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Article

Reducing PMA-induced *COX-2* expression using a herbal formulation in MCF-7 breast cancer cells

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SUMMARY

Cyclooxygenase-2 (*COX-2*) is the inducible form of a group of enzymes that catalyze the conversion of arachidonic acid to prostaglandins. This isoform is often overexpressed in breast cancer cells. *COX-2* overexpression correlates with an aggressive phenotype in breast cancer, including higher histological grade, larger tumor size, estrogen receptor (ER) negativity, and human epidermal growth factor receptor 2 (HER-2)/neu positivity. Another study, via molecular, animal, and human investigations, supported that *COX-2* expression increases as cancer develops, getting progressively worse during the metastatic phase through the release of vascular endothelial growth factor (VEGF). Traditional medicine or herbal compounds have been gaining increasing popularity for alternative treatment for cancer alone and in conjunction with treatments. Green tea and turmeric are famous for their numerous beneficial effects on the body, including anti-oxidative, chemo-preventive, chemo-protective, anti-inflammatory properties. *COX-2* expression was induced using phorbol-12-myristate-13-acetate (PMA). This study tested the effect of the herbal formulation HF1 (mainly composed of (-)-epigallocatechin-3-gallate (EGCG) and curcumin) on the *COX-2* mRNA levels in the breast cancer cell line MCF-7. We hypothesized that PMA-induced overexpression of *COX-2* in MCF7 cells would be eliminated after exposure to HF1. PMA increased *COX-2* levels by 3-fold as compared to the untreated MCF-7 culture (no PMA, no HF1). However, when cells were treated with HF1 alongside PMA, we observed a 60% decrease in *COX-2* levels.

INTRODUCTION

Cancer is the second-leading cause of death globally, creating approximately 9.6 million deaths in 2018 alone (1). Breast cancer, the most frequent type of cancer in women, affects 2.1 million women each year, causing up to 15% of female deaths (2). Hence, research to improve the way such a disease with wide-ranging implications is detected, treated, and managed becomes necessary and significant.

The link between inflammation and cancer was first investigated with Virchow in 1863, and similar inquiry continues to hold steady (1, 3, 4). Inflammation results in a more advanced malignant structure, larger tumor size, ER-negativity, and HER-2/neu-positivity (5, 6). Cyclooxygenase (*COX*) isoenzymes are a group of enzymes that play an active role in triggering an inflammatory response. *COX-2* is the specific inducible isoform shown to be expressed in breast cancer and hence was the focus of this study (7, 8). Increased COX-2 expression is also correlated with stimulation of vascular endothelial growth factor (VEGF) release and increased HER-2/neu expression, progressively worsening the cancer during its metastatic phase (9).

There are many methods through which *COX-2* works towards carcinogenesis after being induced. Mitogenesis occurs when COX-2 stimulates the biosynthesis of the E-series of prostaglandins, specifically PGE-2, triggering a signal cascade for increased cell division (10). Mutagenesis happens when *COX-2* provokes the production of mutagens and other reactive oxygen species (ROS) that can damage DNA (11). *COX-2* can also cause apoptosis with cell death as it stimulates telomerase expression, decreases arachidonic acid, suppresses BAX, and stimulates BCL-2 - all of which block intrinsic apoptosis (12). Immunosuppression is caused by inhibiting proliferation of two immune cell types- dendritic cells and T lymphocytes (13). Angiogenesis is promoted by VEGF levels, which allows for *de novo* formation of blood vessels that supply nutrition and metastatic pathways for tumors to spread systemically (14). Metastatic potential is also supported through the increase of the levels of matrix metalloproteinases (MMP), which aid the invasive potential of the tumor (15). Invasion is the process of malignant cells spreading deeper/becoming more expansive into the tissue of a primary tumor.

Usually, *COX-2*-induced inflammatory response remains tightly controlled and regulated; however, changes caused by sustained inflammation can result in disruption of the feedback loop. Inflammation regularly involves the accumulation of various cell types of both the vascular and immune systems, such as stromal cells, monocytes, and lymphocytes (21). In the *COX-2*-driven inflammatory cascade, cytokines, especially IL-1 and IL-6, play an essential role to stimulate the production of acute-inflammatory-phase proteins and to exert feedback inhibition that stops the response (16, 17). However, during the transition from acute to chronic inflammation, these cytokines bring in monocytes, which interfere with the feedback loop by secreting interrupting pro-inflammatory cytokines (18, 19). The infiltration of monocytes into the inflamed tissue defines chronic inflammation, which directly links to cancer *in vivo*.

Michigan Cancer Foundation-7 (MCF-7) is a breast cancer line that shows an increase in aggressiveness with increased *COX-2* expression (20, 21). Therefore, we selected MCF-7 as the cell line of study to investigate how *COX-2* levels varied in response to different treatments, indicative of metastatic potential.

With the rise in popularity of active compounds from natural, herbal sources as potent molecules in conventional treatments for cancer, Sri Raghavendra Biotechnologies Pvt Ltd in Bangalore developed an herbal formulation, HF1, consisting of green tea (*Camellia sinensis*) and turmeric (*Curcuma longa*). Both spices have a long history in traditional food and medicine, and there are many studies establishing their anti-inflammatory properties and suggesting a potential effect against cancer (22). Curcumin is a polyphenolic compound extracted from the rhizomes of turmeric. It has anti-cancer properties by reducing hypoxia-inducible factors (HIF) transcriptional activities in MCF-7, which is critical because *COX-2* expression is regulated at the transcriptional level by HIF (23, 24). It also suppresses MMP-assisted cell invasion (25). Since there is a positive correlation between this invasion and *COX-2* expression, it is also indicative of suppression of *COX-2*. Additionally, curcumin restores E-cadherin levels (26). There is an association between an increase in E-cadherin levels with a decrease in *COX-2* expression (27). In green tea, (-)-epigallocatechin-3-gallate (EGCG) is the most potent polyphenol catechin, with a wide range of beneficial effects concerning a multitude of inflammatory diseases (28–30). As a pure compound, EGCG has been shown to inhibit *COX-1* and *COX-2* levels (31).

We investigated whether HF1 can restore *COX-2* baseline mRNA levels after *COX-2* overexpression was induced. Due to the anti-inflammatory properties of the compounds used, we hypothesized that the use of HF1 would reduce increased *COX-2* expression back to baseline levels. A variety of inflammatory stimuli can induce *COX-2*; the most common is phorbol ester, phorbol-12-myristate-13-acetate (PMA), a compound used to cause dermal inflammation (32). PMA was used in this study to induce *COX-2* expression in MCF-7 cells to mimic levels observed in an aggressive breast cancer. The results demonstrated that HF1 significantly decreased *COX-2* mRNA levels. PMA activates the protein kinase C (PKC) pathway, which causes a chain reaction of phosphorylating transcription activators within the cell. It results in increased expression of oncogenes like *COX-2* (33).

RESULTS

To test the effect of HF1 on *COX-2* expression, there were three groups of MCF-7 cultures: the untreated control, treated with PMA, and treated with both PMA and HF1. We extracted RNA from the MCF-7 cells grown under these different experimental groups then reverse-transcribed into cDNA. Then, we conducted PCR with *COX-2* and *GAPDH* specific primers. We ran the PCR products through gel electrophoresis and then quantified the intensity of the bands formed using ImageJ, comparing levels of *COX-2* expression between groups.

COX-2 levels in different culture conditions

MCF-7 cells were cultured under three different conditions so that the *COX-2* mRNA could be extracted and measured. Cultures grown in normal, untreated media were considered the negative control and the *COX-2* levels expressed in these cultures were considered as the control base value. PMA was used to induce *COX-2* overexpression in the second set, and a mixture of PMA and HF1 was used in the third group also to test the effect of the herbal formulation. We chose half of

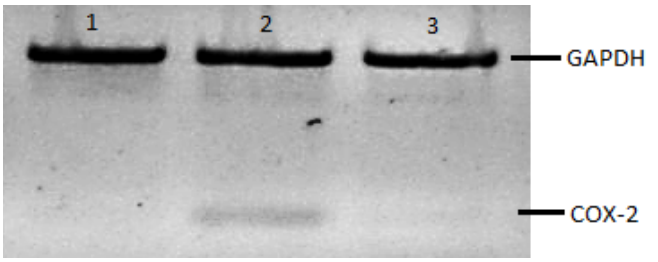

Figure 1: Cumulative gel for *COX-2* expression in three culture conditions (N=3). 1. MCF-7 cells grown under regular, untreated conditions; 2. MCF-7 cells grown with 10 ng/mL PMA for 24 hours; 3. MCF-7 cells grown with 10 ng/mL PMA and 0.3 mg/mL HF1 for 24 hours together.

0.6 mg/mL, the inhibitory concentration (IC_{50}) value of HF1, to ensure the formulation did not cause excessive cell death but did affect gene expression and subsequent protein levels. IC_{50} refers to the concentration that results in 50% cell death and consequently 50% viability. The IC_{50} value was determined from previous experimentation performed within the same lab (34).

The difference in the intensity of the gel electrophoresis bands across the three culture conditions was clearly visible and quantified in terms of fold-increase for comparison (**Figure 1**). Normalized values for *COX-2* were calculated and plotted as fold-change in expression of *COX-2* amongst the three groups. The untreated MCF-7 control was at 1-fold expression, and MCF-7+PMA showed a 3-fold increase in *COX-2* expression. However, MCF-7+PMA+HF1 exhibited a decrease in *COX-2* expression to 1.2-fold expression. Therefore, the addition of HF1 for 24 hours prevented excessive *COX-2* expression by 60% when compared to the culture with PMA-induced *COX-2* levels, resulting in almost baseline levels. (**Figure 2**).

DISCUSSION

Inducing *COX-2* expression in MCF-7 cells using PMA was successful, as those cells expressed a significant 3-fold increase from baseline levels of the MCF-7 control. After the

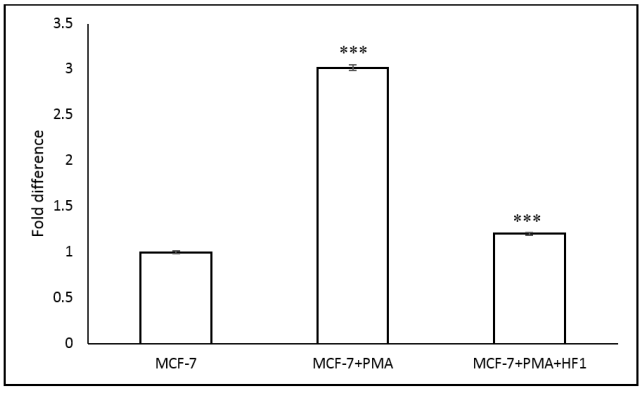

Figure 2: Herbal formulation (HF1) eliminated PMA-induced overexpression of *COX-2* in MCF-7 cells (N=3). Bar graph showing mean *COX-2* mRNA fold change in all three cultures. MCF-7 breast cancer cells were grown under either control conditions, in 10 ng/mL of PMA for 24 hours (*COX-2* overexpression), or in 10 ng/mL of PMA and 0.3 mg/mL of HF1 for 24 hours. Error bars present Standard Deviation. $p < 0.001$ is shown as ***. MCF-7+PMA is significant compared to the control MCF-7 culture. MCF-7+PMA+HF1 is significant compared to MCF-7+PMA.

treatment of HF1 to PMA-treated cells, the *COX-2* mRNA level decreased to 1.2 fold. This datum is the core of the study; it suggests the effect of HF1- reducing *COX-2* expression by 60% after PMA treatment. Consequently, HF1 can be a potential candidate for controlling the metastasis and aggressiveness of breast tumors, which directly supports the hypothesis.

Breast cancer cases have worsening prognosis when detected at later stages, and *COX-2* plays a role in this correlation (35). Previous mouse studies showed that *COX-2* overexpression (induced through transfection using a vector) increased breast cancer metastasis to bone. It may be so because the increased PGE2 production stimulated by *COX-2* may provide a functional advantage in metastasis (36). The increase in *COX-2* when cancer proceeds to later stages means that the tumor increases in size, shows early local invasion into lymph nodes *in vivo*, and demonstrates a disorganized Indian-filing characteristic, which is indicative of a very invasive phenotype (37). Conversely, *COX-2* inhibition of any source resulted in reduced metastatic potential *in vitro* reconstituted extracellular matrix and inhibition of metastasis to the lungs *in vivo* models. Substantially, the malignant phenotype of breast cancer decreased in silenced *COX-2*, which is beneficial to patients and can be used to supplement curative treatments (38).

To better the chance of survival when breast cancer is identified at later stages, *COX-2* inhibition must be studied. This study, although it focuses on raw herbal formulations, is important in hypothetically providing alternative methods in the field of integrative oncology by supporting evidence of suppressing *COX-2* mRNA levels.

While this study was reliably conducted with an appropriate number of trials and repetitions, the limitations of the study include the quantification of the *COX-2* mRNA gel bands as being semi-quantitative. It does not measure the precise quantity of the expression but rather assigns a value that is estimated in relation to the levels of the control. In the future, qPCR measurements can be conducted to ensure quantitative data to support the same conclusion. Additionally, since half the IC_{50} value of HF1 was used, future experiments can investigate a range of concentrations of HF1 to identify a true optimum concentration for *COX-2* expression suppression.

MATERIALS AND METHODS

Cell Culture and COX-2 Induction Using PMA

MCF-7 cells, obtained from NCCS Pune, were cultured in DMEM-F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Thermo Fisher Scientific) in a humidified incubator at 37˚C with 5% CO2. Cells were treated at 40% confluency and were harvested at 80% confluency. Petri dishes (35 mm) were seeded with 2.0 x 105 cells for all sets of treatments, and harvested numbers were counted to be around 1.0 x 106 cells from each petri dish.

The three following experimental groups were conducted. The first group was an MCF-7 control that consisted of untreated cells grown at the conditions mentioned previously. The second was MCF-7+PMA, consisting of MCF-7 cells treated with 10 ng/mL of PMA (Thermo Fisher Scientific) for 24 hours to induce *COX-2* expression. The third was MCF-7+PMA+HF1, consisting of MCF-7 cells co-treated with 10 ng/mL of PMA and 0.3 mg/mL of HF1. All groups were grown for 24 hours.

Expression of COX-2 in MCF-7, MCF-7+PMA, and MCF-7+PMA+HF1

An aliquot of cultured MCF-7 cells was pelleted from the stock culture. This pellet was used for RNA isolation using a spin column (Qiagen). The extracted RNA was quantified at 260 and 280 nm and 3000 ng. RNA was used to synthesize 20 µL cDNA using MuLV Reverse Transcriptase (Thermo Fischer Scientific, Verso). PCR was carried out using Jumpstart (Sigma) and primers to amplify the cDNA (250 ng/50 µL PCR reaction), making *COX-2* expression quantifiable. Forward and reverse primers specific for *COX-2* and *GAPDH*, a housekeeping gene as an internal control, were used. *COX*-2_Fwd: 5' CCACTTCAAGGGATTTTGGA, *COX-2*_Rev: 5' GAGAAGGCTTCCCAGCTTTT, *GAPDH*_Fwd: 5' GGTCGGAGTCAACGGATTTGGTCG, *GAPDH*_Rev: 5' CCTCCGACGCCTGCTTCACCAC. Gel Electrophoresis was carried out and observed under UV-transilluminator.

Statistical Analysis

Image J software was used to calculate the area under the curve for the intensity of the band. The groups were normalized to their respective *GAPDH* levels. GraphPad Prism 5.0 was used to analyze and represent graphical data. One-way ANOVA was performed, followed by Tukey's Test. The experimental data are expressed as mean and standard deviation. P-value < 0.05 was considered statistically significant. Three independent sets of experiments were performed to increase reliability of results (n = 3).

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