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Piperine Acts as an Anticancer Agent by Reducing Cyclooxygenase-2 Activity and Inducing Apoptosis by Activating p53 in HeLa Cells

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ABSTRACT

Cervical cancer is a malignant disease with the highest incidence in women worldwide. Human papillomavirus (HPV) infection causes cancer through degradation and decreased activity of the p53 gene, which is a significant contributor to the apoptosis pathway and a major factor in cancer incidence. In addition to p53, the cyclooxygenase-2 (COX-2) enzyme is essential for tumor formation via inflammatory pathways, particularly in cervical cancer caused by persistent HPV infection. This study aims to investigate how piperine treatment affects the HeLa cervical cancer cell line p53 expression, COX-2 levels, and cell proliferation. HeLa cells were divided into treated groups (65 and 130 µg/mL) and an untreated group as a control group. Its activity on COX-2 levels was further analyzed using the ELISA method and gene expression using the Livak qPCR method. Cell proliferation can be inhibited by piperine, with an IC_{50} value of 66.68 µg/ml. Piperine can also increase p53 gene expression and suppress COX-2 levels in HeLa cells. Piperine has cell growth inhibitory activity, suppresses the inflammatory process by reducing COX-2 enzyme levels, and induces apoptosis through upregulation of the p53 gene.

Keywords: Piperine, Cyclooxygenase-2, Expression of p53, Proliferation, Cervical cancer

Introduction

Cervical cancer is an increasing global health concern, impacting both developed and developing countries.[1] Cervical cancer ranks as the fourth most common and most malignant cancer among women globally, after breast, colorectal, and lung cancer.[1] Human papillomavirus (HPV) is the main cause as shown by epidemiological and molecular evidence.[2] In the general population, 87.0% of women suffer from invasive cervical cancer, and an estimated 4.0% of those women are infected with HPV.[3] In high-risk HPV (hrHPV)–positive cervical cancer, E6 oncoprotein degrades cells and impairs p53 activity by binding to E6-associated protein (E6AP) ubiquitin ligase.[4] In addition, the generality of cervical cancer cells accommodate the *wt*p53 gene, but its expression levels are relatively low.[5]

P53-related targets of apoptosis fall into a variety of categories according to their functions and activation pathways. These gene products possess the capacity to stimulate apoptosis either through the intrinsic (mitochondrial pathway) or extrinsic (death pathway) pathways.[6] Furthermore, stress activates the intrinsic apoptotic pathway (Bcl-2 family proteins)7. There are several types of Bcl-2 family proteins, namely the pro-apoptotic such as Bax, and Bak; anti-apoptotic Bcl-2 and Bcl-XL; and the "BH3-only" pro-apoptotic proteins Bad, Noxa, and Puma (BH3-binding death agonists)[7]

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In contrast to the intrinsic pathway, p53 triggers the induction of death receptors on the plasma membrane, as well as Fas/CD95,[8] DR4, and DR5.[9] This, in turn, inhibits the formation of inhibitors of apoptosis proteins (IAPs), which accelerate the process of apoptosis.[10] In addition, p53 can cause endoplasmic reticulum-dependent apoptosis. p53 will transactivate the production of a protein found in the nuclear membrane and endoplasmic reticulum.[11] In the p53-regulated apoptosis process, the intrinsic apoptotic pathway is the primary means of apoptosis, while activation of the extrinsic apoptotic pathway increases the apoptotic response.[12]

According to previous studies, cyclooxygenase-2 (COX-2) was biochemically proven to be related to p53. p53 can increase COX-2 levels by activating the MAPK/ERK pathway. COX-2 is one of the downstream target genes of p53. In addition, p53 and genotoxic stress–induced apoptosis are inhibited by COX-2. Apoptosis induced by genotoxic stress might be mitigated by overexpressing COX-2. Furthermore, COX-2 inhibits p53 transcriptional activity and may physically interact with p53 both *in vitro* and *in vivo.*[13] During the carcinogenesis, COX-2 plays a crucial role. In cancer, the apoptotic process may be inhibited by an increase in COX-2 expression, either through the intrinsic or extrinsic apoptosis pathway. The inhibition of apoptotic protein activation and the induction of antiapoptotic proteins are associated with this mechanism.[14]

Natural products have long been the subject of ongoing research for the potential of developing new therapeutics.[15,16] A significant proportion of the populace in underdeveloped nations still uses traditional medicine to treat major illnesses like cancer and inflammation[17]. Piperine is derived from an alkaloid extracted from various species of the Piperaceae family.[18] Piperine shows cytotoxic properties against many different kinds, including lung, cervical, prostate, and breast cancer, in *in-vitro* testing.[19] Piperine inhibits the production of many proinflammatory cytokines by tumor cells, disrupting the ability of cancer cells to communicate with each other and shrinking tumors.[20] Additional research has indicated that piperine acts in the apoptotic process.[21] However, there is currently limited knowledge of the

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anticancer mechanism of piperine in cervical cancer cells, so further investigation is required.

Materials and Methods

Materials

Cell culture

HeLa were cultured in Dulbecco's Modified Eagle Medium. Add 10% Fetal Bovine Serum, penicillin and streptomycin 100 U/ml each to the medium. Piperine was obtained from BOC Sciences Inc., USA.

Cell Viability Assay

The potential effect of piperine on HeLa cell viability was measured 72 hours post-treatment using the MTT assay. Cells that were exponentially growing were collected and diluted to 2×10^4 cells/mL. The cells were then seeded at 200 µL each in 96-well plates. Following a 24-hour incubation period at 37 °C, the medium was withdrawn, and piperine was added to the wells at doses of 31.25, 6.25, 125, 250, and 500 µg/mL. After 72 hours of incubation, 25 µL of 5 mg/mL MTT reagent was added. Formazan products generated by live cells were incubated for 4 hours at 37 °C. After washing them in 150 µL of dimethyl sulfoxide, a microplate reader was used to detect their absorbance at 595 nm. Additionally, the viability was assessed using the following formula:

cell viability (%) = [A_{595} (treated cells)/A_{595} (control cells)] × 100.

Cyclooxygenase-2 ELISA

HeLa cells were grown in 24-well plates. Piperine concentrations of 65 and 130 µg/mL were used for COX-2 activity inhibition experiments. An enzyme-linked immunosorbent assay (ELISA) kit was employed in compliance with the manufacturer's guidelines to assess piperine's capacity to inhibit COX-2. Additionally, this enzymatic reaction created a yellow color, which was quantified spectrophotometrically (at 450 nm with a microplate reader).

Gene Expression of the p53 Gene by Real-Time PCR

Piperine concentrations of 65 and 130 µg/mL were added to six-well plates containing 5×10^6 cells each and incubated for 72 hours. Cell RNA was isolated using Trizol. The extracted RNA was then measured using a nanodrop spectrophotometer. Additionally, cDNA synthesis from 1 µL of RNA follows the SensiFAST™ cDNA synthesis kit protocol with the reagents and condition parameters listed in Table 1 and Figure 1. cDNA will be used as a template in the PCR protocol. The primer sequences of p53 and GAPDH employed were forward and reverse (Table 2). For real-time PCR, SYBR green master mix from Thermo Scientific USA was used with the reagent composition as listed in Table 2, and the PCR conditions used were adjusted to the results of primer temperature optimization (Figure 2). P53 is the target gene in this study. The GAPDH gene functions as an internal control, while the untreated group acts as a calibrator. Subsequently, three duplicates of the response were conducted. The relative expression of the p53 gene was ascertained by means of standardizing its Ct value against that of GAPDH. The method used to determine the relative expression of p53 gene expression is ΔΔCt or Livak method.[22]

$\Delta Ct_{(p53)} = Ct_{(p53,\ test)} - Ct_{(GAPDH,test)}$

$\Delta Ct_{(calibrator)} = Ct_{(p53,\ calibrator)} - Ct_{(GAPDH,calibrator)}$

$\Delta\Delta Ct = \Delta Ct_{(p53)} - \Delta Ct_{(calibrator)}$

Relative gene expression or fold change was calculated and compared to the control, which served as a model of the normal state, using the method $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Three distinct independent experiments were tested in triplicate. The parametric test was used to statistically assess the data (ANOVA). Duncan's multiple range test was employed in order to quantify the differences between the treatment groups. A statistically significant difference is defined as $p < 0.05$.

Table 1: Reagents for complementary DNA synthesis

Reagents	Volume (µl)
Total RNA or mRNA	1
5x TransAmp Buffer	4
Reverse Transcriptase	0.4
RNase free-water	1.0

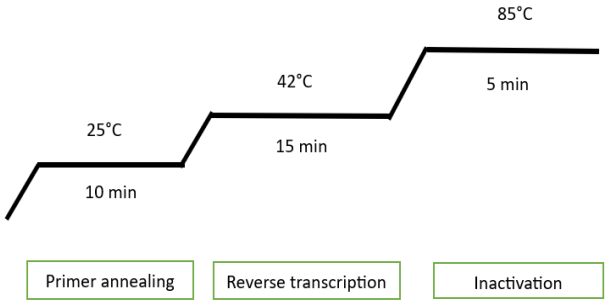

Figure 1: cDNA synthesis procedure (SensiFAST™ kit protocol)

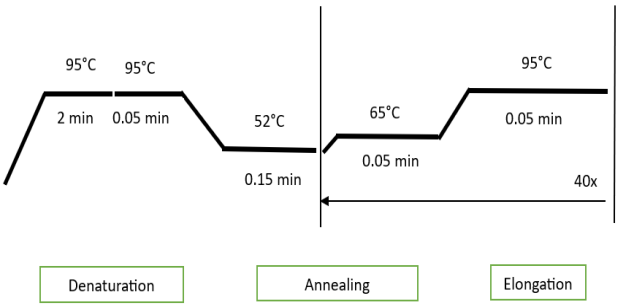

Figure 2: PCR conditions for examining the p53 gene with RT-PCR according to the results of primer temperature optimization.

Table 2: Primer sequences used in examining relative expression using PCR

No.	Name of Gene	Forward Primer	Reverse Primer
1.	P53-human	5′-CATGACGGAGGTTGTGAGG-3′	5′-CAGTAGATTACCACTGGAGCTTCTC-3′
2.	GAPDH - human	5'-CATCATCCCTGCCTCTACTG-3,	5'-CCAAATTCGTTGTCATACCAGG-3'

Result and Discussion

Cell viability assay

Cell viability is the proportion of active cells in a sample.[23] Cell viability was analyzed using the MTT assay. Adding MTT solution to culture cells and letting them sit for several hours is the standard procedure for MTT screening. Yellow tetrazolium will be reduced to purple formazan by the dehydrogenase enzyme found in the mitochondria of active cells. However, cytosolic enzymes may also be needed, including flavin oxidase and nicotinamide adenine dinucleotide reductase.[24] This leads to the generation of insoluble formazan salts in water. Dimethyl sulfoxide (DMSO) is one solvent that can dissolve this formazan ion. Using spectroscopy, the formazan product is measured at 595 nm, and the absorbance of this product is correlated with the quantity of living cells.[25]

After being treated for 72 hours, cytotoxic activity of piperine in HeLa cells significantly and dose-dependently increased. According to test results, as the dose of piperine increased, there was a significant decrease in cell viability (Figure 3). Plotting revealed a statistically significant difference ($p \leq 0.05$) in the reduction of HeLa cell viability between piperine concentrations. Cell viability was observed to be 21.01% at 500 µg/mL piperine. The results indicate that piperine possesses a concentration-dependent antiproliferative effect.

Chemotherapeutic drugs require an understanding of IC_{50} determination.[26] Figure 3 shows that piperine's IC_{50} value was 66.68 µg/mL. The United States National Cancer Institute (NCI) states that an IC_{50} value of 20 µg/ml indicates cytotoxic activity. Conversely, values between 21 and 200 µg/ml indicate moderate cytotoxic activity, 201 to 500 µg/ml indicate mild cytotoxic activity, and values above 500 µg/ml indicate no cytotoxic activity. It is established that piperine exhibits moderate cytotoxic action against HeLa cells.

Cyclooxygenase-2 Level

Inflammation is a condition that initiates the formation and progression of cancer and malignancy. Prostaglandins are lipid compounds that physiologically have various effects. Prostaglandins (PGs) have a role in inflammation, the formation of cancer, regulation of tissue homeostasis, and normal cell proliferation.[27] PG is a protein resulting from the metabolism of arachidonic acid (AA), which is produced through phospholipation of A2, a membrane phospholipid enzyme. COX converts AA into the precursor molecule prostaglandin H2 (PGH2). Prostaglandin transporters (PGTs), proteins belonging to the 12-transmembrane anion transport polypeptide superfamily, rapidly transport prostaglandins (PGs) to the extracellular environment after production. When exported to the microenvironment, PG binds and activates coupled G-protein receptors. PG specifically binds to the receptor, causing receptor stimulation and breaking down the heterotrimeric G protein complex.[27]

Inhibiting the COX-2 enzyme is beneficial for both preventing and treating different kinds of cancer.[29,30] However, due to several kinds of side effects, such as gastrointestinal toxicity, a higher possibility of myocardial infarction, and cardiovascular health problems, the use of COX-2 inhibition and other COX-2 inhibitors is currently limited.[31] As a consequence, PG is the subject of a lot of research that targets COX downstream in an effort to limit the negative consequences of COX-2 inhibition while preserving PG's potential for cancer treatment and prevention. This process was inhibited by piperine in our study due to the decrease in COX-2 levels in HeLa cells after adding piperine. It is thought that piperine prevents the binding of prostaglandins to the receptor and its signal transduction.[28]

Piperine plays a role in the mechanism of inhibiting COX-2 activity, thereby suppressing PG production in HeLa cells. The COX-2 level in the control cells served as a benchmark for the COX-2 level in cancer cells. The average COX-2 value in control cells was 3.92 ng/mL, whereas at 65 µg/mL piperine it was 1.00 ng/mL, and at 130 µg/mL it was 0.76 ng/mL. The most effective piperine concentration was 130 µg/mL, and the more the piperine concentration increased, the greater its activity in reducing COX-2 levels significantly ($p < 0.05$). This study is consistent with earlier studies that state piperin contributes to neoplastic evolution as a proliferator, and a migrator through promoting cell division during the Gap1/Gap0 and Gap2/Mitosis phases, after which apoptosis occurs. This is regulated by downregulating PTGS2 expression, which can increase the release of cytokines and MAPKs, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs), their antagonists. This study highlights the possibility of piperine as an adjunct treatment for cervical cancer.[33]

The ability of piperine to suppress the production of the cyclooxygenase-2 gene was examined in RAW 264. In a dose-dependent manner, piperine lowers the expression of COX-2 and PGE(2) synthesis. Moreover, it suppresses the luciferase reporter activity of COX-2 and the COX-2 promoter. *Activator protein-1* (AP-1), *Nuclear factor-κB* (NF-κB), and CCAAT/enhancer binding protein (C/EBP) are the primary mediators of piperine's actions. Additionally, piperine significantly decreased activation PMA-induced *Extracellular Signal-Regulated Kinase (*ERK) and Akt and reduced COX-2 activity. This study may reveal the signal transduction pathways involved in the antiinflammatory activity of piperine.[34]

Effect of piperine on p53 gene mRNA expression

Cervical cancer originates from lesions of normal cervical epithelial cells infected with hrHPV and experiences progressive development. hrHPV invading the cervical epithelium causes changes in the host genome that can inhibit tumor suppressor genes and increase oncogene activity.[35] HrHPV encodes 3 transformation proteins, namely E5, E6, and E7. E5 protein can inhibit apoptosis, and maintain continuous replication of epithelial cells.[36] However, its expression disappears after it develops into cancer. The HPV E5 protein is thought to contribute to the early stages or initiation process in the progression of cervical cancer.[37] Oncoprotein E6 can induce cell transformation but has weak activity. However, the effect of E6 can increase the transformation of E7 oncoprotein. Initially, E6 oncoprotein forms a trimer bond with p53 by first binding E6AP, before binding to p53 to form a trimer.[38] In this complex, p53 will be removed by proteosomes in cervical epithelial cells through ubiquitination by E6AP.[39]

The p53 protein is one of the proteins that plays the most important role as a tumor suppressor.[40] It was first discovered in 1979 and identified as a protein involved in the transformation process, is known to be found in cancer in the cell nucleus and binds to the simian virus 40 (SV40) antigen.[41] However, 20 years later, research has proven that p53 acts as a powerful tumor suppressor gene and the most potential target for genetic changes in cancer.[42] Cell cycle arrest during the Gap1 and Gap2 phases, correction of genetic abnormalities, and/or ultimate apoptotic cell death can result from p53 activation.[43] It has been revealed that the gene responsible for the oncogenic nature of cancer is mutant p53.[44]

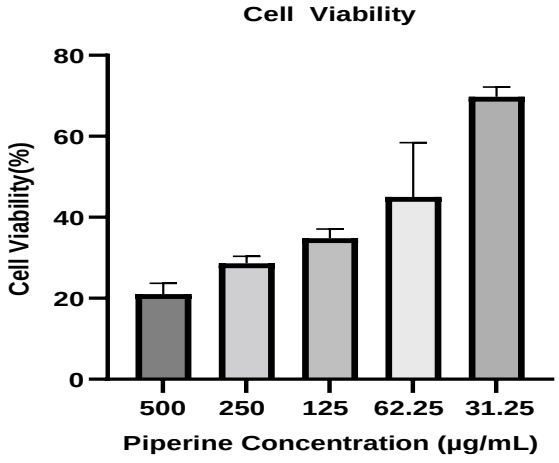

Figure 3: Cell Viability Assay. Cell viability activity of piperine using the MTT method. A piperine concentration of 500 µg/ml can reduce cell viability to 21.01%.

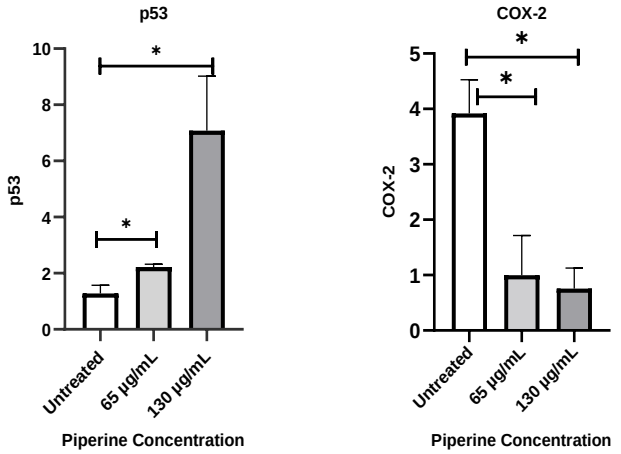

Figure 4: (A) p53 expression. Increased p53 expression after administering piperine concentrations of 65 and 130 µg/ml using the qPCR-Livak method. (B). Cyclooxygenase-2 levels. COX-2 levels were determined using the ELISA method. The addition of piperine can reduce COX-2 levels in HeLa cells (*p< 0.05).

p53 is the gene most frequently mutated in cancer and mutant p53 plays a role in majority of cancer cases. Increasing evidence shows that in addition to losing tumor suppressor function, tumor-associated mutant p53 protein also has activity to promote tumorigenesis, so it is known as "gain-of-function". Mutant p53 may continue increasing in tumors and contribute to the development of malignancy.[45,46]

The function of p53 is replaced by mutant p53 genes in most cancers. Some cancers still have *wild-type* p53 (wtp53), but disruption of p53 regulation can eliminate its functions and play an essential role in cancer development by preserving cells from p53-dependent responses. Thus, restoring p53 function in tumors that retain *wt*p53 is a potential therapeutic target in cancer treatment.[39]

In this study, the presence of piperine in HeLa cervical cancer cells showed increased expression of the p53 gene. This increase in expression occurred along with increasing piperine concentrations (Figure 4). Piperine with a concentration of 130 µg/ml provides optimal results. The same research shows that piperine is thought to activate the p53 protein which acts as the main activator to arrest the cell cycle in the G2/M phase and cause apoptosis in lung cancer A549 cells. Piperine is thought to trigger apoptosis because it can increase Bax protein expression and reduce Bcl-2 expression. These findings reveal that piperine can trigger cell apoptosis mediated by p53, presumably through the mechanism of decreasing Bcl-2 expression and upregulating Bax.[47]

Conclusion

Investigation into the cervical cancer HeLa cell line suggested that piperine has anticancer properties. Piperine decreased the viability of HeLa cells, which prevented their proliferation. Moreover, piperine triggers apoptosis by activating the p53 gene. Furthermore, piperine had the ability to reduce COX-2 levels, which are associated with the growth of cancer. p53 and COX-2 are indicators of the growth of cancer, particularly cervical cancer. The potential of piperine as an anticancer agent can be further explored by focusing on the processes that are significantly involved in cancer progression such as the action of piperine on signaling pathways that control the cell cycle, apoptosis, and angiogenesis, particularly in cervical cancer.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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