Phenotypic Changes in Mammary Adenocarcinoma (4T1) cells *In Vitro* after Treatment with *Carcinosinum*

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Abstract

Objective The present study aimed to identify possible phenotypic changes in 4T1 (murine mammary adenocarcinoma) cells *in vitro*, including viability, HER-2 (human epidermal growth factor receptor-type 2) expression, and metastatic potential, after treatment with *Carcinosinum* in different homeopathic dilutions (12cH, 30cH, 200cH) shaken mechanically in pure, sterile, water from a commercial stock dilution.

Methods Treated cells were cultured in R10 medium, using 24-well plates, 10^5 cells per well, and treated with vehicle, *Carcinosinum* 12cH, 30cH or 200cH; untreated cells were used as the baseline control. After 24 hours of treatment, the percentage of apoptotic cells was analyzed by annexin V. Cell morphology was evaluated by microscopy after hematoxylin-eosin and Giemsa staining, whilst HER-2 expression was assessed using immunocytochemistry. The metastatic potential was determined by the expression and activity of the enzyme matrix metalloproteinase 9 (MMP-9) using zymography. The cytokine profile was established using the cytometric bead array method.

Result Treatment of 4T1 cells *in vitro* with *Carcinosinum* 30cH produced an increase in the number of annexin V-positive cells (apoptosis) and decreased expression of proactivated MMP-9. Cells treated with *Carcinosinum* 200cH presented hyper-expression of HER-2 on the plasma membrane, identified by immunocytochemistry. There were no differences in cytokine production among treatments.

Conclusion The data show promising results for *Carcinosinum* 30cH *in vitro*, but *in vivo* studies are also required to evaluate the role of tumor microenvironment in its effects.

Keywords
- homeopathy
- mammary cancer
- experimental models

Introduction

Cancer is a significant public health problem, second only to cardiovascular diseases. It results from a genetic disorder caused by DNA mutations, which can occur due to intrinsic or extrinsic factors associated with epigenetic alterations that modify the expression or function of essential genes that regulate the fundamental cellular processes of growth, differentiation, survival and senescence. The abnormal and uncontrolled proliferation of cells forms tumors that can ultimately reach distant organs or tissues in secondary tumors or metastases, one of the main signs of highly aggressive cancers.[1]

Among the different neoplasms—other than non-melanoma skin cancer—breast carcinoma is the most common amongst the female population worldwide. However, it is a disease with a significant chance of survival when detected early.[2]

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One of the notable molecular alterations observed in breast cancer is gene amplification and consequent overexpression of the HER-2 protein (human epidermal growth factor receptor-type 2), a membrane receptor with tyrosine kinase activity. In normal amounts, this protein plays an essential role in the growth and development of a wide range of cells; however, its over-expression is associated with a poor prognosis in different types of cancer.[3] Among the valid methods for evaluating HER-2 expression in the clinical situation, immunohistochemistry and fluorescence *in situ* hybridization are the most commonly used.[4]

The 4T1 cell line is an experimental model widely used to assess and better understand the biology of tumors, and is one of the sub-populations of tumor strains isolated from a single mammary tumor that spontaneously arose in a BALB/c mouse. It is a highly tumorigenic and invasive cell line from which there is observed spontaneous metastases to different locations, such as lymph nodes, liver, lungs, bones and brain, behaving similarly to human breast cancer.[5]

Complementary treatments, comprising a group of medical and health products and practices that fall under so-called integrative medicine,[6] can enhance the effectiveness of conventional therapies by reducing side effects.[7] Developed by the German physician, Christian Friedrich Samuel Hahnemann, homeopathy is based on healing by similars.[8] In Brazil, homeopathy was made a medical specialty by the Federal Council of Medicine in 1980 (resolution 1000). Its recognition as a specialization in veterinary medicine took place in 1997 (resolution 622) and, in dentistry, in 2015 (resolution 160).[8–10]

Several studies, using different experimental models, have indicated the efficacy of homeopathic medicines as an adjuvant treatment of tumors. For example, a randomized clinical trial by Sorrentino et al. reported that *Arnica montana* 1000x in patients with recent mastectomies can reduce the formation of post-operative seromas.[7] An *in vivo* study of 4T1 mammary adenocarcinoma treated with *Phytolacca decandra* at different homeopathic potencies showed that the 30cH potency induced tumor growth delay, suggesting that it may be a promising therapeutic resource.[11] Recently, a systematic review of the most used experimental oncology models in basic research of homeopathic treatment showed the need to explore other phenotypic parameters rather than merely the cytotoxicity of the tested products.[12]

Few published studies specifically address *Carcinosinum*, making it a topic of high relevance, given the frequent use of this medicine as a complementary cancer therapy in homeopathic protocols for both human and veterinary practice. Tumor cell changes seen after treatment with *Carcinosinum* have been characterized experimentally in recent years by *in vivo* and *in vitro* models, with mice being the most frequently studied species.[13–15] In 2015, Amaral[16] demonstrated in an *in vivo* trial with Ehrlich tumor that mice treated with *Carcinosinum* 200cH had a lower incidence of symptoms for a shorter period, a more remarkable survival, and a reduction of tumor-induced ascites. Whilst control animals presented a peak of symptoms on day 18 after tumor inoculation, mice treated with *Carcinosinum* 200cH had a significant delay in the manifestation of symptoms (to day 29). In a second phase, the same author with others showed that animals treated with *Carcinosinum* 6cH had a higher incidence and diversity of symptoms than the other groups, demonstrating the importance of homeopathic potency to promote pro- or anti-carcinogenic action.[17]

Among the known matrix metalloproteinase (MMP) enzymes, MMP-9 (also known as gelatinase) has been consistently associated with aggressiveness, metastatic potential and poor prognosis of malignant neoplasms. MMPs play a crucial role in tumor progression, acting directly on basement membrane (BM) components.[18] For this reason, they were chosen in the present experiment as markers of metastatic potential.[19,20]

This study aimed to identify possible phenotypic changes, including apoptosis, HER-2 expression and metastatic abilities, in 4T1 cells after *in vitro* treatment with *Carcinosinum* at different homeopathic potencies. The lineage 4T1, originating from mouse mammary tumor, was used because the relationship with the known biological features of *Carcinosinum's* cellular mode of action[13–17] would be more explicitly relevant.

Methods

Study Design

This work is a descriptive, prospective, randomized, double-blind, experimental *in vitro* study, using interventional analysis, with evaluation between the control and intervention groups. The study is 100% *in vitro*, using murine mammary adenocarcinoma cell lines (4T1): no animals were used in this research.

Preparation of Medications

The stock dilutions of *Carcinosinum* 11cH, 29cH and 199cH were prepared in a homeopathic pharmacy accredited by ANVISA (Lots: 0228245–0002, 0228245–0003 and 0228245–0004, respectively, all valid for 2 years) and dispensed in 30% alcohol. The working samples of *Carcinosinum* 12cH, 30cH and 200cH were prepared from these stock dilutions in pure, sterile, water (obtained by reverse osmosis) and filtered in filters (Millipore, United States) with a 0.22 µm mesh. Succussion was achieved using a Denise mechanical arm (AUTIC, Brazil), according to the procedures established by the Brazilian Homeopathic Pharmacopoeia, 3rd edition,[21] and adopting the Hahnemannian centesimal scale (cH). This scale describes the preparation of drugs in serial dilutions at a rate of 1:100, using hydro-alcoholic solution or water, followed by 100 vertical agitations of the vial, known as succussion.

The manufacture of the latest potencies using sterile water has allowed the alcohol concentration to be reduced 100 times. Multiplying it by the dilution factor (1:5) of the drugs in the cell culture medium, the final concentration of alcohol was 0.06%, which avoided possible non-specific cytotoxic effects of the vehicle on the cells of the experiment, as tested previously.[22]

Table 1 Description of the different treatments applied at 10% ratio into 4T1 cell culture bottles (10^5 cells per bottle, 5 mL of R10 medium)

1	4T1 cell culture with no treatment/untreated sample (US)
2	4T1 cell culture + water (Water)
3	4T1 cell culture + *Carcinosinum* 12cH (Ca 12cH)
4	4T1 cell culture + *Carcinosinum* 30cH (Ca 30cH)
5	4T1 cell culture + *Carcinosinum* 200cH (Ca 200cH)

Controls were performed with the unsuccussed vehicle (pure, sterile water) to avoid the overlapping effects caused by the medicine and the succussed water itself.[12] All preparation and storage procedures and medication handling were performed in a laminar flow cabin to ensure sterility. The control of untreated cells made it possible to verify whether eventual changes in medium osmolarity caused by adding 10% of the water might be involved in possible non-specific effects.

Before starting the study, the drug and vehicle vials had their labels replaced by codes by a laboratory employee not involved in the experiment. The original tags and the respective codes were kept in a sealed envelope throughout the test, and it was only opened after the end of the statistical analysis. Thus, the entire study was conducted in a blinded way.

Treatments

Five treatment groups were used.

Each culture bottle contained 10^5 viable cells in RPMI 1640 medium enriched with 10% bovine fetal serum (BFS) (GIBCO, United States), named R10 medium. Thus, 500 µL of the respective treatment was inserted, and cells were assessed 24 hours later. The cell viability was determined using the Trypan blue method. ►**Table 1** summarizes the treatment groups.

Analyses (apoptosis, HER-2 expression, cytokines, zymography) were performed in quadruplicate from the same cell population. Thus, the results obtained were complementary and comparable to each other.

4T1 Cell Culture and Analysis of the Incidence of Apoptosis

4T1 cells were replicated in culture bottles and incubated in R10 medium (90% RPMI and 10% BSF), at 37°C and 5% CO_2 atmosphere, for 20 minutes, always in a laminar flow cabin. For this, 1.0 mL aliquots were previously kept frozen at a temperature of −80°C in 90% BFS with 10% DMSO (freezing medium).

During cell thawing, performed at room temperature, the entire content of the freezing tube was transferred to 15 mL Falcon tubes containing 9.0 mL of RPMI 1640 (GIBCO, United States). The suspended cells were centrifuged at 1,500 rpm for 5 minutes. After discarding the supernatant, they were resuspended in R10 medium, counted in a Neubauer chamber using the Trypan Blue method, and 1×10^5 viable cells were seeded to adhere to the bottle surface. Then, cells were treated with 500 µL of sterile medicines or the vehicle (10% medium volume). The cells were incubated for 24 hours and analyzed later.

After this period, the supernatant from each bottle was harvested in 1.0 mL samples, distributed in five microtubes, for further analysis. The cells were removed from the bottom of the bottle with the aid of a cell scraper in 1.0 mL of Trypsin-EDTA (0.05%), being immediately washed in cold PBS (phosphate buffer saline).

Then, the counting of cells in apoptosis was performed by the Countess system, using the Alexa Fluor 488—Annexin V apoptosis kit (Life Technologies, United States), following the manufacturer's instructions. This kit uses fluorochrome-linked anti-annexin antibodies for direct fluorescence labeling of positive cells. The Countess cell counter is an automatic fluorescence microscope, which allows the identification and direct counting of fluorescent cells placed in the equipment's counting chambers.

Part of the cell suspension was used to make smears in quintuplicates. Thus, cells were fixed with methanol for 15 minutes, dried, and stored in a freezer.

HER-2 Expression

The previously fixed and coded smear slides were sent to a support laboratory for surgical pathology (APC Laboratory, São Paulo, Brazil), where HER-2 marking was performed.

After a pilot test to determine the origin and optimal antibody dilution and also block the endogenous peroxidase with 5% hydrogen peroxide, the 1:200 dilution of polyclonal anti-HER-2 Dako antibody was chosen (Agilent, United States; lot 20027851B) as default. The secondary antibody linked to peroxidase and DAB (diaminobenzidine) were used to complete the reaction.

Positive cells were photographed in eight to ten random microscopic fields, using a photomicroscope coupled to a digital camera (Nikon E200; Coolpix, Japan). Descriptive analysis of morphology and intensity of labeling was then performed.

Cell Morphology using Hematoxylin & Eosin and Giemsa

The morphology of 4T1 cells was monitored using two staining methods. Cells previously fixed in absolute methanol were stained by the Hematoxylin & Eosin (HE) and Giemsa methods. After washing with distilled water and drying, the slides were mounted in Entellan (Merck, Germany) for analysis under an optical microscope (Nikon E200, Japan). The following cell viability parameters were observed: cytoplasmic vacuolization and cytoplasmic and nuclear degeneration patterns.

Determination of Cytokine Profile by the Cytometric Bead Array Method

Since one of the escape mechanisms of tumor cells from the immune response is the induction of local inflammation, investigating the production of pro-inflammatory cytokines by the tumor cells themselves is another critical tool for phenotypic characterization.

Supernatant samples were previously frozen at −80°C and used to determine cytokines related to Th1/Th2/Th17 patterns (IL-2, IL-4, IL-17, IL-6, IL-10, IFN-γ, TNF-α), using the cytometric bead array technique (BD Biosciences, New Jersey, United States). Thus, 50 µL of each sample was incubated with 50 µL of a mixture of capture beads and secondary antibodies conjugated to the fluorochrome phycoerythrin (PE). The samples were incubated for 2 hours at room temperature and protected from light by covering them with aluminium foil. After washing, flow cytometry was performed using a FACS Accuri device and the obtained data were analyzed by FACS Diva software (BD Biosciences).

Enzymatic Activity of Metalloproteinases Using the Zymography Technique

Zymography is a technique based on denaturing polyacrylamide gel electrophoresis. It allows the detection of the enzymatic activity of MMPs and tissue inhibitors of metalloproteinases from the degradation of their substrate co-polymerized in the gel, with sodium dodecyl sulfate (SDS) as a reaction blocker and with the preferred substrate of the enzyme under analysis. After electrophoresis, the enzymes are reactivated by removing the SDS by washing the gel with a 2.5% aqueous solution of Triton X-100, a non-ionic detergent. Applying an electric current allows the migration of MMPs through the gel, and their separation is due to the differences in molecular size. Since the latent forms have a different molecular weight from the active ones, both forms of MMPs are identified.[18]

Herein, this technique was performed to evidence MMP-2 and MMP-9 isolated from the samples. Since MMP-2 occurs widely in several cell types and performs physiological roles in most cases, it was chosen as the technique's internal control. After the renaturation of MMPs, the gel was incubated for 20 hours at 37°C under constant agitation. A zinc chloride solution containing 50 mM Tris-endopeptidases was added to degrade the substrate. The gel was then stained with 0.5% Coomassie Brilliant Blue solution for 30 minutes; decolorized in a mixture containing 500 mL methyl alcohol solution, 100 mL glacial acetic acid, and 400 mL distilled water for 20 minutes in two repetitions, until the perception of colorless bands, in contrast to the blue gel background. The gel was stored in distilled water at 4°C for 1 day to improve contrast. The capture of gel images was done in high resolution in a Biorad GelDoc XR+ photo documenter with Image-Lab software. Considering that the proteolytic activity band of MMPs is colorless in the fresh gel, in its negative image the higher the optical density the greater is the MMP activity.[20] The proteolytic activity was analyzed qualitatively by inspecting the area and the shade of gray present in each band.

Statistical Analysis

Statistical analysis was performed using Excel 2010 software and GraphPad Prism, version 6.0 for Windows, to assess the effect of treatment on the rate of apoptosis and the concentration of cytokines produced. For this, a one-way analysis of variance (ANOVA) was used, with Tukey's post-test when necessary. Data were previously evaluated to inspect Normality and homogeneity of variances, using the Shapiro-Wilk and Levene's tests respectively. The root mean square error (RMSE) index was used to calculate the effect size and represent the entire model's collective difference standardized by the RMS. For all calculations, a significance level of $p = 0.05$ was adopted. Values of $p \leq 0.05$ were considered statistically significant.

Results

Apoptosis

A higher incidence of apoptosis was observed in 4T1 cells after receiving treatment with *Carcinosinum* 30cH ($F_{(4,15)} = 3.456$, $p = 0.034$; RMSE = 0.930) compared with the control (vehicle) (►**Fig. 1**).

Cell Morphology and HER-2 Expression

The morphological findings obtained by cytology from smears prepared from re-suspended culture cells are shown in ►**Fig. 2**. The 4T1 cells stained by the Giemsa method had a frequently round shape, with evident nucleoli and higher nucleus/cytoplasm ratio, typical characteristics of malignant tumors. However, cytoplasmic spreading over the slide was more pronounced in those treated with *Carcinosinum* 200cH.

The nuclei, in general, had a round shape, sometimes oval, regardless of the treatment. Cells treated with *Carcinosinum* 30cH had typical cells undergoing apoptosis, forming structures like apoptotic bodies. Considering the HER-2 labeling by immunocytochemistry, untreated cells showed an irregular pattern of HER-2 expression in the membrane, which was even more evident in cells treated with *Carcinosinum* 200cH. On the other hand, cells treated with *Carcinosinum* 12cH and 30cH showed a lower pattern of HER-2 expression, sometimes being negative (►**Fig. 2**).

Metalloproteinases

Qualitative analysis of the culture supernatant by the zymography method (►**Fig. 3**) showed that the digestion area of the internal control, pro-activated MMP-2 (pro-MMP-2), was uniform among all the samples. However, a mild increase in its activity was seen after the treatment with

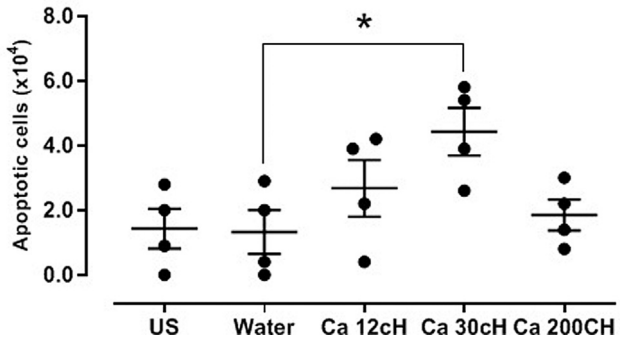

Fig. 1 Incidence of apoptosis in 4T1 cells maintained in monoculture for 24 hours, as a function of different treatments. $F_{(4,15)} = 3{,}456$, $^{*}p = 0{,}034$, RMSE = 0.930. Ca, *Carcinosinum*; US, untreated sample.

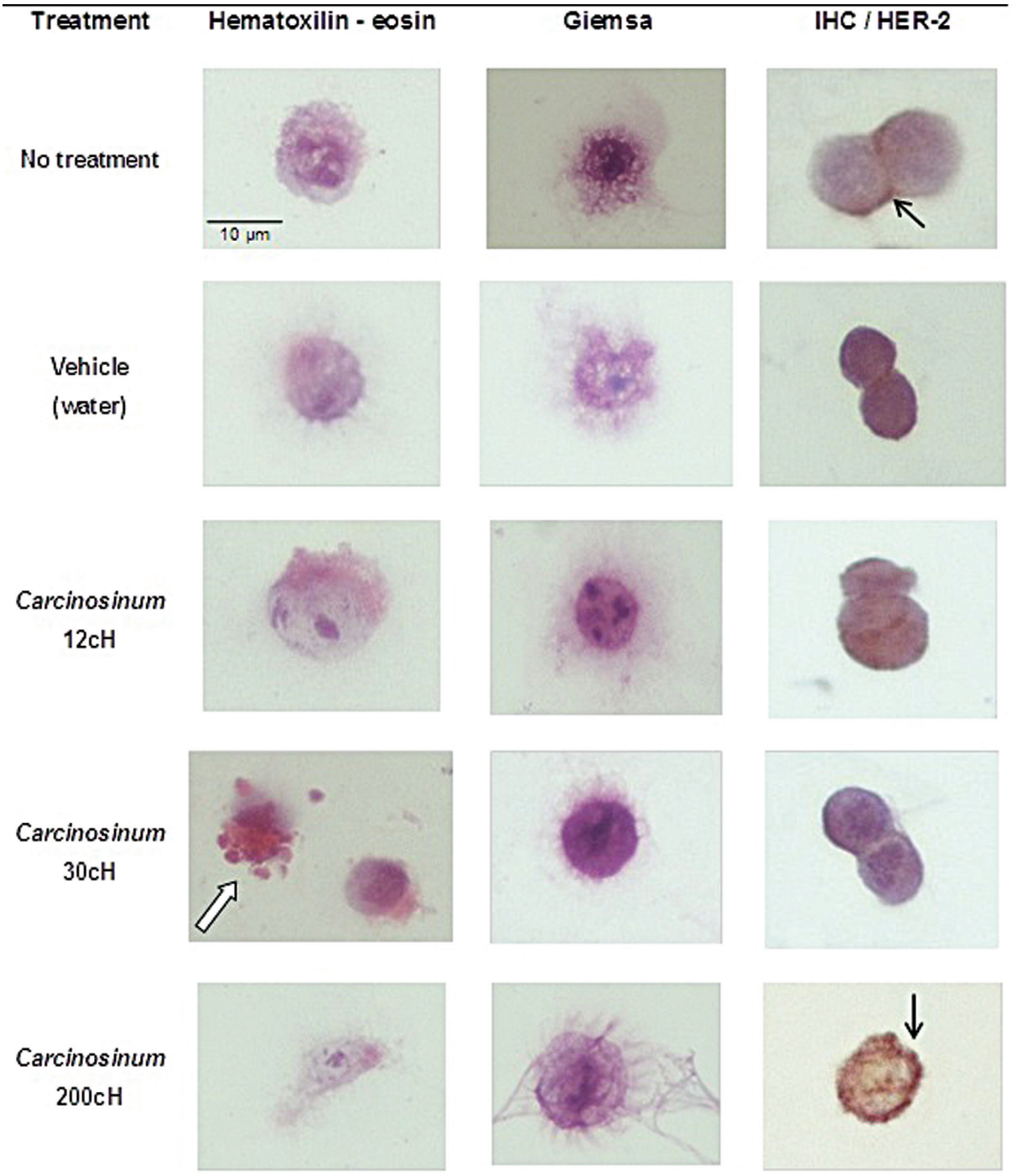

Fig. 2 Photomicrographs of 4T1 cells in smears stained by Hematoxylin-Eosin, Giemsa, and immunocytochemistry for HER-2. Black arrows indicate HER-2 positivity to different degrees. The white arrow indicates a cell with typical apoptotic morphology. Images taken with a 40x objective lens.

Carcinosinum 30cH. The untreated sample (US) had greater area and digestion intensity in the pro-MMP-9 band. In comparison, the *Carcinosinum* 30cH sample had a lower digestion area and intensity in this band, indicating a probable reduction in the expression of this enzyme, still in an inactive form, after the treatment of cells with this medicine.

On the other hand, the area corresponding to the activated MMP-9 was relatively homogeneous among the different treatments, with lower intensity in the sample that received no treatment (US), showing that the activation is a non-specific effect, even observed in the supernatant from cells treated with water only.

Cytokine Production by 4T1 Cells

The following pro-inflammatory cytokines were produced in all treatments: IL-2, IL-4, IL-6, INF-γ, and TNF-α (►**Fig. 4**).

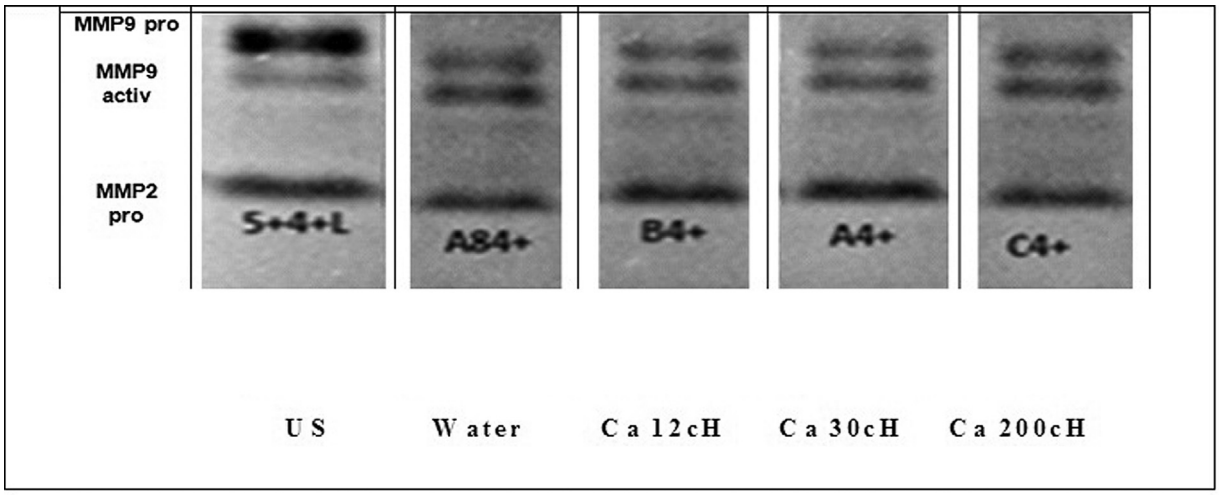

Fig. 3 Gel digestion bands obtained in zymography assay. The activity of gelatinolytic MMP-9 enzyme in the pro-activated and activated stages was compared with internal control (pro-MMP-2) in each treatment. The codes in black represent the identification of the samples in blinded analysis. Ca, *Carcinosinum*; US, untreated sample.

However, there was no statistically significant difference difference between them, given the wide dispersion of results obtained in all treatments (►**Table 2**).

Summary of Findings

A graphical summary of the results is shown in ►**Table 3**.

Discussion

The results observed in this experimental *in vitro* model have demonstrated that the treatment of 4T1 cells with different homeopathic dilutions of *Carcinosinum* is associated with changes in specific phenotypic patterns, including the increased occurrence of apoptosis and changes in the expression patterns of HER-2 and MMP-9, as a function of the dilution used (►**Fig. 4**).

There are indications in the literature for induced apoptosis in tumors after treatment with *Carcinosinum* 200cH *in vivo*, associated with increased expression of the p53 gene. This critical gene induces apoptosis in cells unable to repair DNA damage.[14] However, such effects on the immune response are still controversial.[15]

Frenkel et al[23] performed an *in vitro* study to determine whether medicines prescribed in the homeopathy cancer clinic had effects on different breast cancer cell lines (MCF-7 and MDA-MB-231) and normal breast cells (HMLE). The following medicines were tested: *Carcinosinum* 30cH, *Phytolacca* 200cH, *Conium maculatum* 3cH and *Thuja occidentalis* 30cH. The main results of that study indicated a selective action of medicines on tumor cells, inducing a delay in the process of cell division and apoptosis. Among other cellular changes involved with the inhibition of cell division, these effects were accompanied by changes in the expression of cell cycle regulatory proteins, by activating caspase-7, an important enzyme involved in the apoptotic process. Amongst the four medicines investigated, *Carcinosinum* and *Phytolacca* showed the most evident inhibitory effects.

A previous *in vivo* study showed that mice bearing Ehrlich ascitic tumor and treated with *Carcinosinum* 200cH had better clinical results and increased survival. However, after treating animals with *Carcinosinum* 6cH, some unwanted effects such as local edema, piloerection, and hyperthermia were observed. This finding suggests some local and systemic inflammatory activity, with potential changes in the tumor microenvironment.[16,17] Although they do not imply a reduction in the tumor mass, such effects can lead to a better quality of life.

The biological meaning of the action of each specific homeopathic dilution depends on the context. Thus, the *in vitro* study approach is essential to demonstrate a drug's effects directly on tumor cells without the intervention of the local immune response. On the other hand, the presence of immune cells and other elements of the tumor microenvironment *in vivo* may imply changes in the treatment outcome that could not be observed *in vitro*. The effectiveness of immunotherapies depends on the tumor microenvironment,[24,25] which comprises the set of blood vessels, extracellular matrix and connective tissue cells, capable of secreting growth factors and chemokines that attract other cells such as leukocytes, called tumor-infiltrating leukocytes (TILs). The profile of TILs is frequently associated with prognosis of the disease.[17] Studies have shown that tumor cell interactions with cellular and molecular elements in the tumor microenvironment can influence tumor evolution, growth, and progression.[26] Therefore it is important to know the phenotypic characteristics of tumor cells.

The expression of HER-2, which is a marker for defining prognosis in humans,[3,4] was greater in cells treated with *Carcinosinum* 200cH but less at lower potencies, suggesting that variation in cell differentiation patterns is a function of the homeopathic potency used. However, quantitative studies are still needed to confirm these data. Nevertheless, the qualitative cytological observation obtained here is unprecedented since no data were found in the literature that correlated

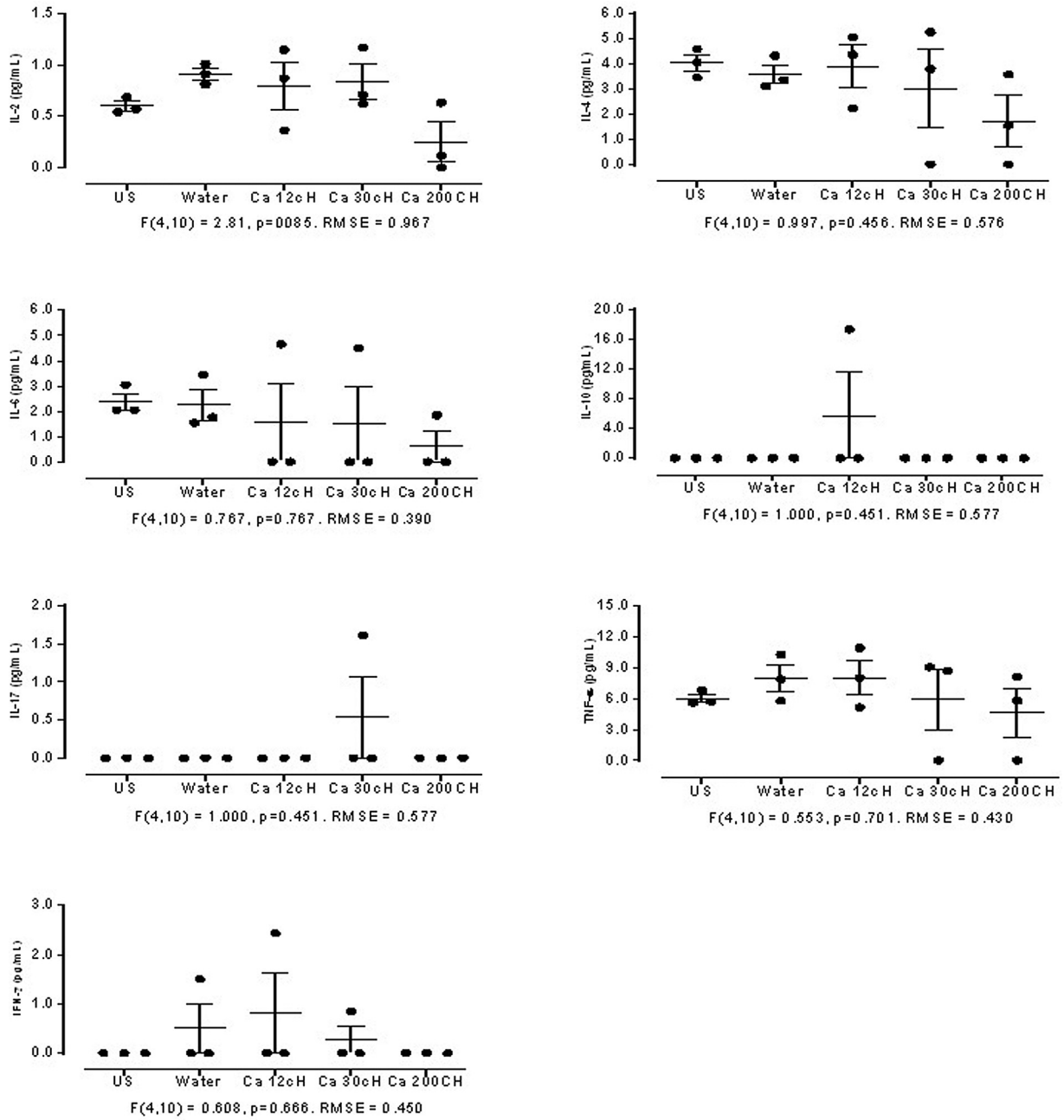

Fig. 4 Representation of cytokine profile (pg/mL) related to Th1/Th2/Th17 patterns (IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, and IFN-γ). Data were obtained from the cytometric bead array technique. Ca, *Carcinosinum*; US, untreated samples.

Table 2 Cytokine concentrations in 4T1 cell culture supernatants treated for 24 hours with various homeopathic dilutions of *Carcinosinum*

Cytokine	Mean ± standard deviation					*p*-Value and RMSE	
	No treatment	Water	*Carcinosinum* 12 cH	*Carcinosinum* 30 cH	*Carcinosinum* 200 cH		
IL-2	0.60 ± 0.10	0.91 ± 0.08	0.80 ± 0.40	0.83 ± 0.30	0.25 ± 0.34	0.085	0.967
IL-4	4.03 ± 0.56	3.59 ± 0.64	3.89 ± 1.47	3.02 ± 2.71	1.71 ± 1.79	0.456	0.576
IL-6	2.38 ± 0.57	2.25 ± 1.03	1.55 ± 2.69	1.50 ± 2.60	0.61 ± 1.06	0.767	0.390
INF-γ	0.00 ± 0.00	0.50 ± 0.87	0.81 ± 1.40	0.28 ± 0.48	0.00 ± 0.00	0.666	0.450
TNF-α	6.02 ± 0.66	7.96 ± 2.24	8.02 ± 2.90	5.93 ± 5.14	4.63 ± 4.18	0.701	0.430

Abbreviations: IFNγ, interferon gamma; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha.
Note: The values shown represent the mean and standard deviation, significance level, and effect size (RMSE) on one-way ANOVA.

Table 3 Graphical summary of the results

	US	Water	Ca 12cH	Ca 30cH	Ca 200cH
Apoptosis	–	–	–	++	–
HER-2	+	–	–	–	+++
MMP-9 Pro	++++	++	++	+	++
MMP-9 Active	+	+++	++	++	+++
Cytokines	–	–	–	–	–

Abbreviations: Ca, *Carcinosinum*; HER-2, human epidermal growth factor receptor type 2; MMP, matrix metalloproteinase; US, untreated sample.

Carcinosinum treatment with the expression of HER-2 in any animal species. Previously, the study by Nogueira,[11] performed with *Phytolacca decandra in vivo*, had shown variations in the expression of this receptor as a function of the tested homeopathic dilution in a non-linear way.

Changes in the expression of specific proteins and other phenotypic variations are common in malignant cells due to gene instability. Intercellular communication by exosomes can modify the phenotype and progression of the 4T1 tumor.[27] In our experiment, the samples were replicated from the same population and the same passage so that the studied cells were as homogeneous as possible.

Another significant result concerns the reduction of bands related to pro-MMP-9 and a mild increase in activated MMP-2, both present in the supernatant of cells treated with *Carcinosinum* 30cH. It suggests lower gene expression of MMP-9, therefore lower metastatic potential, which needs to be confirmed *in vivo*. However, the zymography model was an exact tool for this purpose.[25,28] Nevertheless, non-specific effects on MMP-9 activation were also observed, suggesting that the simple manipulation of cell culture, for example the insertion of water, activated such enzymes. The same could be considered for MMP-2.

Lee et al observed a reduction in the progression of 4T1 cells by inhibiting the expression of MMP-9; it was associated with suppression of pro-inflammatory mediators such as TNF-α and also of the metastatic potential.[28] On the other hand, over-expression of MMP-9 in the lymph nodes themselves is associated with lymphangiogenesis in breast tumors.[19] MMP-2 expression is also significantly higher in malignant breast lesions than in benign lesions.[25]

Metastases are seeded by tumor cells with unique molecular properties, which can function as stem cells in their ability to initiate and propagate tumor growth foci distant from the primary site.[29,30] In 2013, Quail and Joyce[31] demonstrated that tumor progression and metastasis formation also occur due to the interactions of tumor cells with cells or components of the microenvironment where the tumor develops. For the invasion process to occur and breast cancer cells to manifest their malignant potential, they must develop the ability to break down and dissolve the extracellular matrix (ECM), particularly the bounding BM. Degradation of the peri-cellular BM is catalyzed by the combined action of several classes of enzymes.

MMP-9, for example, is over-expressed, mainly in invasive tumors.[28,32–34] When activated, it participates in synthesizing and degrading the ECM and fragments laminin and collagen IV, fundamental components of the BM. It is necessary to detect and quantify their proteolytic activity. Originally, zymography was developed using gelatin as a substrate to measure the enzymatic activity of MMP-2 and MMP-9.[18] Patients with triple-negative breast cancer (one that lacks the three most common biomarkers: estrogen receptor, progesterone receptor, HER-2 protein) have high expression of MMP-9, resulting in a worse prognosis.[35,36] Similar results were also obtained by Cupić et al[37] and Zeng et al.[38]

Although many studies demonstrate the immune system's role in the tumor microenvironment, the mechanisms by which the presence of infiltrating leukocytes favor the occurrence of phenotypic changes in neoplastic cells are not well established.[39] In the tumor environment, cytokines are synthesized both by immune system cells and by tumor and stromal cells (endothelial cells, fibroblasts), in addition to regulating cell proliferation, survival, differentiation, activation, migration and death. In chronic inflammatory processes, cytokines can also induce malignant transformation of various cell types, depending on their concentration, the intensity of receptor expression, and the activation state of adjacent cells.[40,41] In some cases, inflammatory cytokines produced by the tumor cells themselves can impact the inflammatory pattern of tumors and thus the prognosis and the success of treatment,[42,43] which justifies the research of autocrine production of cytokines as a significant phenotypic feature of tumor cells treated with homeopathic products. Here, the results obtained showed no change in cytokine production by 4T1 cells as a function of treatment, suggesting that the regulation of phenotypic characteristics by *Carcinosinum* does not involve this specific profile.

This work demonstrates phenotypic changes in tumor cells treated with *Carcinosinum* in single-cell culture. However, these results still need to be reproduced in other laboratories, in different conditions, and using different cell lineages, with more repetitions. It is mandatory to know how these changes can be considered universal or specific to each experimental situation.

Conclusion

The treatment of 4T1 cells *in vitro* with homeopathic dilutions of *Carcinosinum* produces significant phenotypic changes in the regulation of tumor development, such as increased number of apoptotic cells, and decreased proteolytic activity related to pro-MMP-9, observed after the treatment with *Carcinosinum* 30cH. Variations in the expression of HER-2 on the membrane surface were also seen, being over-expressed in cells treated with *Carcinosinum* 200cH. The characterization of these phenotypic features *in vitro* is essential for understanding its effects when this medicine is used clinically, showing a promising result for *Carcinosinum* 30cH. The role of the tumor microenvironment on tumor progress must, however, also be investigated *in vivo*.

Highlights

- Murine breast cancer cells (4T1 cells) were treated with *Carcinosinum in vitro* for phenotype characterization.
- *Carcinosinum* 30cH increased the incidence of apoptotic cells and decreased the expression of MMP-9.
- *Carcinosinum* 200cH increased the positivity to HER-2 on the cell surface.
- No change in cytokine production by tumor cells was identified.
- *Carcinosinum* modulates phenotypic relevant features of 4T1 cells as a function of the potency.

Conflict of Interest

None declared.

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