Original article

In vitro study activity proliferative fibroblast human submitted to treatment Zincum metallicum and Calendula officianlis in homeopathic ultra dilutions

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Abstract

Cicatrizization can be divided into three phases: inflammation, fibroblastic, maturation and remodeling [1]. The extracellular matrix may be replaced by a stronger and more elastic connective tissue. In a scar collagen is the major component of the mature connective tissue [2]. In homeopathic area, the greater is the investigated segment ultra dilutions [6]. However, little research has been done to explore the effect of dynamized drugs in in vitro cell culture [4]. Using the Zincum metallicum 6CH and Calendula officinalis 6CH applied independently in different concentrations in fibroblast cultures sought to determine the increase in proliferative activity using techniques such as IC50, MTT, flow cytometry and quantification of collagen. As expected from the literature, ie both homeopathic according to the literature are used for treatments that Require skin healing, both showed increased proliferative activity, having Calendula most cellular response, presenting as cell cycle stimulating checked via flow cytometry.

Keywords: Zincum metallicum, Calendula officinalis, ultra-dilutions, proliferative activity, fibroblasts..

Introduction

Of immense complexity, as indicated by Carvalho, 2001, cicatrization has gained the attention of researchers over the years, focusing on the factors that delay or hinder it. It is a non-linear but dynamic process that involves the integration of several cellular and biochemical events that can be divided into three phases: inflammation, fibrioblastic phase, maturation and remodeling (Ringles, 200)

The extracellular matrix can be replaced for a stronger and more elastic connective tissue, stage know as fibrioblastic phase. In a scar, collagen is the main component of mature connective tissue. Thus, in an injury that is in the process of regeneration, it is possible to affirm that the principal component are collagen producing fibroblasts, which are stimulated by signals for greater collagen biosynthesis as a consequence of increased fibroblastic activity. (Ryan and Majno, 1977)

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Topical administration of substances to improve the cicatrization process has been widely studied (Santos et al, 2006). Of great attractiveness, the administration of compounds on the skin is due to the fact that it is the local of action (topical administration) with good effectiveness and safety because it has no systemic effects, has no first-pass metabolism or adverse systemic effects. (Prausnitz, Mitragotri and Langer, 2004).

One of the most popular forms of integrative practice, Homeopathy has been studied over more than 200 years and is based on three pillars: Dynamized medications, experiment in sane man and similarity principle. To be considered homeopathic medicine, this should have been potentialized through dynamization, tested in healthy individuals and have been chosen by the principle of similarity. (Sunila et al, 2007). Confidence in the Principles of Homeopathy and the various theoretical approaches based on Scientific bases collaborated for observed evidences in Laboratory cell lines. (Bellavite, 2006).

In the homeopathic area, the largest segment searched is the ultra-high dilutions of substances, usually allergenic or irritating to prevent or cure. When such substances are submitted to the homeopathic

pharmacotechnical process (ultra-high dilutions and dynamization), these resulting compounds are referred to as biotherapeutics. Biotherapeutics have a many specific applications in other homeopathic branches but in clinical practice they are recommended for improving mental, emotional and physiological aspects. The effects generated by the treatment are investigated by numerous researchers, who report on the progression of the clinical condition.

Advances with experiments in laboratory conditions in several areas, such as in immunology and toxicology, have been reported. As with *Arsenicum album*, in mice as well as in clinical trials used to improve arsenic-induced toxicity by lowering the high titer of antinuclear antibodies and hematological toxicity. However, few investigations have been done to explore the effect of dynamized drugs on in vitro cell culture. (Sunila, 2007).

Batello (2002) Found that when in vitro, *Cuprum metallicum* C30 and the *Arsenicum album* C30, both diluted above the Avogadro number, achieved a significant inhibition of lipoperoxidation when compared to melatonin and thus can infer the antioxidant action of these two homeopaths following the law of Similar.

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Following the Similar Law, the protective effect on lymphocytes was verified against the toxic effects of Arsenic. After the in vitro administration of *Arsenicum album*, indicating that even above the Avogadro Number, the cytoprotective effects of *Arsenicum album* were potentiated and the culture was preserved. (Ive, E.C. Couchman, I.M.S. e Reddy, Lanini, 2012).

Homeopathic medicines were also tested to evaluate their effectiveness in the immune system and so in 2012, Bonamin et al, verified the immunomodulatory activity of *Thymulin* 5CH in inducing the formation of granulomas in test subjects after induction of BCG. (Guimaraes et al 2009) Showed the immunological response of homeopathic complexes to tumors, suggesting that homeopathic complexes are great candidates in combined therapies to induce and enhance the immune response to tumors.

Saha et al, 2013, using the *Calcarea carbônica* in In different centesimal dilutions and ministering in test subjects and cell culture, verified the action of this homeopathic in the reduction of tumors through the rejuvenation of the immune system without causing some systemic toxicity

The *Zincun metallicum*, originating from Zinc is a metallic element of atomic number 30 and atomic mass of 65,38 and symbol Zn. It forms an essential part of many enzymes, having essential function in protein synthesis and cell division, thus being a necessary trace element in the diet. Its deficiency is associated with hormonal problems and the weakness in wound healing. *Zincun metallicum* is extremely common in the earth's crust and is obtained directly from the chemical industry

Calendula officinalis, from the *Asteraceae* family and originally from the Mediterranean, is an annual plant of alternate leaves, without stipules and with a group of flowers without pendulum and terminals Orange-yellow. In its chemical composition can be found: essential oils, carotenoid pigments, mucilages, flavonoids, saponins, resins, organic acids and minerals. (VAZ and JORGE, 2006). The part used is the major flowering and the preparation of the mother tincture through the methods of percolation or maceration (BRASIL, 2011). It is indicated for cases where there is a need for scarring, when there are superficial burns and erysipelas, and a more favorable healing occurs with less suppuration (BOERICKE, 2003).

The dermatological and dermocosmetic area has been attractive because it is the site

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of effect (topical administration) that is effective and safe (Mano, 2013). For this reason, it is useful for research in fibroblast strains by applying the principle of simillimum, seeking to increase cell proliferative activity through applications of small doses of ultra dilution of 6CH of *Zincum metallicum* and *Calendula officinalis*.

Methodology

Experimental delimitation

This project was developed in the Laboratory of Molecular Biology and Cell Culture of the School of Health Sciences at Anhembi University. The CCD - 1072 Sk fibroblast lineage, which corresponds to the human fibroblasts of a newborn male, provided by the organization ATCC (<http://www.atcc.org>), was submitted to treatment with different volumes of *Zincum metallicum* 6CH and *Calendula officinalis* 6CH, which was kindly provided by the Interactive Pharmacy Homeopathic Pharmacy located at Rua Dr. Jesuino Maciel, 1008 Campo Belo - São Paulo, Brazil. After the treatments were performed, cell viability and proliferation assays where it was sought to elucidate molecular mechanisms that may be involved in this process

Human fibroblast cell culture

Cells of the CCD-1072Sk strain were cultured in ISCOVE™ medium with 10% fetal bovine serum, 0.292 g / l L-glutamine, 1.0 g / l D-glucose, 2.2 g / l NaHCO₃, 10,000 IU Penicillin and 0.060 g / l Streptomycin. Cells were maintained in 25 cm² flasks (1 × 10⁵ cells / mL) in a humid oven with 5% CO₂ atmosphere at 37°C. In all experiments, fibroblast cultures were submitted to the cell viability test using trypan blue dye and were read in a haemocytometric chamber by light microscopy. All experiments described were performed when the cell viability is equal to or greater than 95%.

Cytotoxicity assessment (determination of IC₅₀)

The concentration curve provided by the IC₅₀ of the test compounds was determined by the method of incorporation of the trypan blue dye, where after 24 hours of incubation with the different homeopathic and control volumes the contents of each well of the cultures of Fibroblasts were collected in 15 ml tubes, and centrifuged at 1500 rpm for 5 minutes. The pellets were resuspended in 1 ml of RPMI medium. Thereafter, the cell solutions were diluted (1: 2) in trypan blue dye and the counting of viable and non-viable cells was performed in a hemacytometric chamber by light microscopy. The sum of the total cell count

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(viable and unviable from each well) was considered as 100% cells, and the calculations were performed to verify the percentages corresponding to the viable and unviable cells analyzed in the experiment.

MTT to formazan reduction assays.

The reduction test MTT ([3-(4,5-Dimethylthiazol-2-yl) 2,5-Diphenyl Tetrazolium Bromide]) is used with great success to estimate the number of viable cells in an initial screening of drugs. Its interpretation is indicative of the cellular metabolic activity, and the place of occurrence of redox reactions includes both mitochondria and the cytosol. The reduction of the salt of MTT to formazan is carried out mainly by the enzyme succinate dehydrogenase, and results in insoluble formazan crystals in violet color. The intensity of staining is used to measure mitochondrial activity and consequently cell viability. Thus, cells treated with different volumes (10 µL, 20 µL, 30 µL, 40 µL and 50 µL) of the 6 centesimal dilution according to the Hahnemann (CH) methodology of *Zincum metallicum* and *Calendula officinalis* and at a concentration of 5×10^4 cells per well were 10 µL of the 5mg / ml MTT solution (Sigma-Aldrich) was added to each well.

After 4 hours the plate was centrifuged, the

supernatant from each well discarded, and the pellet with the crystals formed on the bottom of the plate dissolved with 100 µL of pure ethanol and then homogenized on a plate shaker for 15 minutes. The optical density was measured by the microplate reader (FlexStation® 3 multimode benchtop reader) at 540 nm. The percentage of cell survival in the presence of the compounds under study was calculated as follows:

$$\% \text{ Viable cells} = 100 * \text{absorbance per well (with homeopathy)} / \text{absorbance of control (without homeopathic)}$$

Propidium iodide (PI) incorporation assay

PI incorporation assays were performed to evaluate cell cycle phases by flow cytometry. Briefly, the cells were plated in twenty-four well plates (initial cell density / well, 2×10^5) and added to the hypotonic fluorocromatic solution (HFS - 0.1% w / v trisodium citrate, , 5% w / v Triton-X 100 and 50 µg / ml propidium iodide). After a four hour incubation period at 4 ° C and sheltered from light, cells and supernatant were collected and analyzed. The FACScan flow cytometer, the CELLQuest program and the data obtained, considering 20,000

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events per analysis in each test, were analyzed in the WinMDI 2.8 program.

Sirius Red - collagen quantification

Cells were cultured and treated as described above. After culturing, the medium will be withdrawn and the wells washed three times with 0.1M PBS. 100 μ L of Bouin Fluid (picric acid, formaldehyde and glacial acetic acid) was added for fixation for 1 h. The samples were washed with PBS, then the Sirius Red dye was added. After the time of 1 h, the dye max was removed and washed with 150 μ L of 0.01 M hydrochloric acid solution for 30 sec. Removal of the dye that did not bind to collagen. The dye was then removed from the cell layers with the addition of 0.1 M NaOH solution for 30 min. Aliquots of 100 μ L of the solutions contained in the wells were transferred to a new plate. Absorbance was measured on an Elx-800-UV microplate reader (Bio-Tek Instruments, USA) at 570 nm.

Statistical analysis

The results were presented as mean + SEM (standard error of the mean). The results were submitted to statistical analysis by analysis of variance (ANOVA) of one way, followed by Tukey a posteriori test. Semi-quantifications were analyzed by Student's t-test. Values of $P < 0.05$ were considered

significantly different. The analyzes were carried out in the program GraphPad Prism version 5.0.

Results

Cytotoxicity assessment (determination of IC50)

According to the results obtained in the quintuplicata of samples by the trypan blue incorporation test, Zinc metallicum 6CH and Calendula officinalis 6CH did not present IC 50 when applied the volume of 50 μ L (5%) when compared to the control group, figure 1 A linear increase in the number of viable cells was also observed in both cultures treated with 5% Z. metallicum and C. officinarum as compared to the number of viable cells present in the control group, Figure 2. the microscopic analysis of the treated fields And control fields diverged relative to the individual amounts of cells present. When analyzed in Neubauer's chamber, it was possible to verify that cell viability was maintained in the meanwhile the total number of cells was much higher in treated wells when compared to the control group. In addition to treatment of the results by ANOVA with Tukey's correction, the Student's T test was applied assuming equivalent variances, ie, the percentage of viable cells was not altered by homeopathic treatment and confirming that there were

no changes in the percentage cell viability, Table 1. Figures 3 and 4 illustrate boxplots for better visualization of the variations and differences between the averages, corroborating with Figure 2 regarding cell volume differences found in treated wells versus wells with control cells.

Table 1 - Student's T Test Results

	Zincum metallicum	Calendula officinallis
Average	97,8%	98,6%
P- one-tailed	0,15	0,00
t-one-tailed	2,13	2,13
critical

Figure 1. Results of the percentage of cell viability by the blue trypan technique after 24 hours of exposure with 50uL of Zinc metallicum 6CH and Calendula officinalis 6CH. Prior to the start of the tests, the cells were depleted of fetal bovine serum. (*) $P > 0.05$ - significant in relation to control, ANOVA, Tukey. (**) $P > 0.05$ - significant in relation to control, ANOVA, Tukey.

Figure 2. Results of the cell viability by blue trypan technique after 24 hours exposure with 50uL of Zinc metallicum 6CH and Calendula officinalis 6CH. Prior to the start of the tests, the cells were depleted of fetal bovine serum. (*) $P > 0.05$ - significant in relation to control, ANOVA, Tukey. (**) $P > 0.05$ - significant in relation to control, ANOVA, Tukey..

Figure 3. Results of cell viability by blue trypan technique after 24 hours exposure with 50uL of Zinc metallicum 6CH with mean and variance in boxplot, indicating significant statistical differences between the control and the treated lineage Z. mettalicum 6CH. Prior to the start of the

tests, the cells were depleted of fetal bovine serum.

Figure 4. Results of cell viability by blue trypan technique after 24 hours exposure with 50uL of Calendula officinalis 6CH with mean and variance in boxplot, indicating significant statistical differences between the control and the treated lineage C. officinalis 6CH. Prior to the start of the tests, the cells were depleted of fetal bovine serum

MTT reduction to formazan

In relation to the MTT proliferation test, the proliferation tendency of *Z. metallicum* and *C. officinarum* on the CCD-1072SK fibroblasts tested in this study, no reduction in the oxidative activity of the quadruplicate of samples was observed, however an increase in the metabolic rate. In this test, 10 µL (1%), 20 µL (2%), 30 µL (3%), 40 µL (4%) and 50 µL (5%) volumes were applied

in order to verify in which percentage the best response of culture. For *Zincum metallicum*, a linear increase in fibroblast proliferation was observed from 2%, and this increase was significant, in relation to the control group, for the percentage of 4% and 5%, figure 5. Regarding *Calendula officinalis*, This same linear increase in proliferation was verified, however, there was an increase already in the culture treated with 1% of the drug, however significant significance was observed in this proliferative increase from cultures treated with 3%, figure 6. Tests F to verify the variances were applied the Results of 4% (40µL) 5% (50µL) for *Zincum metallicum* and for the results of 3% (30µL), 4% (40µL) and 5% (50µL) for the results of *Calendula officinalis* comparing them with the results Of the control groups, as shown in Table 2, confirming the Tukey Correction ANOVA results (Figures 5 and 6) for the relationship between variations in results with statistical significance.

Table 2 - Results of Test F with Verification of Variance

	Z.me	Z.me	C.ofi	C.ofi	C.ofi
	t 4%	t 5%	c 3%	c 4%	c 5%
P	0,56	14,7	0,10	0,37	0,75
	8				
t	0,11	9,28	0,11	0,11	0,11

P: P-one tailing; t: t-one tailing critical

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Figure 5. Results of cell viability through the MTT technique after 24 hours exposure with different volumes of Zinc metallicum 6CH. Prior to the start of the tests, the cells were depleted of fetal bovine serum. (*) $P > 0.05$ - significant in relation to control, ANOVA, Tukey. (**) $P > 0.05$ - significant in relation to control, ANOVA, Tukey.

Figure 6. Results of cell viability through the MTT technique after 24 hours exposure with different volumes of Calendula officinalis 6CH. Prior to the start of the tests, the cells were depleted of fetal bovine serum. (*) $P > 0.05$ - significant in relation to control, ANOVA, Tukey. (***) $P > 0.05$ -

significant in relation to control, ANOVA, Tukey.

Sirius Red - quantification of collagen

The quantification of the collagen produced by the CCD-1072SK fibroblasts was performed by the technique using the Sirius Red acid dye. The results in the triplicate of samples showed that the exposure of the fibroblasts to the homeopathic was only able to significantly increase the collagen content for the treated culture With Zincum metallicum at the highest concentration used after single exposure in 24 hours, however, the results for Calendula officinalis indicate a significant increase in the production of collagen at the concentration of 40µL (4%), reaching up to 50µL (5%), (Figure 7). Figure 8 and 9 outline the results in bloxplot for differences in means between the control groups and the results of 50µL of Zincum metallicum and 40µL and 50µL for Calendula officinalis. Table 3 is a result of F test for the applications of Z. metallicum at 5% and C. officinalis at 4% and 5% when compared to the control group.

Table 3 - Results of Test F with Verification of Variances

	Z.met	C.off	C.off
	5%	4%	5%
Average	121%	115%	121%
P- one-tailing	0,46	0,08	0,26
T - one tailing	0,05	0,05	0,05
critical

Figure 7. Result of total collagen quantification through incorporation of the Sirius Red acid dye after 24 hours exposure to Zinc metallicum (6CH) and Calendula officinalis (6CH). Prior to the start of the tests, the cells were depleted of fetal bovine serum. (*) And (***) $P > 0.05$ - significant in relation to the control, ANOVA, Tukey.

Figure 8. Result of total collagen quantification by incorporation of the Sirius Red acid dye after 24 hours exposure to Zinc metallicum (6CH) application of 50 µL, mean and variances different with statistical significance in boxplot. Prior to the start of the tests, the cells were depleted of fetal bovine serum.

Figure 9. Result of total collagen quantification by incorporation of the Sirius Red acid dye after 24 hours exposure to Callendula oficinalis (6CH) application of 40 µL and 50 µL, mean and variances different with statistical significance in boxplot. Prior to the start of the tests, the cells were depleted of fetal bovine serum.

Propidium iodide (PI) incorporation assay

Finally, from the results obtained above, which are related to cell proliferation after in vitro treatment with the homeopathic tested, we used flow cytometry and the incorporation of the propidium iodide to quantify the cell fractions that are in the S-G2-M phases Of the cell cycle (Figure 4A and 4B).

Figure 4A shows the graphical representation of the percentage of homeopathic treated cells that are in the S-G2-M phases of the cell cycle as compared to the untreated control.

Figure 10 - Effects of homeopathic exposure on human fibroblast lineage. (A) Percentage of cells in S-G2-M phase. * P < 0.05 when compared to control. (ANOVA, tukey). (B) Histograms representative of the cell populations studied when exposed for 24 hours, after the qualifying period.

Conclusion

Considering the above results, it is concluded that both Zincum metallicum 6CH and Calendula officinalis 6CH, when applied to 5% by CCD-1072SK fibroblast culture for 24 hours was sufficient to develop reset cell to induce the increase in the number of viable cells of oxidative metabolism and collagen production. It was observed response to increasing level of S-G2-M phases of the cell cycle in all cultures treated with Zincum metallicum and Calendula officinalis 6CH at 4% and 5%, however this increase of cells in DNA synthesis activity was only statistically significance to group CCD-1072SK cells treated with Calendula officinalis 6CH 5% for 24 hours, suggesting a possible dermocosmetic application.

It is recommended to be made more studies in the area, the style developed by Mano (2014) using cosmetic bases as merger means the two homeopathic medicines tested.

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Conflict of Interest: None declared.

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