18  |
|------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| **Absolute Tumor Volumes** |     |     |     |     |     |     |     |     |
| 0                |     |     | 1259| 720 | 833 | 2020| 1067| 1184|
| 7                |     |     | 1028| 690 | 922 | 2964| 1612| 3561|
| 14               |     |     | 1149| 777 | 996 | 8487| 1777| -   |
| Necrosis         |     |     | 15% | <5% | 10% | 20% | 50% | -   |
| **Relative Tumor Volumes** |     |     |     |     |     |     |     |     |
| 0                |     |     | 1.00| 1.00| 1.00| 1.00| 1.00| 1.00|
| 7                |     |     | 0.82| 0.96| 1.11| 1.47| 1.51| 3.01|
| 14               |     |     | 0.91| 1.08| 1.20| 4.20| 1.67| -   |
| **Specific Growth Rate (SGR)** |     |     |     |     |     |     |     |     |
| 7                |     |     | −0.029| −0.006| 0.015| 0.055| 0.059| 0.157|
| 14               |     |     | −0.007| 0.005| 0.013| 0.103| 0.036| -   |
Table 3. Quantitative characterization of tumor growth for rats on W+Cur diet with EP+Cur treatment. The relative tumor volumes and SGR were calculated for days 7 and 14 after treatment, compared to the tumor volume before start of the treatment (at day 0).

| Day | Rat     | 29   | 30   | 35   | 26   | 31   | 34   | 40   |
|-----|---------|------|------|------|------|------|------|------|
| 0   |         | 1580 | 2702 | 2630 | 866  | 1223 | 1323 | 808  |
| 7   |         | 1118 | 1859 | 3165 | 1935 | 2472 | 2773 | 1459 |
| 14  |         | 294  | 2087 | 2378 | 2909 | 1780 | -    | 2236 |

Necrosis: 10% 5% 25% 15% 30% 30% 15%

| Day | Rat     | 29   | 30   | 35   | 26   | 31   | 34   | 40   |
|-----|---------|------|------|------|------|------|------|------|
| 0   |         | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 7   |         | 0.71 | 0.69 | 1.20 | 2.23 | 2.02 | 2.10 | 1.81 |
| 14  |         | 0.19 | 0.77 | 0.90 | 3.36 | 1.45 | -    | 2.77 |

Specific Growth Rate (SGR)

| Day | Rat     | 29   | 30   | 35   | 26   | 31   | 34   | 40   |
|-----|---------|------|------|------|------|------|------|------|
| 7   |         | −0.049 | −0.053 | 0.026 | 0.115 | 0.101 | 0.106 | 0.084 |
| 14  |         | −0.120 | −0.018 | −0.007 | 0.087 | 0.027 | -    | 0.073 |

Table 4. Quantitative characterization of growth of second (T2) and third (T3) tumors only, after treatment with EP+Cur for both West and W+Cur diet. The relative tumor volumes and SGR were calculated for days 7 and 14 after treatment, compared to the tumor volume before start of the treatment (at day 0).

| Day | Rat     | 6–T2 | 6–T3 | 29–T2 | 35–T2 | 35–T3 | 31–T2 | 31–T3 |
|-----|---------|------|------|-------|-------|-------|-------|-------|
| 0   |         | 780  | 514  | 2331  | 1176  | 992   | 982   | 1360  |
| 7   |         | 879  | 520  | 1633  | 1391  | 1134  | 168   | 1386  |
| 14  |         | 604  | 193  | -     | -     | -     | 204   | 1981  |

Necrosis: 5% 5% 30% 5% 5% 10% 10%

| Day | Rat     | 6–T2 | 6–T3 | 29–T2 | 35–T2 | 35–T3 | 31–T2 | 31–T3 |
|-----|---------|------|------|-------|-------|-------|-------|-------|
| 0   |         | 1.00 | 1.00 | 1.00  | 1.00  | 1.00  | 1.00  | 1.00  |
| 7   |         | 1.13 | 1.01 | 0.70  | 1.18  | 1.14  | 0.17  | 1.02  |
| 14  |         | 0.77 | 0.38 | -     | -     | -     | 0.21  | 1.46  |

Specific Growth Rate (SGR)

| Day | Rat     | 6–T2 | 6–T3 | 29–T2 | 35–T2 | 35–T3 | 31–T2 | 31–T3 |
|-----|---------|------|------|-------|-------|-------|-------|-------|
| 7   |         | 0.017 | 0.002 | −0.051 | 0.024 | 0.019 | −0.252 | 0.003 |
| 14  |         | −0.018 | −0.070 | -     | -     | -     | −0.112 | 0.027 |

Further, we also determined a correlation between tumor size at treatment on day 0 and tumor response for rats on W+Cur diet and EP+Cur treatment. Fisher’s exact test (two tail) revealed that there was a significant ($p$-value = 0.0286) correlation between tumor response and tumor size (>1500 mm$^3$) for W+Cur diet with EP+Cur treatment.
Tumors that showed a lack of response to treatment on W+Cur diet were not associated with differences in pre-treatment growth rate. To support this, we compared the growth rate of all the tumors (including second and third tumors) before treatment (before day 0) for the rats. For example, in rats 31 and 40 which did not respond to the treatment, tumor sizes were 50% and 61% at 7 days before treatment compared to the day of treatment. In comparison, at 7 days before treatment, the sizes were 52% and 35% for rats 35 and 30, which responded to the treatment. This indicates that the tumor growth rate before treatment was similar in tumors irrespective of the response to EP+Cur treatment. This was confirmed by the repeated measure ANOVA analysis on all tumors, which showed that there was no significant difference between these two groups ($p$-value = 0.9314).

The extent of visual necrosis in the tumor tissues was also analyzed in the tumor tissues collected post euthanasia. Tables 2–4 show the percentage of necrotic tumor tissue. There was no correlation between the percentage of necrosis and tumor response for both treatment groups, indicating that the levels of necrosis did not influence the tumor response.

Together, these results suggest that EP+Cur treatment results in a better response, when the rats are fed W+Cur diet, with larger nodules showing significantly higher response rates compared to smaller nodules, despite same level of growth in both, before treatment.

4. Discussion

In the present study, we investigated the effect of electrochemotherapy with curcumin on the spontaneous mammary tumor growth in female SD rats on West or W+Cur diet. The SD rats-MNU system used in this study is a widely accepted model of human breast cancer [47–49]. The mammary tumor histology in this model resembles strongly to that of human breast tumors [48,49]. Similar to humans, rat model mammary tumors are primarily ductal in origin, as compared to mouse lesions, which are mainly alveolar [52]. The MNU induced mammary tumors remains one of the most frequently used models to study mammary tumor carcinogenesis and treatment [48,52,53]. The MNU model offers several advantages, including easy application without needing irradiation or metabolic activation for tumorigenesis, reliability of tumor induction, organ specificity, and ductal origin of the tumors [50,52].

We observed that the final incidence of palpable mammary carcinoma was 60% in West diet and 70% in W+Cur diet after MNU administration. This is in agreement with previous SD rat studies, where 60–76% of the incidences of palpable mammary carcinomas were observed upon MNU administration [52,53]. In our study, the first palpable mammary tumors were observed during the 9th week (57 days) of the MNU application in both diet groups (rat age 127 days), which matches extremely well with previous observation, where palpable tumors appeared 9 weeks after the first application of MNU in SD rats [47]. These observations are in line with the existing literature and establish the validity of the model to demonstrate the efficacy of ECT with curcumin.

We did not observe any significant difference between West and W+Cur diet groups in tumor onset, incidence, multiplicity, and growth. These results were expected, as we intentionally added a low concentration of curcumin in West diet (1% w/w) to avoid confounding in our study, as, a delay in tumor onset and growth due to curcumin diet alone could impact tumor treatment response. This correlates with previous studies, where a higher (2% w/w) concentration of dietary curcumin did not reduce the tumor incidence, latency, and multiplicity in carcinogen induced mammary tumorigenesis [54,55].

Herein we demonstrate that EP+Cur treatment resulted in a better tumor response, when the rats were fed with W+Cur diet (57.14% response vs. 16.67% response in West diet). Further, we showed that the larger tumor nodules (>1500 mm$^3$) within Cur diet were more likely to exhibit significantly higher response rates compared to smaller nodules upon EP+Cur treatment, despite same level of pre-treatment growth in both.

Regarding curcumin's actions, despite the low bioavailability of curcumin, its metabolites could positively influence the effect of EP+Cur treatment we observed in this study. Curcumin is rapidly and efficiently transformed into metabolites, which reduces the bioavailability of parent compound curcumin upon entering the body [56]. However, in circulation, curcumin undergoes conjugation,
including glucuronidation and sulfation in tissues, mostly in the liver [57]. These conjugates are now known to exert antioxidative, anti-inflammatory, and anti-cancer effects [58]. In addition, we and others have quantitively shown the enhanced uptake and cytotoxicity effects of curcumin when delivered with electrical pulses [41,42,46,59].

In this study, we used 1% curcumin concentration in the diet. However, it will be of interest to further study the effect of various curcumin concentrations in diet on the outcome of EP+Cur. Previously, a study showed that a 2% w/w dietary curcumin with paclitaxel decreased the breast cancer metastasis to lung by downregulating paclitaxel-induced NF-κB-regulated gene products [60]. Considering this, we hypothesize that a higher concentration of curcumin in diet (2% or more) may further enhance the effects of EP+Cur treatment.

This is the first study, demonstrating the enhanced effects of ECT with curcumin on rat mammary tumors with W+Cur diet. Although, in this study the tumor initiation and progression under these novel conditions were studied, the limitation of this study is that a detailed investigation on histopathological phenotype of the treated tumors with respect to the controls was not included. Future work will investigate histopathological features and immunohistochemistry analyses of apoptosis, cell proliferation, and angiogenesis.

However, these results do provide a foundation for further studies, such as characterizing the association between W+Cur diet and the effectiveness of EP+Cur. Previous studies have reported that curcumin exhibits a diverse array of metabolic, cellular, and molecular activities including potent anti-inflammatory actions due to its ability to target pro-inflammatory pathways, such as NF-κB, MAPK, and JAK/STAT signaling pathways [4,61]. The chronic inflammation promotes cancer cell growth through direct or indirect interactions between inflammatory cells and cancer cells, including release of growth factors, removal of growth suppressors, and enhanced resistance to cell death [4]. The inflammatory tumor microenvironment is also linked with the metabolic reprogramming to aerobic glycolysis (Warburg effect). The pro-inflammatory cytokine TNF-α induces aerobic glycolysis, lactate export, and expression of the glucose transporter 1 (GLUT1), which is reversed by dietary curcumin [61,62]. In connection with these documented actions of curcumin, our group has previously demonstrated that the EP+Cur treatment targeted glycolysis with reduced glucose uptake and intracellular lactate in MDA-MB-231 breast cancer cells to trigger apoptotic cell death [42]. Thus, a possible explanation of the better tumor response observed in Cur diet group upon EP+Cur treatment in the present study could be the result of the combination of these long-term and related mechanisms. In future, it will be worthwhile investigating other promising plant derived compounds, such as lycopene and resveratrol [63], which could have positive influence on better response of EP+Cur.

**Author Contributions:** R.S. conceptualized and supported the study. R.S. and I.G.C. designed the study. L.M. prepared and maintained all reagents for tumorigenesis and treatment. I.G.C. provided expertise with animal model and administered MNU. L.M. performed data analysis, statistical analysis, and drafted the first version of the manuscript. All authors participated in data interpretation. All authors commented and approved the final version of the manuscript.

**Funding:** This research received no external funding.

**Acknowledgments:** We are grateful to Bennett David Elzey, Sandra Torregrosa-Allen, and Melanie Currie for their great help with the design and conducting this study. We also are grateful to Sakkarapalayam Mahalingam for his assistance with MNU injection. We also thank the reviewers and the editor for their insightful comments, which enhanced the quality of the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA 2018, 68, 394–424. [CrossRef] [PubMed]
Biomedicines 2020, 8, 498

2. Aggarwal, B.B.; Sundaram, C.; Malani, N.; Ichikawa, H. Curcumin: The Indian solid gold. In The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease; Aggarwal, B.B., Surh, Y.-J., Shishodia, S., Eds.; Springer US: Boston, MA, USA, 2007; pp. 1–75. ISBN 978-0-387-46401-5.

3. Schraufstatter, E.; Bernt, H. Antibacterial Action of Curcumin and Related Compounds. Nature 1949, 164, 456–457. [CrossRef] [PubMed]

4. Banik, U.; Parasuraman, S.; Adhikary, A.K.; Othman, N.H. Curcumin: The spicy modulator of breast carcinogenesis. J. Exp. Clin. Cancer Res. 2017, 36, 619–621. [CrossRef] [PubMed]

5. Kunnumakkara, A.B.; Bordoloi, D.; Harsha, C.; Banik, K.; Gupta, S.C.; Aggarwal, B.B. Curcumin mediates anticancer effects by modulating multiple cell signaling pathways. Clin. Sci. 2017, 131, 1781–1799. [CrossRef]

6. Varghese, E.; Samuel, S.; Abotaleb, M.; Cheema, S.; Mantani, R.; Büsselberg, D. The “Yin and Yang” of Natural Compounds in Anticancer Therapy of Triple-Negative Breast Cancers. Cancers 2018, 10, 346. [CrossRef]

7. Oppenheimer, A. TURMERIC (CURCUMIN) IN BILIARY DISEASES. Lancet 1937. [CrossRef]

8. Pubmed.gov Curcumin—Search Results—PubMed. 2020. Available online: https://pubmed.ncbi.nlm.nih.gov/?term=curcumin (accessed on 9 June 2020).

9. Search of: Curcumin—List Results—ClinicalTrials.gov. Available online: https://www.clinicaltrials.gov/ct2/results?cond=&term=curcumin&cntry=&state=&city=&dist= (accessed on 13 September 2020).

10. Heger, M. Don’t discount all curcumin trial data. Nature 2017, 543, 40. [CrossRef]

11. Search of: Curcumin | Breast Cancer—List Results—ClinicalTrials.gov. Available online: https://www.clinicaltrials.gov/ct2/results?cond=Breast+Cancer&term=curcumin&cntry=&state=&city=&dist= (accessed on 13 September 2020).

12. Ryan, J.L.; Heckler, C.E.; Ling, M.; Katz, A.; Williams, J.P.; Pentland, A.P.; Morrow, G.R. Curcumin for radiation dermatitis: A randomized, double-blind, placebo-controlled clinical trial of thirty breast cancer patients. Radiat. Res. 2013, 180, 34–43. [CrossRef]

13. Bayet-Robert, M.; Kwiatkowski, F.; Leheurteur, M.; Gachon, F.; Planchat, E.; Abrial, C.; Mouret-Reynier, M.-A.; Durando, X.; Barthomeuf, C.; Chollet, P. Phase I dose escalation trial of docetaxel plus curcumin in patients with advanced and metastatic breast cancer. Cancer Biol. Ther. 2010, 9, 8–14. [CrossRef]

14. Saghatelayan, T.; Tananyan, A.; Janoyan, N.; Tadevosyan, A.; Petrosyans, H.; Hovhannisyan, A.; Hayrapetyan, L.; Arustamyan, M.; Arnhold, J.; Rotmann, A.-R.; et al. Efficacy and safety of curcumin in combination with paclitaxel in patients with advanced, metastatic breast cancer: A comparative, randomized, double-blind, placebo-controlled clinical trial. Phytomedicine 2020, 70, 153218. [CrossRef]

15. Pignanelli, C.; Ma, D.; Noel, M.; Ropat, J.; Mansour, F.; Pupulin, S.; Larocque, K.; Wu, J.; Liang, G.; Wang, Y.; et al. Selective Targeting of Cancer Cells by Oxidative Vulnerabilities with Novel Curcumin Analogs. Sci. Rep. 2017, 7, 1105. [CrossRef] [PubMed]

16. Marty, M.; Sersa, G.; Garbay, J.R.; Gehl, J.; Collins, C.G.; Snoj, M.; Billard, V.; Geertsen, P.F.; Larkin, J.O.; Miklavcic, D.; et al. Electrochemotherapy—An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. Eur. J. Cancer Suppl. 2006, 4, 3–13. [CrossRef]

17. Sersa, G.; Cemazar, M.; Rudolf, Z. Electrochemotherapy: Advantages and Drawbacks in Treatment of Cancer Patients Review Article. 2003. Available online: https://pdfs.semanticscholar.org/ef4a/d524d99c62b9fd5a3b7fecc3c3c99660df7.pdf (accessed on 29 September 2018).

18. Cadossi, R.; Ronchetti, M.; Cadossi, M. Locally enhanced chemotherapy by electroporation: clinical experiences and perspective of use of electrochemotherapy. Futur. Oncol. 2014, 10, 877–890. [CrossRef] [PubMed]

19. Sersa, G.; Stabuc, B.; Cemazar, M.; Jancar, B.; Miklavcic, D.; Rudolf, Z. Electrochemotherapy with cisplatin: potentiation of local cisplatin antitumour effectiveness by application of electric pulses in cancer patients. Eur. J. Cancer 1998, 34, 1213–1218. [CrossRef]

20. Sersa, G.; Stabuc, B.; Cemazar, M.; Miklavcic, D.; Rudolf, Z. Electrochemotherapy with cisplatin: the systemic antitumour effectiveness of cisplatin can be potentiated locally by the application of electric pulses in the treatment of malignant melanoma skin metastases. Melanoma Res. 2000, 10, 381–385. [CrossRef]

21. Sersa, G.; Stabuc, B.; Cemazar, M.; Miklavcic, D.; Rudolf, Z. Electrochemotherapy with cisplatin: clinical experience in malignant melanoma patients. Clin. Cancer Res. 2000, 6, 863–867.

22. Rebersek, M.; Cufer, T.; Cemazar, M.; Kranjc, S.; Sersa, G. Electrochemotherapy with cisplatin of cutaneous tumor lesions in breast cancer. Anticancer. Drugs 2004, 15, 593–597. [CrossRef]
23. De Giorgi, V.; Scarfi, F.; Saquer, E.; Gori, A.; Tomassini, G.M.; Covarelli, P. The use of cisplatin electrochemotherapy in nonmelanoma skin cancers: A single-center study. *Dermatol. Ther.* 2020, e13547.

24. Belehradek, J.; Orlovska, S.; Poddevin, B.; Paoletti, C.; Mir, L.M. Electrochemotherapy of spontaneous mammary tumours in mice. *Eur. J. Cancer Clin. Oncol.* 1991, 27, 73–76. [CrossRef]

25. Lanza, A.; Pettorali, M.; Baldi, A.; Spugnini, E.P. Surgery and electrochemotherapy treatment of incompletely excised mammary carcinoma in two male pet rats (Rattus norvegicus). *J. Vet. Med. Sci.* 2017, 79, 623–625. [CrossRef]

26. Tozon, N.; Sersa, G.; Cemazar, M. Electrochemotherapy: Potentiation of local antitumour effectiveness of cisplatin in dogs and cats. *Anticancer Res.* 2001, 21, 2483–2488. [PubMed]

27. Campana, L.G.; Falci, C.; Basso, M.; Sieni, E.; Dughiero, F. Clinical electrochemotherapy for chest wall recurrence from breast cancer. In *Electroporation-Based Therapies for Cancer*; Woodhead Publishing: Sawston/Cambridge, UK, 2014; ISBN 978-1-907568-15-2.

28. Campana, L.G.; Mocellin, S.; Basso, M.; Puccetti, O.; De Salvo, G.L.; Chiarion-Sileni, V.; Vecchiato, A.; Corti, L.; Rossi, C.R.; Nitti, D. Bleomycin-Based Electrochemotherapy: Clinical Outcome from a Single Institution’s Experience with 52 Patients. *Ann. Surg. Oncol.* 2009, 16, 191–199. [CrossRef] [PubMed]

29. Campana, L.G.; Valpione, S.; Falci, C.; Mocellin, S.; Basso, M.; Corti, L.; Balestrieri, N.; Marchet, A.; Rossi, C.R. The activity and safety of electrochemotherapy in persistent chest wall recurrence from breast cancer after mastectomy: A phase-II study. *Breast Cancer Res. Treat.* 2012, 134, 1169–1178. [CrossRef] [PubMed]

30. Witkiewicz, M.; Murawa, D.; Litwiniuk, M.; Kufel-Grabowska, J.; Bocian, A.; Witkiewicz, W. Initial experiences of the use of electrochemotherapy in the treatment of skin metastases. *Nowotwory* 2016, 66, 127–135.

31. Matthiessen, L.W.; Keshtgar, M.; Curatolo, P.; Kunte, C.; Grischke, E.-M.; Odili, J.; Muir, T.; Mowatt, D.; Clover, J.P.; Liew, S.H.; et al. Electrochemotherapy for Breast Cancer-Results From the INSPECT Database. *Clin. Breast Cancer* 2018, 18, e909–e917. [CrossRef] [PubMed]

32. Whelan, M.C.; Larkin, J.O.; Collins, C.G.; Cashman, J.; Breathnach, O.; Soden, D.M.; O’Sullivan, G.C. Effective treatment of an extensive recurrent breast cancer which was refractory to multimodal therapy by multiple applications of electrochemotherapy. *Eur. J. Cancer Suppl.* 2006, 4, 32–34. [CrossRef]

33. Larkin, J.O.; Collins, C.G.; Aarons, S.; Tangney, M.; Whelan, M.; O’Reily, S.; Breathnach, O.; Soden, D.M.; O’Sullivan, G.C. Electrochemotherapy: Aspects of Preclinical Development and Early Clinical Experience. *Ann. Surg.* 2007, 245, 469–479. [CrossRef]

34. Cabula, C.; Campana, L.G.; Grilz, G.; Galuppo, S.; Bussone, R.; De Meo, L.; Bonadies, A.; Curatolo, P.; De Laurentiis, M.; Renne, M.; et al. Electrochemotherapy in the Treatment of Cutaneous Metastases from Breast Cancer: A Multicenter Cohort Analysis. *Ann. Surg. Oncol.* 2015, 22, 442–450. [CrossRef]

35. Campana, L.G.; Galuppo, S.; Valpione, S.; Brunello, A.; Ghiotto, C.; Ongaro, A.; Rossi, C.R. Bleomycin electrochemotherapy in elderly metastatic breast cancer patients: Clinical outcome and management considerations. *J. Cancer Res. Clin. Oncol.* 2014, 140, 1557–1565. [CrossRef]

36. Benevento, R.; Santoriello, A.; Perna, G.; Canonico, S. Electrochemotherapy of cutaneous metastases from breast cancer in elderly patients: A preliminary report. *BMC Surg.* 2012, 12 (Suppl 1), S6. [CrossRef]

37. Campana, L.G.; Testori, A.; Curatolo, P.; Quaglino, P.; Mocellin, S.; Framarin, M.; Borgognoni, L.; Ascierto, P.A.; Mozillo, N.; Guida, M.; et al. Treatment efficacy with electrochemotherapy: A multi-institutional prospective observational study on 376 patients with superficial tumors. *Eur. J. Surg. Oncol.* 2016, 42, 1914–1923. [CrossRef]

38. Overview | Electrochemotherapy for Primary Basal Cell Carcinoma and Primary Squamous Cell Carcinoma | Guidance | NICE. 2014. Available online: https://www.nice.org.uk/guidance/IPG478 (accessed on 2 June 2019).

39. Gehl, J.; Sersa, G.; Matthiessen, L.W.; Muir, T.; Soden, D.; Occhini, A.; Quaglino, P.; Curatolo, P.; Campana, L.G.; Kunte, C.; et al. Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. *Acta Oncol.* (Madr.) 2018, 57, 874–882. [CrossRef]

40. Thulasidas, J.S.; Varadarajan, G.S.; Mittal, L.; Sundararajan, R. The Combined Effect of Aqueous Tulasi Extract and Electrical Pulses on the Viability of HeLa Cells. *IETE J. Res.* 2019. [CrossRef]

41. Mittal, L.; Raman, V.; Camarillo, I.G.; Sundararajan, R. Ultra-microsecond pulsed curcumin for effective treatment of triple negative breast cancers. *Biochem. Biophys. Res. Commun.* 2017, 491, 1015–1020. [CrossRef] [PubMed]

42. Mittal, L.; Aryal, U.K.; Camarillo, I.G.; Raman, V.; Sundararajan, R. Effective electrochemotherapy with curcumin in MDA-MB-231-human, triple negative breast cancer cells: A global proteomics study. *Bioelectrochemistry* 2020, 131, 107350. [CrossRef] [PubMed]
43. Mittal, L.; Camarillo, I.G.; Varadarajan, G.S.; Srinivasan, H.; Aryal, U.K.; Sundararajan, R. High-throughput, Label-Free Quantitative Proteomic Studies of the Anticancer Effects of Electrical Pulses with Turmeric Silver Nanoparticles: An in vitro Model Study. *Sci. Rep.* 2020, 10, 7258. [CrossRef] [PubMed]

44. Mittal, L.; Aryal, U.K.; Camarillo, I.G.; Ferreira, R.M.; Sundararajan, R. Quantitative proteomic analysis of enhanced cellular effects of electrochemotherapy with Cisplatin in triple-negative breast cancer cells. *Sci. Rep.* 2019, 9, 13916. [CrossRef] [PubMed]

45. Camarillo, I.G.; Xiao, F.; Madhivanan, S.; Salameh, T.; Nichols, M.; Reece, L.M.; Leary, J.F.; Otto, K.; Natarajan, A.; Ramesh, A.; et al. Low and high voltage electrochemotherapy for breast cancer: An in vitro model study BT. In *Electroporation-Based Therapies for Cancer*; Woodhead Publishing: Sawston/Cambridge, UK, 2014; pp. 55–102. ISBN 978-1-907568-15-2.

46. Mittal, L.; Raman, V.; Camarillo, I.G.; Garner, A.L.; Sundararajan, R. Viability and cell cycle studies of metastatic triple negative breast cancer cells using low voltage electrical pulses and herbal curcumin. *Biomed. Phys. Eng. Express* 2019, 5, 025040. [CrossRef]

47. Perše, M.; Cerar, A.; Injac, R.; Štrukelj, B. N-methylnitrosourea Induced Breast Cancer in Rat, the Histopathology of the Resulting Tumours and its Drawbacks as a Model. *Pathol. Oncol. Res.* 2009, 15, 115–121. [CrossRef] [PubMed]

48. Tsubura, A.; Lai, Y.-C.; Miki, H.; Sasaki, T.; Uehara, N.; Yuri, T.; Yoshizawa, K. Animal Models of N-Methyl-N-nitrosourea-induced Mammary Tumorigenesis and Retinal Degeneration with Special Emphasis on Therapeutic Trials. *In Vivo* 2011, 25, 11–22. [PubMed]

49. Roomi, M.W.; Roomi, N.W.; Ivanov, V.; Kalinovsky, T.; Niedzwiecki, A.; Rath, M. Modulation of N-methyl-N-nitrosourea induced mammary tumors in Sprague-Dawley rats by combination of lysine, proline, arginine, ascorbic acid and green tea extract. *Breast Cancer Res.* 2005, 7, R291–R295. [CrossRef] [PubMed]

50. Thompson, H.J.; Adalaka, H. Dose-responsive induction of mammary gland carcinomas by the intraperitoneal injection of 1-methyl-1-nitrosourea. *Cancer Res.* 1991, 51, 3411–3415. [PubMed]

51. Mehrara, E.; Forssell-Aronsson, E.; Ahlman, H.; Bernhardt, P. Specific growth rate versus doubling time for quantitative characterization of tumor growth rate. *Cancer Res.* 2007, 67, 3970–3975. [CrossRef] [PubMed]

52. Thompson, H.J.; McGinley, J.N.; Rothhammer, K.; Singh, M. Rapid induction of mammary intraductal proliferations, ductal carcinoma in situ and carcinomas by the injection of sexually immature female rats with 1-methyl-1-nitrosourea. *Carcinogenesis* 1995, 16, 2407–2411. [CrossRef] [PubMed]

53. Liska, J.; Galbavy, S.; Macejova, D.; Zlatos, J.; Brtko, J. Histopathology of mammary tumours in female rats treated with 1-methyl-1-nitrosourea. *Endocr. Regul.* 2000, 34, 91–96. [PubMed]

54. Siddiqui, R.A.; Harvey, K.A.; Walker, C.; Altenburg, J.; Xu, Z.; Terry, C.; Camarillo, I.; Jones-Hall, Y.; Mariash, C. Characterization of synergistic anti-cancer effects of docosahexaenoic acid and curcumin on DMBA-induced mammary tumorigenesis in mice. *BMC Cancer* 2013, 13, 418. [CrossRef]

55. Huang, M.T.; Lou, Y.R.; Xie, J.G.; Ma, W.; Lu, Y.P.; Yen, P.; Zhu, B.T.; Newmark, H.; Ho, C.T. Effect of dietary curcumin and difenzoylmethane on formation of 7,12-dimethylbenz[a]anthracene-induced mammary tumors and lymphomas/leukemias in Sencar mice. *Carcinogenesis* 1998, 19, 1697–1700. [CrossRef]

56. Sharma, R.A.; Steward, W.P.; Gescher, A.J. Pharmacokinetics and pharmacodynamics of curcumin. *Adv. Exp. Med. Biol.* 2007, 595, 453–470.

57. Toden, S.; Goel, A. The Holy Grail of Curcumin and its Efficacy in Various Diseases: Is Bioavailability Truly a Big Concern? *J. Restor. Med.* 2017, 6, 27–36. [CrossRef]

58. Prasad, S.; Tyagi, A.K.; Aggarwal, B.B. Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: The golden pigment from golden spice. *Cancer Res. Treat.* 2014, 46, 2–18. [CrossRef]

59. Lu, C.-H.; Lin, S.-H.; Hsieh, C.-H.; Chen, W.-T.; Chao, C.-Y. Enhanced anticancer effects of low-dose curcumin with non-invasive pulsed electric field on PANC-1 cells. *Onco Targets Ther.* 2018, 11, 4723–4732. [CrossRef] [PubMed]

60. Aggarwal, B.B.; Shishodia, S.; Takada, Y.; Banerjee, S.; Newman, R.A.; Bueso-Ramos, C.E.; Price, J.E. Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB pathway in breast cancer cells and inhibits lung metastasis of human breast cancer in nude mice. *Clin. Cancer Res.* 2005, 11, 7490–7498. [CrossRef] [PubMed]

61. Aggarwal, B.B.; Gupta, S.C.; Sung, B. Curcumin: An orally bioavailable blocker of TNF and other pro-inflammatory biomarkers. *Br. J. Pharmacol.* 2013, 169, 1672–1692. [CrossRef] [PubMed]
62. Vaughan, R.A.; Garcia-Smith, R.; Dorsey, J.; Griffith, J.K.; Bisoffi, M.; Trujillo, K.A. Tumor necrosis factor alpha induces Warburg-like metabolism and is reversed by anti-inflammatory curcumin in breast epithelial cells. *Int. J. Cancer* 2013, 133, 2504–2510. [CrossRef]

63. Mitra, S.; Dash, R. Natural Products for the Management and Prevention of Breast Cancer. *Evid. Based Complement. Altern. Med.* 2018, 2018, 8324696. [CrossRef]

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