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RESEARCH ARTICLE

Orally Administered Curcumin Inhibits Breast Cancer *In vivo* and Reduces Cell Proliferation *In vitro* in an Iron Dependent Manner

Marce Inzunza-Soto[1], Emir Leal-León[2], Marcela Vergara-Jiménez[1], Samuel López-Moreno[2], Maria Cedano-Prieto[2], Moises Franco[3], Jesús Enrique Jiménez-Ferrer[4], Elian Yuritzi Alegría Herrera[4] Jonathan Cortés Alvarez[5], David Alemán-Hidalgo[1] and Eli Teran-Cabanillas[1,*]

[1]Facultad de Nutrición y Gastronomía, Universidad Autónoma de Sinaloa, Avenida Cedros y Calle Sauces S/N, Culiacán, Sinaloa, 80019, México; [2]Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa, Ciudad Universitaria, Culiacán, Sinaloa, 80030, México; [3]Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Ciudad Universitaria, San Nicolás de los Garza, Nuevo León, México; [4]Centro de Investigaciones Biomédicas del Sur-IMSS, Calle República Argentina 1, C.P 62790 Centro Xochitepec, Morelos, México; [5]Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Morelos. Avenida Universidad 39, C.P. 62210, Cuernavaca, Morelos, México

Abstract: ***Background:*** Increased intracellular iron metabolism is a hallmark of breast cancer. Curcumin is an iron chelator with suggested anti-proliferative effects in breast cancer cell lines. However, preclinical studies in murine models are required to validate these important benefits.

Aims: Therefore, this study aimed to determine if the iron-chelating properties of curcumin are responsible for its anti-proliferative effect in breast cancer cells and to investigate the translation of this effect to *in vivo* models.

Methods: For *in vitro* experiments, human MCF-7 and mouse 4T1 breast cancer cells were tested. Cell proliferation was assessed in the presence and absence of different concentrations of FAC (ferric ammonium citrate) and curcumin. For *in vivo* studies, 4T1 cells were implanted into BALB/c mice. After tumor development, animals were divided into four groups (n=5); control, curcumin, optimized curcumin (OC) and chemotherapy group. Tumor volumes were calculated prior and posterior oral gavage treatments.

Result: Curcumin inhibited cell proliferation in both MCF-7 and 4T1 cell lines in a seemingly iron-dependent manner. FAC addition inhibited the anti-proliferative effect exhibited by curcumin. Moreover, curcumin group showed a significantly decreased in tumor growth; interestingly, treatment with OC supplement induced the opposite effect.

Conclusion: These results suggest that curcumin may have an important positive impact on breast cancer, due to its iron-dependent and anti-proliferative properties.

ARTICLE HISTORY

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1. INTRODUCTION

Breast cancer is the most diagnosed cancer in women worldwide. Approximately one in four female cancer cases is breast cancer [1]. It also stands as one of the leading causes of morbidity and mortality in women [2]. Breast cancer is described as a multifactorial disease [3], where 5% of the risk factors are of genetic origin and the rest are modifiable factors. The latter include obesity, alcohol and tobacco consumption, physical inactivity, hyperinsulinemia, and more importantly, the diet, which accounts for 30-35% of these factors [4-6].

Several studies have proved the connection between increased cellular iron metabolism and breast cancer [7-10]. Furthermore, deregulated iron homeostasis is associated with cancer phenotype. In this matter, iron-regulating genes in charge of cellular iron uptake have been found over-expressed in cancer, which in turn promotes cellular division and survival. Tumor cells adapt the intracellular metabolism of iron in their favor in order to accumulate a greater amount of iron absorption and storage while limiting the export [11]. Previous *in vitro* and *in vivo* experiments suggest that the application of iron chelators may successfully deprive malignant cells of iron. Therefore, a balance of iron levels in cancer cells must be established that can meet metabolic needs but does not yet cause cell damage, alter oncogenic

*Address correspondence to this author at the Facultad de Ciencias de la Nutrición y Gastronomía, Universidad Autónoma de Sinaloa, Culiacán Sinaloa, 80019; Email: eteranc@uas.edu.mx

signaling, or induce ferroptosis to maintain or stop the progression of cancer disease [12].

Iron chelators are small naturally occurring or synthetic molecules that bind to iron with a high affinity. Curcumin acts as a natural iron-chelator [13]. Curcumin ((1E, 6E)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is considered a dietary polyphenol and is the main active compound in *Curcuma longa* (turmeric). Curcumin has been studied for its antiangiogenic, anti-inflammatory, antioxidant and anticancer effect, as well as for its chemo-preventive effects, chemo-sensibilization and radio-sensitization [14, 15]. *In vivo* studies have demonstrated that curcumin, alone or in combination with chemotherapeutic agents, induces apoptosis in tumor cells [14]. In the same fashion, *in vitro* studies demonstrated that curcumin induces apoptosis in several cancer cell lines such as lung cancer, myeloma, melanoma, prostate, leukemia, human neuroblast, oral and breast cancer [16]. More importantly, curcumin is a safe and well-tolerated compound both in animals and humans [17].

Treatments involving non-cytotoxic agents can improve results and overcome chemoresistance without increased toxicity to the organism [18, 19]. Understanding the impact of curcumin iron chelating properties in *in vivo* and *in vitro* cancer cells may provide new insights for better prognosis and therapeutic strategies for breast cancer.

2. METHODS

2.1. Cancer Cell Lines

MCF-7 (human breast cancer, non-metastatic and epithelial with ER-positive) and 4T1 (mouse breast cancer, metastatic, similar to the stage IV in human breast cancer) breast cancer cell lines were purchased by the Universidad Autónoma de Nuevo León from the American Type Culture Collection (ATCC). Cell maintenance was performed in T75 flasks filled with DMEM (Gibco™) and supplemented with 10 mM pyruvate, 10 mM glutamine, 10 mM non-essential amino acids, penicillin/streptomycin (100 UI/0.1mg/mL) and 10% of heat-inactivated fetal bovine serum. Cells were maintained in a humidified incubator at a concentration of 5% CO_2 and 37 °C.

2.2. *In vivo* Model

Female BALB/c mice, 6-8 weeks old and weighing 20-25 g, were supplied by Centro de Investigaciones Biomédicas del Sur-IMSS, Xochitepec, Morelos. Mice were maintained under controlled temperature, humidity and 12:12 light:dark periods with *ad libitum* food and sterile water, with a maximum of 5 animals per cage. Mice were depilated in the right flank and subcutaneously inoculated with $5x10^5$ 4T1 cells suspended in 200 µL of RPMI-1640 medium. They were randomized into 4 groups (n =5), and received by oral gavage the upcoming treatments: control group received saline solution daily for 3 weeks, curcumin group was treated with 5 mg/kg curcumin, optimized curcumin (OC) group [20] was treated with 2.5 mg/kg optimized curcumin (Optim Laboratoire) and chemotherapy group received a single dose of 15 mg/kg taxol at the beginning of all treatments. Tumor size was measured once a week with a digital Vernier caliper and tumor volume was calculated with the formula: $(ab^2)/2$, where a and b refer to the longest and shortest dimensions, respectively. Animal weight was measured once per week at the same time as tumor measurements. Mice were euthanized 5 weeks after tumor inoculation, in a CO_2 chamber in accordance with the ethical guidelines for the care and use of animals in CIBIS-IMSS Laboratory.

2.3. Iron Chelating Experiment

The methodology described by Bernabé-Pineda *et al*. [21] was performed to assess the formation of curcumin-Fe complexes. In brief, we incubated 50 mM curcumin with different concentrations of FAC (0, 10, 50, and 100 mM) in a 96-well plate, and the absorbance was measured at 430 nm.

2.4. MTT Proliferation Assay

The MTT proliferation (Thermo Fisher) assay was performed to assess cellular viability. MCF-7 and 4T1 breast cancer cells ($5x10^5$) were cultured in phenol red free RPMI-1640 medium in 96-well plates and incubated in a humidified incubator at a concentration of 5% CO_2 at 37°C overnight. Thereafter, the medium was replaced with fresh RPMI-1640 medium, containing different treatments: ferric ammonium citrate (FAC) (Sigma-Aldrich Co.) at concentrations of 10, 50 and 100 µM and curcumin (Sigma-Aldrich Co.) at concentrations of 0, 20 and 50 µM for the MCF-7 cell line, and 0, 50 and 100 µM for the 4T1 cell line. Ratio of combinations was as follows: 1:1 (50:50 µM) FAC: curcumin, 1:2 (50:100 µM) FAC:curcumin, 1:3 (50:150 µM) FAC:curcumin, 2:1 (100:50 µM) FAC:curcumin and 3:1 (150:50 µM) FAC:curcumin, all with incubations for 48 h, followed by 50 µl of MTT (2 mg/ml) added to each well and a 4 h incubation. The medium was discarded and 50 µl of dimethylsulfoxide was added to each well and incubated for 20 min. The absorbance was measured at an optical density (OD) of 540 nm. The percentage of cellular viability was calculated according to the formula: (experimental OD value/control OD value) - 100%.

2.5. RNA Extraction and PCR

Total RNA was extracted from 1 x 10^6 cells using the Qiazol lysis reagent (Qiagen, Valencia, CA, USA) following manufacturer instructions. RNA concentration and purity were analyzed by absorbance at 260/280 nm in a spectrophotometer (NanoDrop, Wilmington, DE, USA). The resulting RNA was stored at - 70 °C until needed. Real-time qRT-PCR was performed using the One-step Brilliant II SYBRGREEN QRT-PCR Master Mix kit (Stratagene, La Jolla, CA, USA) in a Step One system (Applied Biosystems, Foster City, CA, USA). The primers for TfR1 were TfRF1-Forward AGCAGAGACCACCGAAGACT and TfR1-Reverse AGACAAACCCTCCATCCAAG. The primers for Ireb2 were Ireb2-Forward ACCAGAGGTGGTT-GGATGT-GAGTT and IReb2-Reverse ACTCCTACTT-GCCTGAGG-TGCTTT. Cycle threshold (Ct) values were normalized against endogenous β-actin gene and compared

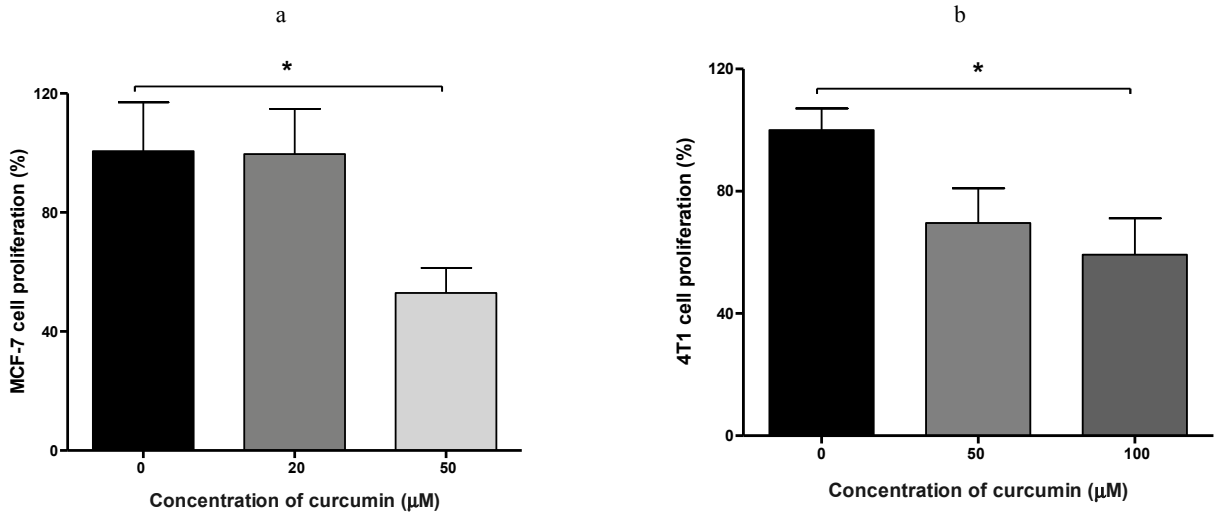

Fig. (1). Effect of curcumin in cell proliferation of breast cancer cell lines. The percentage of cell proliferation was measured by MTT assay at different concentrations of curcumin in MCF-7 (**a**) and 4T1 cells (**b**). Bars represent mean ± SEM; * indicates $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

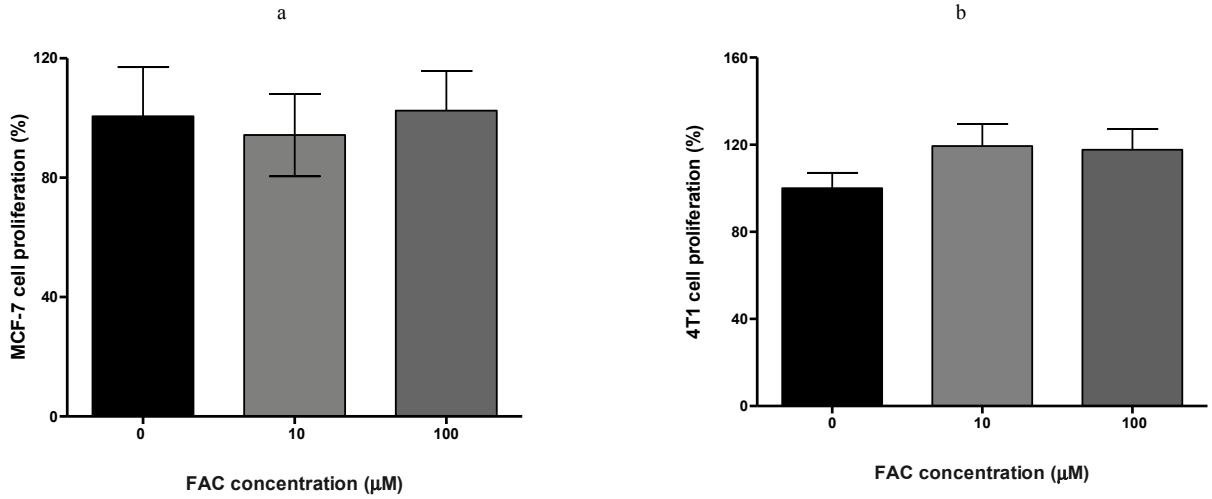

Fig. (2). Effect of FAC in cell proliferation of breast cancer cell lines. The percentage of cell proliferation was measured by MTT assay at different concentrations of FAC in MCF-7 (**a**) and 4T1 cells (**b**). Bars represent mean ± SEM. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

with the non-stimulated cells; values are represented as mean fold relative to β-actin (2^{DCt}).

2.6. Statistical Analysis

All data are presented as mean ± SEM. Comparisons between control and experimental groups were made by unpaired Student's t-test, as each experimental group was independently compared against control group. Differences were considered statistically significant when the *p*-value was < 0.05.

3. RESULTS

3.1. Curcumin Inhibits Cell Proliferation of Breast Cancer Cell Lines

To analyze the anti-proliferative effects of curcumin in MCF-7 and 4T1 breast cancer cell lines, the MTT assay was performed. The proliferation of MCF-7 cells treated with 20 μM curcumin was unchanged when compared with non-treated cells ($p > 0.05$). However, cells treated with 50 μM curcumin, showed a 47% decrease in proliferation when compared with non-treated cells ($p < 0.05$) (Fig. **1a**). On the other hand, 4T1 cells treated with 50 μM curcumin displayed a 30% inhibited proliferation in comparison with non-treated cells, however, no statistical difference was observed ($p > 0.05$). Nevertheless, 100 μM curcumin significantly inhibited 41% cell proliferation when compared with non-treated cells ($p < 0.05$) (Fig. **1b**).

3.2. Cell Proliferation of Breast Cancer Cell Lines is Unaltered by Iron

MCF-7 and 4T1 cells were treated with different concentrations of FAC. MCF-7 cells treated with 10 and 100 μM FAC exhibited non-statistically decrease in proliferation

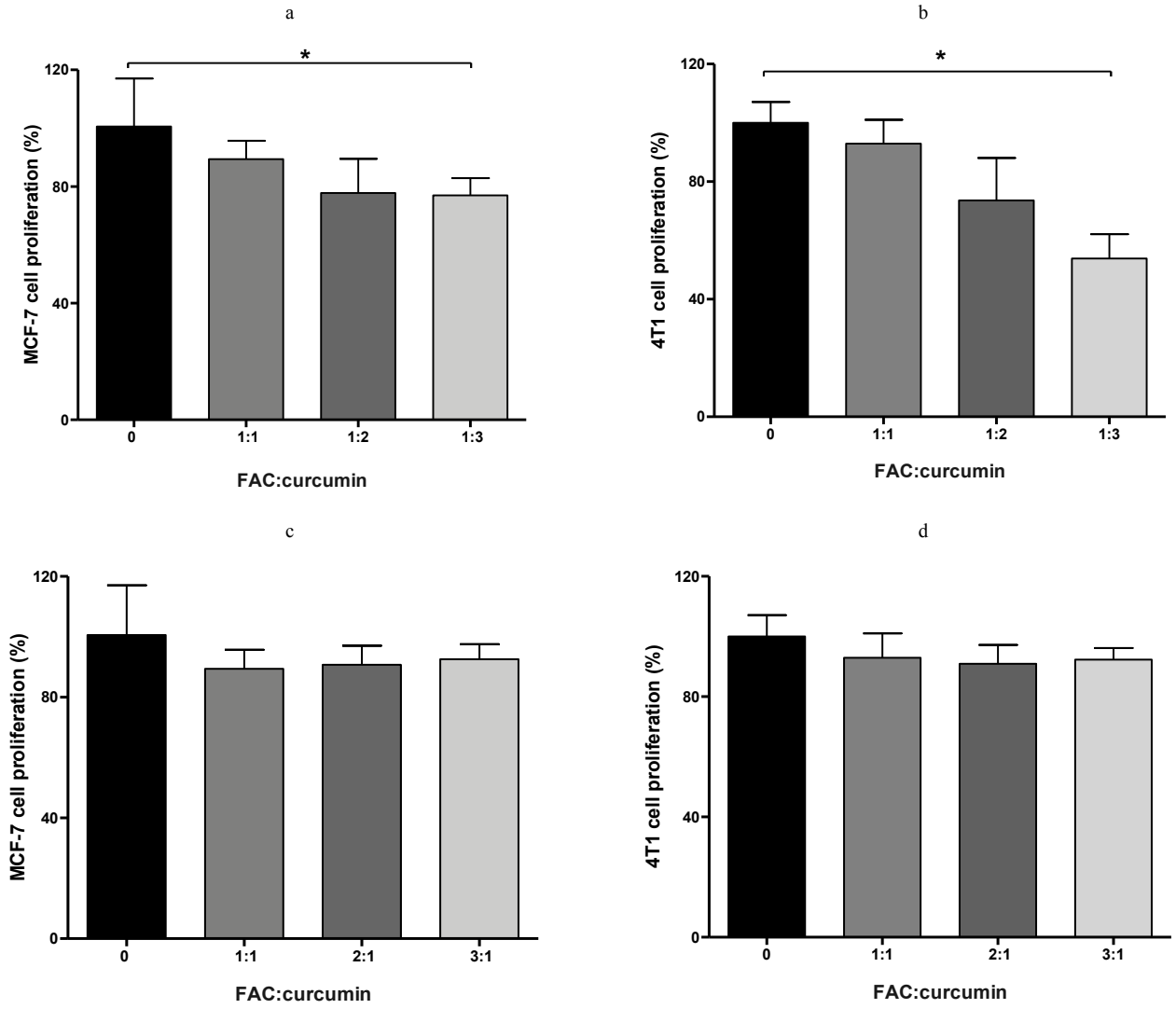

Fig. (3). Effect of co-incubating different ratios of FAC:Curcumin in the proliferation of breast cancer cell lines. The percentage of cell proliferation was measured by MTT assay at different concentrations of curcumin and a fixed concentration of FAC in MCF-7 (**a**) and 4T1 cells (**b**). In a similar fashion, the percentage of cell proliferation was measured using a fixed concentration of curcumin and increasing concentrations of FAC in MCF-7 (**c**) and 4T1 cells (**d**). Bars represent mean ± SEM; * indicates $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

when compared with non-treated cells ($p > 0.05$) (Fig. **2a**). Similar results were observed for 4T1 cells, where 10 and 100 μM FAC showed a non-statistically significant increase in cell proliferation with respect to non-treated cells (Fig. **2b**).

3.3. Anti-Proliferative Effects of Curcumin are Antagonized by Iron

MCF-7 cells treated with 1:1 ratio of FAC:curcumin showed an anti-proliferative effect of 11% when compared with non-treated cells ($p > 0.05$). For the 1:2 ratio of FAC:curcumin, a 22% decrease in cell proliferation was observed, although, this was statistically not significant. However, MCF-7 cells treated with the 1:3 ratio of FAC:curcumin, exhibited a significant 23% inhibition of cell proliferation when compared with non-treated cells ($p < 0.05$) (Fig. **3a**). With respect to 4T1 cells, results showed statistical similarity to those from MCF-7 cells, given that for 1:1, 1:2 and 1:3 ratios of FAC:curcumin anti-proliferative effects of 7, 26 and 46% were observed when compared with non-treated cells ($p > 0.05$, > 0.05 and < 0.05, respectively) (Fig. **3b**).

According to the increment of the iron source concentration, MCF-7 cells treated with 2:1 and 3:1 ratios of FAC:curcumin, presented similar proliferation when compared to non-treated cells ($p > 0.05$ in both cases) (Fig. **3c**). In addition, as well as MCF-7 cells, 4T1 cells treated with 2:1 and 3:1 ratios of FAC:curcumin displayed similar proliferation when compared with non-treated cells ($p > 0.05$) (Fig. **3d**).

Direct interaction between curcumin and iron was demonstrated as per the procedure described in Bernabé-

Pineda *et al*. [21]. Absorbance of the 50 mM curcumin solution decreased in a concentration dependent manner when increasing concentrations of FAC were added to the solution, reflecting the formation of curcumin–Fe complexes (Fig. **4**).

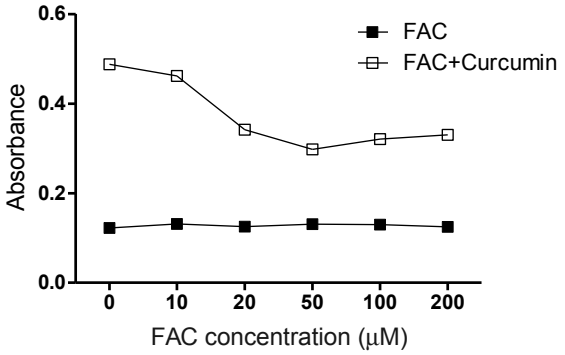

Fig. (4). Curcumin chelates iron. Incubation of 50 mM curcumin with different concentrations of FAC (0, 10, 50, and 100 mM). Absorbance was measured at 430 nm. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.4. Curcumin Modulates Iron Related Genes in Breast Cancer Cell Lines

MCF-7 and 4-T1 cells were treated with curcumin in the presence and absence of FAC, and both *TfR1* and *Ireb2* mRNA expression were analyzed. Curcumin increased the *TfR1* relative expression in both MCF-7 and 4T1 cells (Figs. **5a** and **5b**, respectively), however, *Ireb 2* relative expression remained unaltered (Fig. **5c** and **5d**). In contrast, FAC inhibited the *TfR1* relative expression in both MCF-7 and 4T1 cells (Fig. **5a** and **5b**, respectively) while demonstrating no effect on *ireb2* relative expression in both cell lines (Figs. **5c** and **5d**). Moreover, the effect of curcumin in *TfR1* relative expression was depleted when an equal concentration of FAC was added to cells.

3.5. Curcumin Inhibits Tumor Growth in Mice

During the study, mice weight range was from 16.6-24.2 g. After five weeks of treatment, mice in OC group exhibited an increased in body weight when compared with control. On the contrary, mice in chemotherapy and curcumin groups showed a decreased in body weight (Fig **6a**). Regarding tumor volume, all groups showed similar results at the second week. However, tumor volume from each group began to

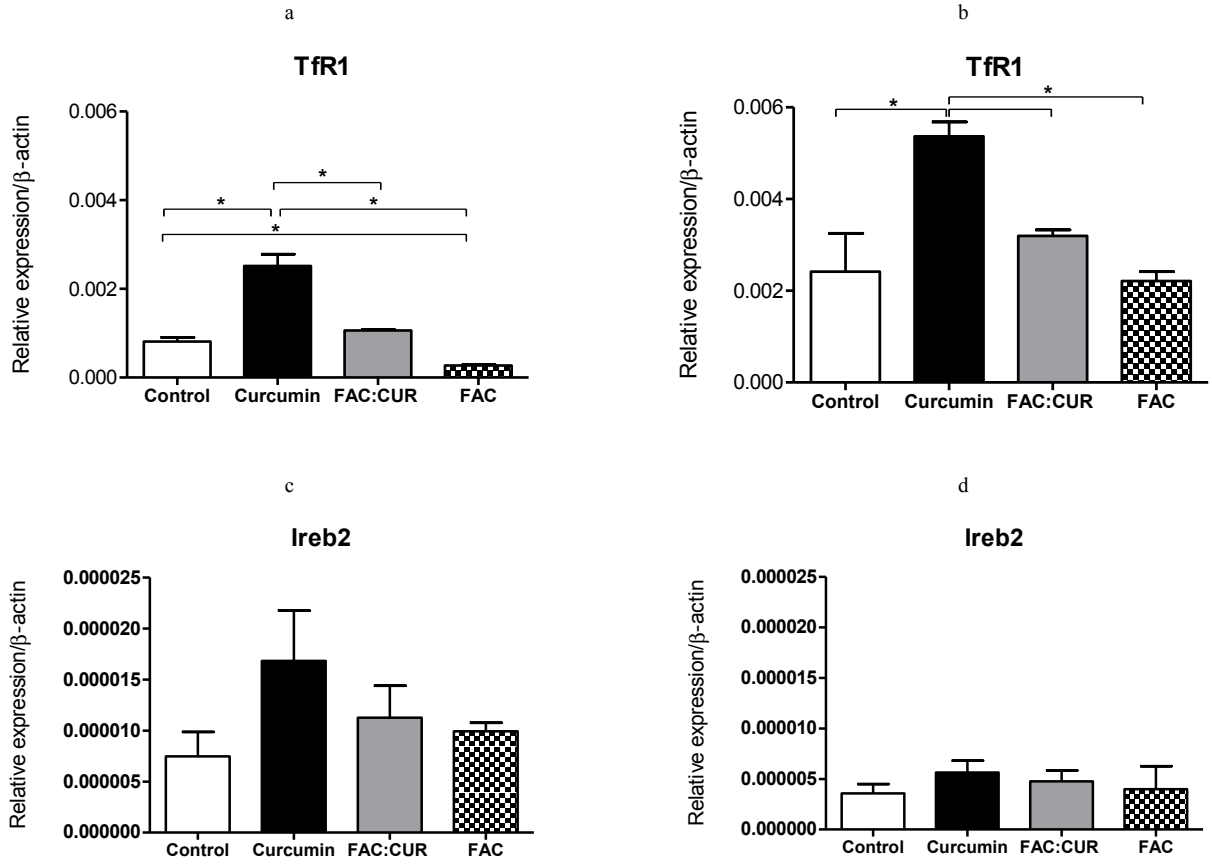

Fig. (5). Effect of iron, curcumin and co-incubation of iron:curcumin in the relative expression of Tfr1 and Ireb2. Relative expression of TfR1 in MCF-7 cells (**a**) and relative expression of TfR1 in 4T1 cells (**b**). Relative expression of Ireb2 in MCF-7 cells (**c**) and relative expression of Ireb2 in 4T1 cells (**d**). Bars represent mean ± SEM; * indicates $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

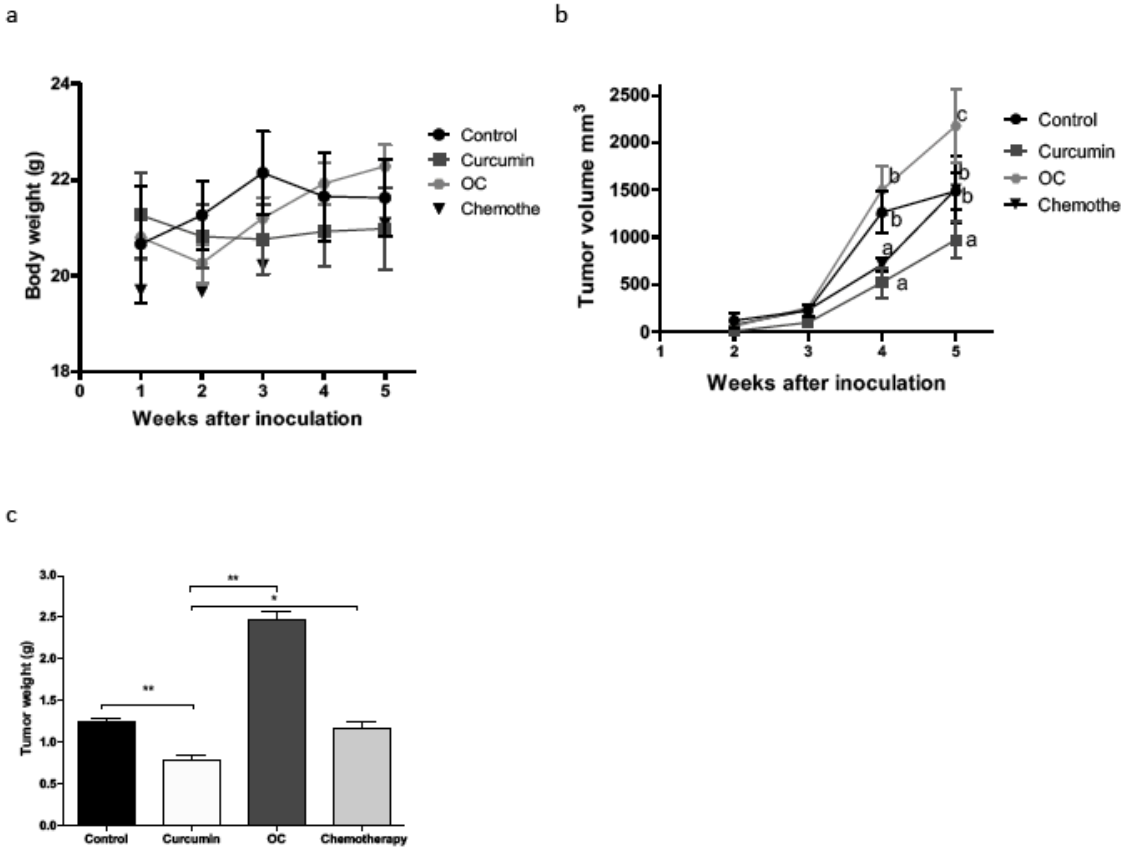

Fig. (6). Effect of oral treatments on body weight, tumor volume and tumor weight. The body weight of all animals was measured once weekly after cell inoculation (**a**). Tumor volume was measured starting on the second week after inoculation (**b**). Animals were euthanized five weeks after cell inoculation and tumors were weighted (**c**). Marks represent mean ± SEM; Bars represent mean ± SEM; * indicates $p < 0.05$; ** indicates $p < 0.01$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

display different values after three weeks of treatment; these changes became more prominent towards the fifth week. In this sense, curcumin group presented a decreased tumor volume when compare with control group ($p < 0.05$) and OC group ($p < 0.05$). Interestingly, after five weeks of treatment, chemotherapy group exhibited very close results to those of control group ($p > 0.05$). Moreover, contrary to expectations, OC group showed a significant increase in tumor volume when compared to control ($p < 0.05$) (Fig. **6b**).

Tumors were removed and weighted after five weeks of treatment. Groups had average tumor weights of 1.23, 0.78, 2.46 and 1.16 g for control, curcumin, OC and chemotherapy groups, respectively. In accordance to tumor volume measurements, curcumin group yielded statistical differences when compared with control ($p < 0.01$), OC ($p < 0.01$) and chemotherapy ($p < 0.05$) groups. As per chemotherapy group, no statistical difference was observed when compared with control group ($p > 0.05$). Lastly, OC group displayed no statistical difference in the average tumor weight when compared with control and chemotherapy groups ($p > 0.05$ in both cases) (Fig. **6c**).

4. DISCUSSION

Breast cancer remains one of the most lethal neoplasms worldwide. Current treatments are insufficiently effective and present a wide range of side effects; thus, it is essential to develop new and better therapeutic strategies with fewer side effects. In this study, we analyzed the effect of curcumin both *in vitro* and *in vivo*. Previously, Lv *et al*. [14] conducted *in vitro* experiments to determine curcumin growth inhibition in MCF-7 and MDA-MB-231 cell lines, and observed a time- and dose-dependent decrease in cell viability. Furthermore, in aggressive cell lines, such as MBCDF-T and EA.hy926 (both triple-negative breast cancer cells), Garcia-Quiroz *et al*. [22] also observed that cell proliferation decreased when adding different curcumin concentrations. In the same manner, we demonstrate that curcumin decreases cell proliferation in MCF-7 cells and the advanced stage breast cancer cell line, 4T1.

Previous studies have suggested that curcumin acts as a biologically active iron chelator and may induce cytotoxicity through this mechanism [16]. In fact, curcumin possesses a high affinity for iron, particularly Fe^{+3}, with a formation constant of 10^{22} M^{-1}[21]. In our *in vitro* experiments, iron antagonized curcumin in a dose-dependent manner, which indicates that the anti-proliferative effect brought about by curcumin, involves direct interaction with iron (Fig. **3**). Yang *et al*. [16] used different FAC concentrations with or without 50 μM curcumin in castration-resistant prostate cancer cells (DU145 and PC3) and observed that the anti-proliferative

effect of curcumin was neutralized when an equal concentration of FAC was added to the cells. Moreover, it is interesting to notice that in our experiments, curcumin induced TfR1 expression in MCF-7 and 4T1 cell lines and that this effect was dumped when iron was added; suggesting that curcumin may target intracellular iron metabolism and exert its activity through iron chelating-properties.

Curcumin has been associated with anti-tumor properties, which extend to several cancer cell lines. Curcumin is relatively safe and well-tolerated both in animals and humans, even at high doses [17]. However, usage of curcumin for medical purposes is greatly limited, as it has been associated with low bioavailability and poor water solubility [19]. Therefore, further investigation was required in this field. To investigate the translation of these promising *in vitro* results to a whole organism, we used a murine model of breast cancer. We administered two different types of curcumin, a research-grade, 95% curcumin and an over-the-counter (OTC) curcumin. OTC curcumin, an optimized curcumin supplement, has shown an increased absorption in clinical studies. This curcumin supplement possesses an enhanced absorption as encapsulated by nanoparticles of lecithin and fatty acids [20, 23]. With that in mind, we compared the anti-tumor activities of both curcumin presentations. Interestingly, our results showed that research-grade curcumin significantly decreased the volume and weight of tumors whereas, unexpectedly, OTC curcumin increased both parameters. Our results suggest that despite its enhanced absorption, OTC curcumin may not be as easily released into plasma, therefore, plasmatic concentrations of free curcumin may be much lower than those reached with the other research grade curcumin.

Previous studies involving xenograft models have suggested the effectiveness of curcumin as a therapeutic compound in breast cancer treatment. Co-treatment with calcitriol and curcumin caused tumor reduction, delayed tumor development and decreased micro-vessel density [22]. These results are consistent with *in vitro* studies that demonstrate that treatment with curcumin results in a dose-dependent and time-dependent apoptosis [14]. However, in these xenograft studies, curcumin was administered by the parenteral route, which conveniently avoids first pass metabolism. Moreover, the parental route is very invasive and requires a trainee expert, which represents a great problem if it is planned to be administrated on the daily basis. Here, we demonstrated the effectiveness of curcumin when administered by oral gavage in a murine model, in an attempt to emulate its most likely route of administration in humans. Nevertheless, it is important to highlight that positive results obtained in animal models are not always replicated in clinical studies, as species differences are an important matter to consider. It is established that the consumption of curcumin is relatively safe. According to JECFA (The Joint FAO/WHO Expert Committee on Food Additives) [24] and EFSA (European Food Safety Authority) [25] reports, a person may receive a dose of 0-3 mg/kg per day without presenting negative side effects. We have envisioned the use of curcumin as an adjuvant in the treatment of breast cancer, and perhaps other types of tumors. Therefore, curcumin should not replace the usage of standard treatments instead, it should be included as a supplement in the diet. This might result in the possibility of using lower doses of standard treatment, and in consequence, decreasing the negative side effects of chemotherapy.

CONCLUSION

In summary, our *in vitro and in vivo* experiments demonstrate that curcumin plays an inhibitory role in the proliferation of MCF-7 and 4T1 breast cancer cells and that the antiproliferative effects of curcumin are antagonized by the addition of iron. Curcumin was able to chelate iron and modulate the expression of the intracellular iron importer TfR1. In *in vivo* experiments, we proved that 5 mg/kg of curcumin decreased breast tumor growth.

LIST OF ABBREVIATIONS

EFSA	=	European Food Safety Authority
JECFA	=	The Joint FAO/WHO Expert Committee on Food Additives
ATCC	=	American Type Culture Collection
FAC	=	ferric ammonium citrate
OC	=	optimized curcumin

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Research procotol was read and approved by the ethics committee of Centro de Investigación en Ciencias Biomédicas del Sur-IMSS, Mexico.

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. All animal research procedures were followed in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals (published by the National Academy of Sciences, the National Academies Press, Washington, D.C.).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available within the article.

FUNDING

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CONFLICT OF INTEREST

The author(s) declare no conflict of interest, financial or otherwise.

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Declared none.

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