A STUDY ON THE ROLE OF ANTIOXIDANTS IN CERVICAL CANCER

ABSTRACT
OF THE
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SUBMITTED FOR THE AWARD OF THE DEGREE OF
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BY
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ABSTRACT

Globally, cervical cancer takes the lives of more than 2,50,000 women each year, particularly in under-resourced areas of low-, middle-, and high-income countries. Options for cancer control and treatment have reached a point that there are interventions for control that could be adopted for virtually every resource and demographic situation. Emerging evidence demonstrates that targeting the tumor proteasome is a promising strategy for cancer therapy. Natural products with proteasome-inhibitory effects, such as green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG), soy isoflavone genistein, and the spice turmeric compound curcumin, have been studied alone and in combination with traditional chemotherapy and radiotherapy against various cancers. As a variety of antioxidants and chemopreventive agents are cytotoxic to cancer cells, thus, there is also interest in developing these natural compounds as potential chemopreventive agents.

As the active role-played by reactive oxygen species (ROS) in the cervical cancer is well established, thus an attempt was made in the present study to probe natural compounds having antioxidant properties in arresting ROS in cancer cells. Various workers on cervical cancer HeLa cell lines have extensively carried out such a study, but no or little work has been done on cervical cancer monocytes. Thus, comparative efficacy of natural antioxidants was carried out on cervical cancer monocytes versus HeLa cell lines. Thus, in the present study, EGCG – a natural antioxidant and anti-mutagenic polyphenolic agent was employed to explore the comparative potential chemopreventive mechanism in cervical cancer monocytes versus cervical cancer cell lines i.e. HeLa cell lines.

The most striking finding of the present study is the model system involving monocytes and biopsies from cervical cancer patients showed high magnitude anti-inflammatory as well as apoptotic effect of EGCG (0-100 μg/ml), while on the other hand, EGCG (0-100 μg/ml) exerted high magnitude anti-inflammatory but low apoptotic effect on HeLa cell lines.

Glutathione directly reacts with ROS, and GPx catalyzes the removal of hydrogen peroxide. Decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms. In the present study, a decline in GPx activity

was observed in cervical cancer monocytes and cervical HeLa cells that were untreated or treated either with H_2O_2 thereby correlating with earlier reports that substantial amounts of ROS are being generated in cancerous cells due to cellular activation. Enhancement of GPx activity in cervical cancer monocytes and cervical HeLa cell cultures after addition of NAC, a precursor of in vivo antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Surprisingly, here slightly augmented GPx activity was observed when EGCG was co-cultured instead of NAC, indicating EGCG to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in cancerous cell. Thus, our study shows enhanced GPx activity by EGCG, which correlated inversely with the downregulation of TNF-alpha, IL-1 and IL-6 protein expressions and ROS in cervical cancerous cells.

Although the chemopreventive effect of EGCG has been extensively studied by a number of workers in cancerous cell lines, but its apoptotic effect and mechanism in biopsies and monocytes of cervical cancer patients still remains to be properly understood. Thus, in the present study, we show the mechanism of EGCG mediated apoptosis in monocytes of cervical cancer patients, which involved activation of caspase-3, the executor of apoptosis and cleavage of PARP - a specific marker of apoptosis as well as suppression of IL-6. EGCG was highly effective in inhibiting the viability of cervical cancer monocytes by PARP cleavage in response to activation of caspase-3. This resulted in the loss of normal PARP function, which irreversibly commits the cell to die.

Our present study demonstrated the activities of caspase-3, caspase-8 and caspase-9 in cervical cancer monocytes were activated by EGCG, indicating that both death receptor-related apoptotic pathway and the mitochondria-related pathway were activated. On the contrary, suppression or reduction in cell viability was inhibited by inhibitors of caspase-3, 8 and 9 namely Z-VAD-FMK, Z-IETD-FMK and Z-LEHD-FMK respectively, which specifically blocked PARP cleavage.

We further observed that either specific caspase-8 or caspase-3 inhibitor blocked the EGCG-induced apoptosis by an appreciably high magnitude, whereas the caspase-9 inhibitor only partially inhibited it. This implies that apoptosis

THESIS

induced by EGCG in cervical cancer monocytes were initiated by a pathway involving the activation of caspase-8, leading to the activation of caspase-3 and the cleavage of PARP. The activation of caspase-9 is probably a secondary consequence of the activation of caspase-8 through the cross communication between the two apoptotic pathways.

Cytosmears from non-neoplastic ectocervical biopsy cultures with EGCG also showed only keratinization promoting effects of EGCG and no apoptotic cells could be seen. On the contrary, cytosmears from cervical cancer biopsy cultured with EGCG showed distinguished nuclear condensation and rounded contours with deep eosinophilia of cell cytoplasm, thereby indicating for EGCG-induced apoptotic. Histopathological sections from EGCG treated test samples also showed evidences of apoptosis, showing one apoptotic cell per 2-3 high power fields to more than one dispersed apoptotic cells in a single high power field. Occasionally, apoptotic cells were seen in groups implying foci of apoptotic changes.

Next, we also report suppression of high levels of IL-6 in cultures of cervical cancer monocytes by EGCG to be dose-dependent. In accordance with its potential role in cancer progression, various types of cancer show elevated level of IL-6, and that, the strong ability of malignant cells to escape immune surveillance is a well known phenomenon. The anti-tumor immune response has been reported to be regulated by several factors, which includes cytokines produced by tumor and other cells of tumor stroma. It seems likely that the local cytokine microenvironment, acting on tumor cell or on the adjacent cells, can either block or facilitate tumor growth, and that proinflammatory cytokines strongly influence the immunologic state.

Treatment with EGCG increased the proportion of cells in the G_1 phase of the cell cycle and induced apoptosis. The results in the present study indicated EGCG to decrease the anti-apoptotic Bcl-2 protein expression, and activation of Caspase-3, 8 and 9, suggesting that EGCG induces apoptosis via a both cellular as well as mitochondrial pathway, although the major pathway was cellular. The observations made in the present study demonstrate that EGCG, a major component of green tea, possesses cervical cancer cell growth inhibitory

properties. It is likely that EGCG inhibits cancer cell growth through many different regulatory pathways, along with apoptosis and cell cycle arrest. The results of the present study are consistent with EGCG inhibiting cell growth and inducing apoptosis in cervical cancer biopsies and HeLa cell lines. The cytotoxic properties of EGCG seemed to be more effective against cervical cancer monocytes than HeLa cells. At this time, there is no explanation for these differences and more experiments are needed for further analysis. PARP-cleavage and FITC-Annexin assays in the present study clearly confirmed apoptosis induction in EGCG-treated cervical cancer cells. EGCG-induced apoptotic death in cervical cancer monocytes was mediated in part by activation of caspase-3.

In summary, EGCG, a natural antioxidant, is a potent inducer of apoptosis in monocytes, biopsies of cervical cancer patients as well as HeLa cell lines, and that, induction of EGCG-mediated apoptosis involved activation of caspase-3 / CPP32, caspase-8 and caspase-9 as well as IL-6 suppression. The apoptotic as well as anti-inflammatory / anti-proliferative effects of EGCG was more prominent in cervical cancer monocytes than in HeLa cell lines. Why EGCG is more effective in cervical cancer monocytes than in HeLa cell lines warrants further in-depth investigation. It is hoped that in view of the above aspects, whether EGCG can be promising candidate for the chemo-prevention of cervical cancer might be an interesting topic for further investigation.

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Certificate

This is to certify that **Mr. Sohail Hussain** has carried out this work for Ph.D. thesis on the topic entitled "A study on the role of antioxidants in cervical cancer" under my supervision. To the best of my knowledge, this is Mr. Hussain's original work, which is suitable for the award of **Ph.D.** degree in **Biochemistry** of the Faculty of Medicine, Aligarh Muslim University, Aligarh, India.

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(Sohail Hussain)

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALP	:	Alkaline phosphatase
ATP	:	Adenosine triphosphate
ACS	:	American Cancer Society
AICR	:	American Institute for Cancer Research
BSA	:	Bovine serum albumin
BPB	:	Bromophenol blue
CBB	:	Coomassie brilliant blue
CDC	:	Centers for Disease Control
CIN	:	Cervical Intraepithelial Neoplasia
DC	:	Dendritic cell
DEPC	:	Diethyl pyrocarbonate
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
DTT	:	Dithiothrietrol
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked immunosorbant assay
HPV	:	Human papillomavirus
IARC	:	International agency for research on cancer
GPx	:	Glutathione peroxidase
GSH	:	Glutathione

GSSH	:	Oxidized GSH
H_2O_2	:	Hydrogen peroxide
IC_{50}	:	Concentration for 50% inhibition
IgG	:	Immunoglobulin G
IFN-γ	:	Interferon gamma
IL	:	Interleukin
LPS	:	Lipopolysaccharide
LEEP	:	Loop Electrosurgical Excision Procedure
LPO	:	Lipid peroxidation
IκB	:	Inhibitor of NF-kappa B
M-CSF	:	Macrophage- Colony Stimulating Factor
ml	:	Millilitre
mM	:	Millimolar
MN	:	Monocyte
MTT	:	3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium-bromide)
MMP	:	Matrix metalloproteinase
NAC	:	N-acetylcysteine
NF-κB	:	Nuclear factor kappa B
NO	:	Nitric oxide
NADPH	:	Reduced β-nicotinamide adenine dinucleotide phosphate

NHS	:	Normal human sera
nM	:	Nanomolar
oATP	:	Oxidized ATP
PAGE	:	Polyacrylamide gel electrophoresis
PBMC(s)	:	Peripheral blood mononuclear cell(s)
PBS	:	Phosphate buffered saline
PMSF	:	Phenylmethylsulphonyl floride
p-NPP	:	Para-nitro phenyl phosphate
PAH	:	Polycyclic aromatic hydrocarbon
PMSF	:	Phenylmethylsulphonyl fluoride
pRb	:	Retinoblastoma protein
PVDF	:	Polyvinylidine Fluoride
RANK	:	Receptor activator of NFκB
RNA	:	Ribonucleic acid
ROI	:	Reactive oxygen intermediates
ROS	:	Reactive oxygen species
RPMI	:	Roswell Park Memorial Institute
rRNA	:	Ribosomal RNA
SDS	:	Sodium dodecyl sulphate
SNP	:	Sodium nitroprusside
sTNF-α	:	Soluble TNF- α
STIs	:	Sexually transmitted infections

TEMED	:	N,N,N',N'-tetramethylethylene diamine
TGF-β	:	Transforming growth factor beta
TNF α	:	Tumor necrosis factor alpha
TNFR	:	TNF receptor
TRAF	:	TNFR associated protein
TRAP	:	Tartarate resistant acid phosphatase
Tris	:	Tris (hydroxymethy) aminomethane
TS	:	Tumor suppressor
uPA	:	Urokinase-type plasminogen activator
WCN	:	Women's Cancer Network
WCRF	:	World Cancer Research Fund
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar

INTRODUCTION

In 1974, the Government of United Sates declared a "war on cancer" with the hope and anticipation that death from cancer be greatly reduced by the end of century. In spite of advances in cancer research and treatment, it still remained a major global health issue. According to American Cancer Society (ACS) on cancer statistics, cancer of the lung, prostrate, colon, rectum continues to be most common causes of cancer deaths (Jemal et al., 2002).

Cancer is the leading cause of the death in the world and second in the United States, with one out of four deaths being cancer related World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR, 1997). More than 1 million new cases of cancer and over 500,000 deaths occur each year in the U.S. due to cancer (Breslow, 1997). According to the American Cancer Society, in 2006 an estimated 1,399,790 males and females in the United States were diagnosed with cancer (American Cancer Society, 2006) Moreover, It is projected that 14.7 million people globally will be diagnosed with cancer by the year 2020 (WCRF/AICR, 1997).

Carcinogenesis is a complex, multi-step process in which signal transduction pathway involved in various normal cellular physiology are qualitatively or quantitatively altered (Vogelstein and Kinzler, 1993). Under normal conditions, these tightly controlled excitatory or inhibitory pathways regulate cellular functions like cell division, differentiation and senescence. Cancer is a group of diseases characterized by uncontrolled growth and proliferation of abnormal cells causing a total loss of cell function (American Cancer Society, 1998). Cancer was considered to be an unpredictable disease because treatments, even aggressive invasive procedures, often come with unpredictable outcome and reoccurrence (Gerster, 1993) For this reason it is important to emphasize the implications of education to prevent cancer.
Cancer is the result of both genetic and environmental factors, which may act together or in sequence to initiate carcinogenesis (American Cancer Society, 1998). In addition to elucidating some of the genetic and environmental causes of cancer, currently researchers has also engaged to uncover the mechanisms by which carcinogenesis occurs.

In this era of modernization and technology, large number of chemicals enters into our environment through occupational exposure, automobiles exhaust, pesticides, industrial wastes and contamination of food and water. These chemicals exist in the

environment in a relatively stable condition until taken in by an exposed individual and activated. Thus, these chemicals appear to pose a great concern and threat to mankind. Polycyclic aromatic hydrocarbon (PAH), nitrosamine, mycotoxins, pesticides and metallic contaminants such as arsenic and cadmium are some of xenobiotics that can enter in our food chain during processing, storage, preservation and cooking. According to International Agency for Research on Cancer (IARC), now there are about 88 chemicals, with sufficient evidences of carcinogenic potential in human (IARC, 1994). Physical agent such as ultra-violet rays (UV) and the ionizing radiation (X-rays, gamma-rays) are known to be hazardous in nearly all tissue or organs of human or experimental animals depending upon the radiation dose and exposure schedule (IARC, 1992). The scenario is becoming alarming with the application of nuclear technology in science, medicine and industries as well as in the expanding use of these radiations in diagnosis and therapy of the disease itself (Trichopoulus et al., 1996).

Biological agent, such as retrovirus, bacteria and parasitic infections, the oncogenic virus such as papillomas viruses, feline- leukemia viruses and bovine-leukosis virus are linked with increased incidence of various types of Neoplasia. The Epstein-Barr virus has shown to be implicated with the occurrence of Burkitt's lymphoma, Hodgkin and non- Hodgkin lymphoma and pharyngeal carcinoma (Cordova et al., 2003; Young and Murray, 2003). Certain bacteria like *Helicobacter pyroli* are also implicated in development of cervical, esophageal, head, neck and stomach cancer (Peto, 2001).

Various factors that can play crucial role in establishment of cancer are host (internal) factors comprised of hormonal disturbance, immune functions and inherited predisposition of certain cancers. The genetic manipulation such as activation of cellular proto-oncogenes and alteration in tumor suppressor genes also results in aberrant proliferation of cells (Ames et al., 1995; Peto et al., 2001). Many cancer chemotherapeutic drugs, particularly Vinca alkaloids, Alkylating agent, immunosuppressive agent and certain cyclosporins are also reported to increase cancer risk. (Ames et al., 1995; Trichopoulus et al., 1996; Cheuvenet et al., 2003).

According to famous British pathologist, R.A. Wills (1960), tumor can be defined as, "An abnormal mass of tissue the growth of which is in excess and uncoordinated with the normal tissue and persists in the same excessive manner, even after the cessation of the stimuli that evoked the change. "The six major alterations in cell physiology that collectively dictate malignant growths are (Hanahan and Weinberg, 2000) are:

- Self-sufficient in growth signals.
- Insensitivity to growth inhibitor signals.
- Evasion of apoptosis (programmed cell death).
- Limitless replicative potentials
- Sustained angiogenesis and adjoining tissue invasion.
- Metastasis.

Each of these physiological changes acquired during tumor development represent the successful breaching of an anticancer defense mechanism. These six features are shared in common by most types of human and animal tumors. Aberrant DNA causes cancer. First, genetic mutations in critical genes can lead to tumors. In fact, in approximately one-half of human tumors, mutations exist in the tumor-suppressor gene, p53. This is important because inactivation of the p53 gene allows for uncontrolled cell division. Another mechanism involved in carcinogenesis is DNA lesions. This refers to damaged bases or chromosome breaks that have a certain probability of producing a mutation upon cell division. The "effectiveness" of a particular lesion depends on the rate of excision by DNA repair enzymes, and on the probability that a mutation will occur when the cell divides. Cellular apoptosis or "programmed cell death" provides the body with a mechanism for removal of cells with damaged DNA. The effect that a particular insult has on the cell is dependent on the level of each of these defenses, which in turn are actually dependent on the past history of exposure. These defenses can also be disabled by lack of micronutrients such as antioxidants in the diet.

At a molecular level, antioxidant deficiency is considered to be a risk factor for cancer, as it can lead to "unrepaired or misrepaired endogenous oxidative DNA damage" (Sahu and Washington, 1992) and membrane lipid peroxidation (LPO). Free

radicals may mediate many events in carcinogenesis by producing damage to DNA, lipids, proteins, and carbohydrates. External sources of free radical include polycyclic aromatic hydrocarbons in food, polluted air, cigarette smoke, background radiation, and internal sources include oxidative transformations in prostaglandin synthesis, redox cycling of quinones, and oxidative phosphorylation (Block, 1992)

Still another mechanism that may underlie for cancer is the excess free radical production in chronic infection and inflammation. Leukocytes and other phagocytic cells combat bacteria, parasites, and virus-infected cells by destroying them with nitrogen oxide and superoxide. Both of these compounds can lead to formation of peroxynitrite, hypochlorite, and hydrogen peroxide, all of which are mutagenic oxidizing agents. So, while these oxidants protect the body from immediate harm, they can cause oxidative damage to DNA, DNA mutation, and chronic cell death with compensatory cell division. All of these processes then contribute to cancer development.

Cancer, one of the three major diseases in the U.S, is becoming more common in the Western world and also in many populations of developing countries. With modern medicine, some treatments have met with success, but they have been very expensive. It is becoming evident that the most cost-effective control of these diseases is through prevention. This is mainly achieved through the diet and other lifestyle changes (Argiles et al., 1998). In addition, cancer chemoprevention is a new field that has great potential to affect cancer incidence rates. Cancers can be prevented through lifestyle modifications; dietary measures have been estimated to have the potential to prevent about 30-40% of all cancers worldwide (WCRF/AICR, 1997). Five of the ten leading causes of death for Americans are associated with diet: heart disease, some cancers, stroke, diabetes, and atherosclerosis (Glanz, 1997) The World Cancer Research Fund now recommends a plant-based diet rich in a variety of fruits, vegetables, and legumes along with reduction in the amount of processed starchy staple foods (WCRF/AICR, 1997).

Strong epidemiological and experimental evidence inversely correlate plant-based diets with the incidence of several types of cancers (WCRF/AICR, 1997). Specifically, the North American diet, associated with the highest incidence of

cancer, correlated to high levels of saturated fat and low levels of plant-based foods. The lowest incidences of cancer were observed in populations with Mediterranean and Asian diets that were characterized as having a lower content of saturated fats and a higher rate of consumption of plant based foods such as legumes, vegetables and fruits (Simopoulos, 2001). It has been suggested that several distinctive phytochemicals (vitamins, fibers and micronutrients) may be responsible for the protective effect of fruits and vegetables (Steinmetz and Potter, 1996: Flood et al., 2002; Uzcudun et al., 2002; Littman et al., 2001). Conversely, another well-supported hypothesis is that high consumption of fruits and vegetables is protective against many cancers (Steinmetz and Potter, 1996). Block (1992), cites approximately 120 of 130 studies conducted that show a statistically significant reduction of risk of cancers of the lung, larynx, esophagus, oral cavity, pancreas, stomach, rectum, cervix, colon, ovary, endometrium, breast, and bladder with increased antioxidant intake. More recently, scientists have been interested in investigating micronutrients, carotenoids and polyphenolic compounds, because of their potential as inhibitors of tumor growth.

In in-vitro studies, phytochemicals have been found to inhibit leukemia cancer cell growth and induce apoptosis at different phases. Carotenoids have been shown to cause a G_0/G_1 arrest in HL-60 myeloid leukemia cells (Palozza et al., 2002; Palozza et al., 2001; Amir et al., 1999). In addition, certain polyphenols have been responsible for a G_0/G_1 arrest in CEM leukemic T-cells (Yoshida et al., 1992) and a G_2 arrest in HL-60 myeloid leukemic cells (Kang and Liang, 1997; Palozza et al., 2002) demonstrated that carotenoids can induce apoptosis in HL-60 cells at the same concentration required for cell cycle arrest. For polyphenols, in general, there is a dose and time dependent induction of apoptosis in human leukemia cells (Csokay et al., 1997; Ferry-Dumazet et al., 2002).

One important factor in the prevention on cancer is to assess the risk for cancer, which can be influenced by exposure to radiation, chemicals, hormones, infectious agents, chemicals, genetics and nutrition. It is expected that about one-third of the number of cancer deaths will be associated with poor nutrition, physical inactivity and other lifestyle factors that can be changed (WCRF/AICR, 1997)

ANATOMY OF CERVIX

The cervix is situated in the lower part of the uterus and forms a narrow canal between the vagina and the uterine body cavity (Fig. 1). Cervical squamous epithelium consists of stratified epithelium (multiple layers typically 10-15 cell layers thick) and extends from the endocervical canal to the epithelium of the vagina (fig. 2). The layers of the epithelium are divided based on increasing maturation away from the basement membrane. Squamous (cubiodal) cells close to the basement membrane have a larger nuclear to cytoplasmic ratio than other squamous cells of the cervix; they are separated from the stroma by the basement membrane (fig.3). Cells undergo maturation as they move from the basal layer to the epithelial surface where they become more flattened and their cell volume increases. Tight junctions are dominant in the superficial layer, which reduces the extracellular space. Cell attachments in less mature cells consist of microfilaments that hold the cells together (Walker et al., 2003). Cervical cancer arises in the basal cells of the cervical epithelium at a site called the transformation zone. This zone is sensitive since it forms a border between endocervical cylindrical epithelium and multilayered ectocervical squamous epithelium (Burghardt et al., 1983).

There are two main types of cervical cancer called squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma derives from squamous cells that represent the majority of the cells in the lower cervix. This is the most common form of cervical cancer and constitutes 75% of all cases (Vizcaino et al., 1998). Adenocarcinoma which arise from cervical glandular cells are more rare and represent 10-15% while cervical cancer with other or without specified histology, make up the remaining 10-15%. (Euscher et al., 2001; Vizcaino et al., 1998).

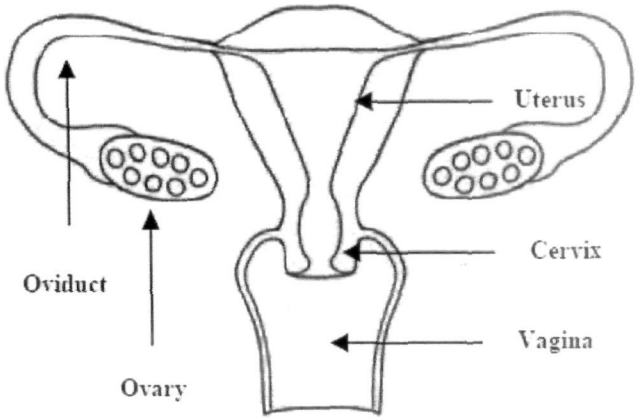

Figure 1: The Basic anatomy of female reproductive organ.

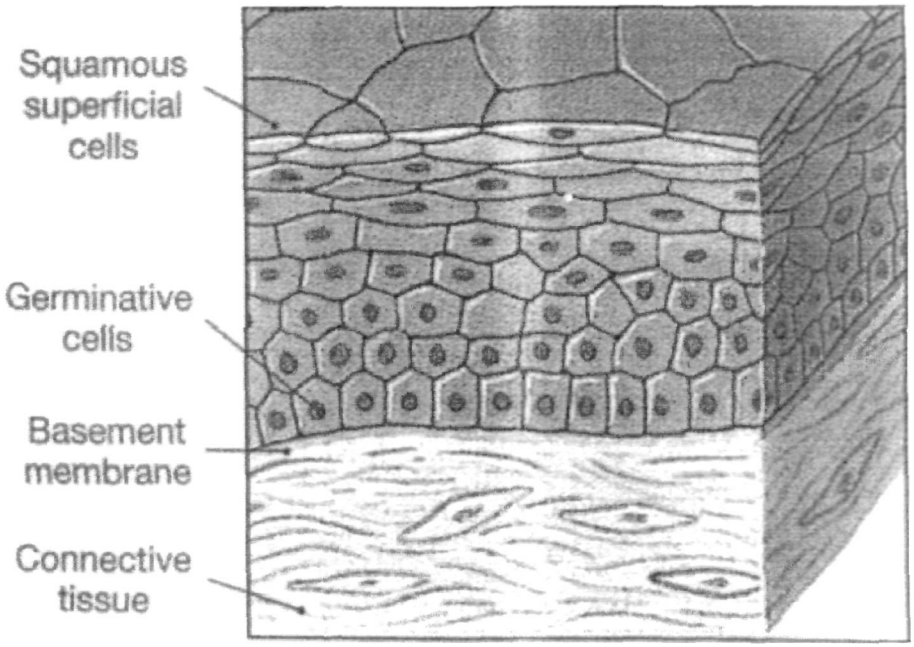

Figure 2: Epithelium structure of the cervix.

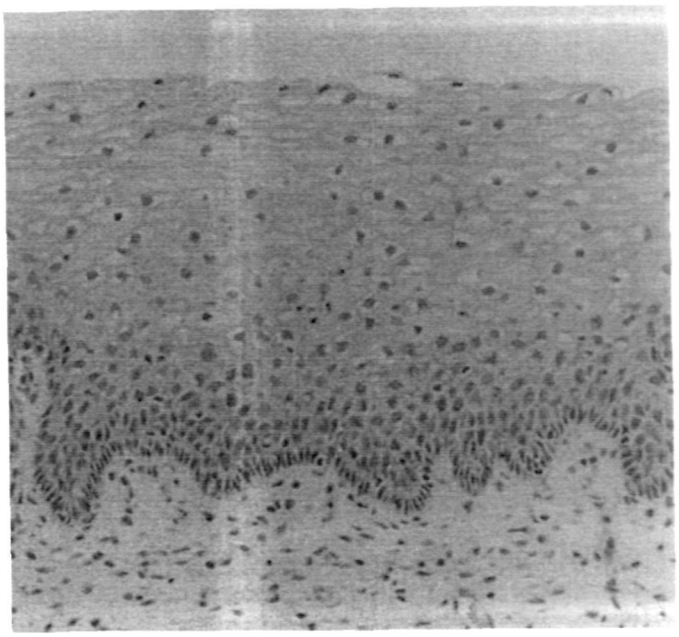

Figure 3: Histological image of cervical epithelium

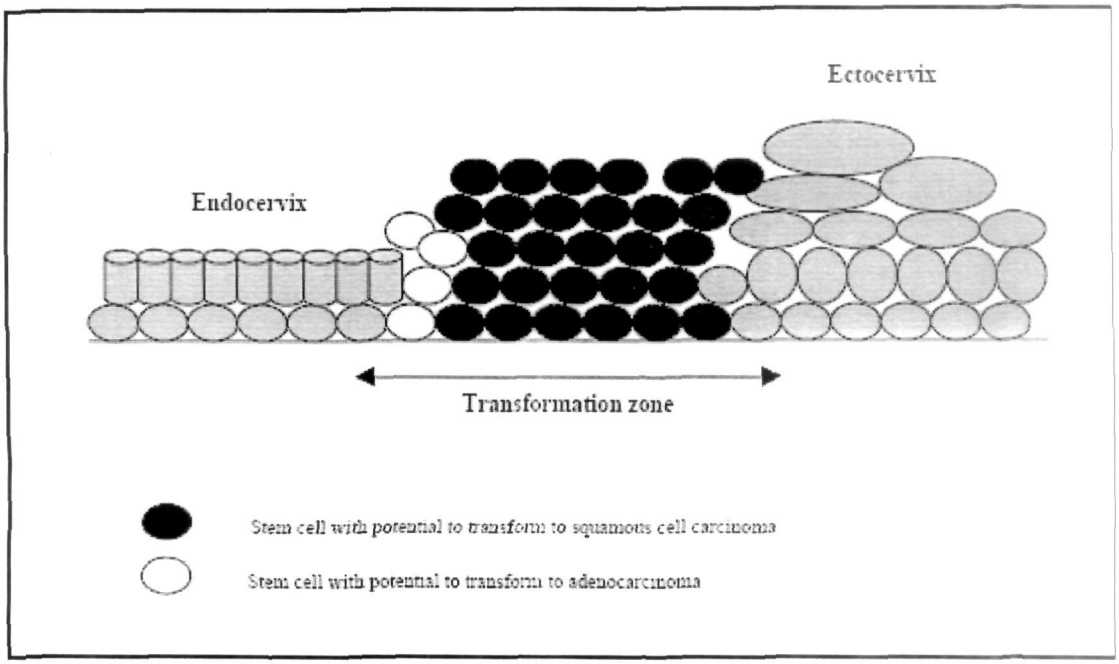

Figure 4: Schematic illustration of the cervical epithelium. The fragile transformation zone forms a border between endocervical and ectocervical epithelium.

Cervical carcinoma develops progressively through different stages from normal epithelium to cervical cancer in situ. The final step, invasive cervical carcinoma, is characterized by adhesion to and penetration of the basement membrane by the primary *in situ* tumor. After penetration, the cancer has potential to spread and form metastatic daughter tumors at other sites of the body.

Histopathology of Cervical Cancer

When HPV infection was detected in the epithelium, it may span a spectrum ranging from normal to condyloma planum (flat warts) or acuminatum (acuminate warts), to intraepithelial neoplasia and invasive cancer. Disease may occur at a wide variety of sites, including the conjunctiva, oral cavity, keratinized skin, and anogenital mucosa. HPV infection occurs in the basal cell layer of the epithelium, which constitutes the dividing cell population (Fig. 4) (Pfister, 1984; Howley et al., 1986). The importance of this observation is that the basal cells may then constitute a continuous reservoir of HPV DNA, and the viral genome is then partitioned to all daughter cells when the cells divide. HPV gene expression appears to be limited in the basal cell layer (Durst, 1992). However, as the cells begin to mature and rise through the epithelium, the viral DNA molecules continue to reproduce themselves in the absence of basal cell division, resulting in a higher HPV DNA copy number per cell. This process continues such that the highest DNA copy numbers per cell are found in the most mature cell layers, as indicated by *in situ* hybridization of HPV-infected tissues (Durst, 1992, Fig. 2). As a result of increased viral copy number and/or alterations in keratinocyte factors that modulate HPV gene expression within mature keratinocytes, viral activity increases as the cells mature.

Epidemiology

In the United States, 85% of the annually reported communicable diseases are sexually transmitted infections (STIs), with the large majority consisting of those affecting men and women during their reproductive years. Eighty-six percent of these STIs occur among persons in the 15-29 year range. Human papillomavirus (HPV) was the most commonly reported STI in North America in the years 1998- 1999 and persistent cervical HPV infection was found to be a precursor for (as well as the most significant cause of) cervical cancer (Brackbill et al., 1999; Division of STD

Prevention 1997; Moscicki et al., 1989; Smith et al., 2003; Taira et al., 2004) In 1999, Cervical cancer posed a significant public health concern, due to the fact that it was the second most prevalent cancer among women in the United States – after breast cancer – and the third most prevalent cancer among women worldwide, with a death rate of approximately 50%. One year later, it was found to be the second-leading cause of cancer deaths in women worldwide, with HPV having been determined as the primary etiologic agent in more than 95% of cervical cancers (Parkin et al., 1999; Pisani et al., 1999; Stoler, 2000). In 2000, the estimated prevalence of HPV in the United States was 15% to 20% (Gerhardt et al., 2000). In 2004, the American Cancer Society estimated that 10,520 women would be diagnosed with cervical cancer and 3,900 women would die from the disease (Centers for Disease Control [CDC], 2004a). Genital HPV infection is widespread, estimates show that as many as 75% of women will become infected with one or more of the sexually-transmitted HPV types during her lifetime (Baseman and Koutsky, 2005)

In addition, roughly 80 percent of individuals who acquire sexually transmitted HPV infections clear the virus rapidly without ever developing warts or other clinical symptoms (Dunne and Markowitz, 2006). Uterine cervical tumors kill several thousand people each year and represent a major disease burden in women worldwide (Schiffman and Castle, 2003). The presence of HPV in the vast majority of all cervical cancers worldwide offers the highest attribute reported for a specific cause of any of the major human cancers (Walboomers et al., 1999). Globally, approximately 500,000 new cases are identified yearly (World Health Organization, 1999).

Screening programmes

The incidence of cervical cancer is generally low in developed countries with age-standardized rates less than 14.5 per 100,000 (Parkin et al., 2002). The main reason for the low incidence rates and decreased mortality of cervical cancer is the long-standing screening programmes initiated in the 1960's and 1970's. The birth of the screening for pre-stages of cervical cancer took place in year 1943 when Dr. Papanicolaou (Pap) invented a new histological staining procedure of vaginal smears. This new assay made it easier to detect abnormal cell differentiation in the cervix and

thereby also to detect lesions in the cervix and uterus (Papanicolaou, 1943; Frisch, 1989).

There are different Pap smear diagnoses, which reflect distinct types of morphological lesions observed during cancer development. The nomenclature describing the cytological status of a smear depends on the classification system used. There are four main systems for Pap smear diagnosis. The Papanicolaou system was presented in 1954 and is based on certainty that malignant cells are present in a vaginal smear (Kurman et al., 1994). This system was followed by a descriptive system in 1968 based on morphological criterias (Burghardt, 1973). The cervical intraepithelial neoplasia (CIN) system was introduced in 1978 and is applied by cytologists although derived from histology (Nguyen and Nordqvist, 1999). Finally, the Bethesda system was introduced in 1988 with the aim to increase the reproducibility within and between laboratories by reducing the number of categories. (Kurman et al., 1994; Nguyen and Nordqvist, 1999).

The cytological screening for cervical cancer has generally been a success. Countries with widely accessible screening have experienced a 40-80% decrease in cervical cancer related mortality since the screening started. (Sigurdsson, 1999; Bergstrom et al., 1999). At present, women diagnosed with invasive cervical cancer in developed countries are mostly the ones who are screened irregularly or failed to attend screening (Geirsson, 1986; Janerich et al., 1995).

The origin of cervical cancer (HPV)

In 1843, Rigoni-Stern, an Italian physician in Rome, made the fist observation pointing to an infectious component in the etiology of human cancer (Hausen and De Villier, 1994b). He noted a difference between the calibrated nuns and prostitutes with respect to cervical cancer. The observation that prostitutes had much higher incidences of cervical cancer suggested that cervical cancer was sexually transmitted (Hausen and De Villier, 1994b). Recent epidemiological studies have confirmed this observation, and sexual activity (both male and female) represent the prime risk factor for cervical cancer (Bosch et al., 2002; Bosch and De Sanjose, 2002). It had been suggested that the Herpes Simplex type 2 (HSV 2) could be the causative agent. however Zur Hausen, were unable to demonstrate HSV DNA in the biopsies of

cervical cancer (Hausen, 1977). The establishment of cloning techniques allowed for the recognition of the first genital HPV type, HPV type 6 isolated from genital wart (Gissmann and Hausen, 1980). This was followed by the identification of another low risk HPV type, type 11 (Gissmann et al., 1982) as well as the high HPV type HPV 16 (Durst et al., 1983) and HPV 18 (Boshart et al., 1984).

During the past 20 years, it has been proved beyond reasonable doubt that certain types of sexually transmitted human papillomaviruses (HPVs) are necessary for the development of cervical cancer (Hausen, 1996, Bosch et al., 2002). HPV was found in close to every cervical cancer biopsy and the World Health Organization (WHO) has recognized cervical carcinoma as the first cancer to be 100% attributable to infection (Bosch et al., 2002; Walboomers et al., 1999).

Morphology of Human Papillomavirus

Papillomaviruses are common and widespread among higher vertebrates but exhibit strict species and tissue specificity. The viruses belong to the family of *Papovaviridae* (De Villiers, 1994, Hausen, 1994) and are characterized by a small, enveloped 72nm capsomere capsid and 55nm diameter with an icosahedral symmetry containing the viral genome (Klug and Finch, 1965, Klug, 1965). HPVs are relatively stable and because they have no envelope they can remain infectious for months in a moist environment. (Orth et al., 1978).

Human papillomaviruses fall into two broad groups: low risk types, associated with cervical condylomas and CIN 1; and high risk types that are associated with cancer. Using data from 11 different case-control studies conducted in many areas of the world, the list of carcinogenic HPV types now includes HPV 16, 18, 31, 33, 35, 45, 51, 52, 58 and 59 (Munoz, 2000).

The clinical manifestations of these viruses are warts (papillomas and condylomas), which are small epithelial tumors. The majority of these are benign tumors that regress spontaneously in immunocompetent individuals. Modern classification of HPV types is based on DNA sequence differences within the coding

regions of the early proteins E6 and E7 and the late protein L1. Different genotypes have <90% homology in these regions.

HPV genomes are maintained as episomes in the nucleus of normal infected cells. However, in cervical intraepithelial neoplasias (CINs) and even more frequently in cancers, HPV genomes are found integrated into the human chromosomes (Badaracco et al., 2002; Nagao et al., 2002; Peitsaro et al., 2002). During integration of the viral genome, a portion of the E2 gene is frequently deleted.

HPV and Cancer development

There are approximately 40 mucosal HPV types and these are frequently found in the anogenital tracts of men and women. Mucosal HPV types are often referred to as low-risk types and high-risk types, based on their carcinogenic potential. HPV16 is the most prevalent among the oncogenic HPV types and is together with HPV18 the type most commonly associated with invasive cervical cancers (Bosch et al., 1995; Clifford, 2005), these two high-risk types have been recognized by the WHO as carcinogenic agents for humans (Bosch et al., 1995, Bosch et al., 2002). HPV16 and HPV18 are together with HPV31 found in approximately 75% of all cases of progressive cervical cancer (Bosch et al., 1995).

The main mechanisms behind the steps toward cancer involve the HPV E6 and E7 proteins and are shown in (Figure 5). In the early 1970's it was recognized that E6 and E7 turn cells into mutator phenotypes by hindering anti-cancer pathways (Loeb et al., 1974). Since then it has been shown in tissue culture and animal models that E6 and E7 have ability to immortalize and transform epithelial cells (Hawley-Nelson et al., 1989; Halbert et al., 1991; Munger et al., 2002). The means of damaging the human cell division regulation system and destroying the defense against tumor development by E6 and E7 are highly efficient. Transformed cells are prone to accumulation of mutations and chromosomal abnormalities, insensitive to anti-proliferative stimuli and brakes, which normally act to control cell division, and self sufficient in growth signals (Hanahan and Weinberg, 2000). Genomic instability

is a common characteristic in many progressed epithelial cancers (Lengauer et al., 1997; Lingle et al., 2002; Pihan et al., 2003).

The key action of the high-risk HPV E6 protein is inhibition of the tumor suppressor protein 53 (p53). In contrary to low-risk HPV, the high-risk E6 binds to p53 and promotes proteolytic degradation via the ubiquitin proteolysis pathway (Scheffner et al., 1990; Werness et al., 1990; Huibregtse, et al., 1991; Scheffner et al., 1993; Thomas et al., 1996; Hengstermann et al., 2001). The p53 protein works in the G1-S interphase and activated in stressful conditions for the cell such as DNA damage, hypoxia and low levels of ribonucleoside triphosphate (Linke et al., 1996). Two major events are induced by p53, (1) cell growth arrest in the G1 phase followed by DNA repair and survival or, (2) apoptosis (Figure 5). The consequence of p53 removal in the cell by the E6 protein is insensitivity to DNA damage and evasion of apoptosis. Mutations can accumulate when this DNA damage repair mechanism is negatively affected, an event similar to that in other human cancers.

The HPV E7 is the major transforming protein with the same function in both low and high-risk types. The oncogenic potential of the E7 protein results from interference with the tumor suppressor (TS) retinoblastoma protein (pRb) (Dyson et al., 1989). Genes required for DNA replication and entry into S-phase is activated by the E2F-family of transcription factors. The pRb protein acts as a break in cells about to progress into S-phase by binding to E2Fs and E7 can bind and inactivate pRb (Dyson et al., 1989). Binding of pRb to the E7 oncoprotein results in release of E2Fs, which stimulate cell cycle S-phase entry and lead to cell replication (Munger et al., 2001). Low-risk HPV E7 proteins have affinity to pRb but the E6 proteins are unable to interfere with p53 (Havre et al., 1995). It is currently unclear how low-risk HPV types overcome the p53-mediated apoptosis but the search for further cellular partners of E6 and E7 proteins is ongoing.

Factors directly related to HPV

In most women HPV infections lasts no more than a couple of years (Evander et al., 1995; Moscicki et al., 1998; Ho et al., 1998). Yet, some individuals are not able to clear infection and the virus persists many years in the cervical epithelial basal

layer. Prolonged duration of a HPV infection, also called persistence, is strongly associated with risk of developing cervical cancer. The persistence of an HPV infection, particularly high-risk types, is associated with higher risk for abnormal Pap smear (Ho et al., 1998) malignant cervical epithelial neoplasia (Ho et al., 1995; Schlecht et al., 2001), and invasive carcinoma (Wallin et al., 1999). The relative risk (RR) of incident squamous intraepithelial lesion (SIL) is about ten times higher for persistent infections with oncogenic HPV type relative to non-oncogenic, low-risk HPV types. Especially for HPV16 and HPV18 there is a strong relationship between persistence and SIL (Schlecht et al., 2001). Persistence is now a widely established risk marker for the disease but the number of years required for development of cancer is not known exactly. Persistence of oncogenic HPV is also associated with high viral load (Beskow et al., 2002; Dalstein et al., 2003).

Factors indirectly related to HPV

HPV infections are transmitted through sexual contact and the probability of exposure to HPV is therefore directly related to an individual's sexual activity. Many parameters regarding the sexual behavior have been linked to cervical cancer. However, most of them are just indirect measures of HPV exposure and are therefore not direct risk factors for cervical cancer. For instance, the risk of HPV infection increases with earlier age at sexual debut, number of sexual partners, frequency of intercourse and anal sex (Koutsky et al., 1992, Schiffman et al., 1993, Andersson-Ellstrom et al., 1996). In other words, there must be some further risk factors or cofactors that take part in the process of cervical carcinogenesis.

Cigarette smoking

Although studies show no direct association between smoking and cervical neoplasia (Zaninetti et al., 1986) but there are some evidence that shows cigarette smoking increases the risk (Becker et al., 1994; Reeves et al., 1987; Ylitalo, 1999). Some observations have also suggested that there may be an interaction between HPV and smoking in the causation of cervical neoplasia (Herrero, 1989; Hildesheim et al., 2001). However, reports are available that smoking is significantly associated with cervical carcinoma (52% of cases vs. 27% controls) but is not associated with HPV infection (Reeves et al., 1987).

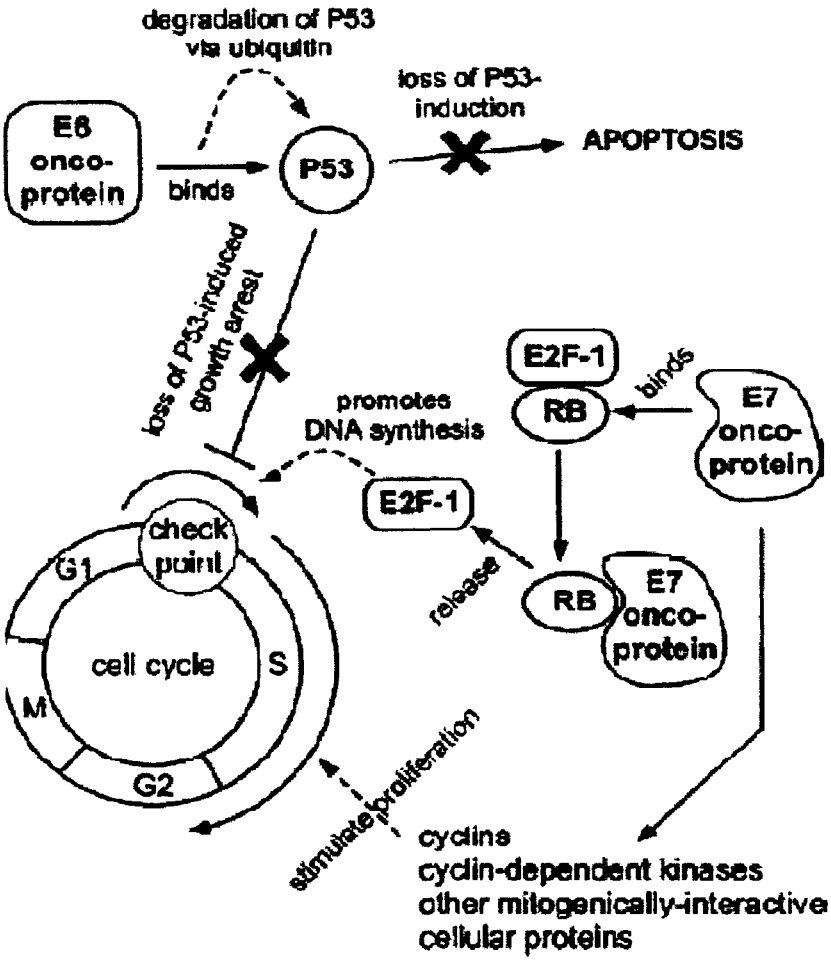

Figure 5: The mechanisms of high-risk HPV E6 and E7 proteins. The proteins interact primarily with p53 and pRb, which leads to transformation of the cell. Illustration by Dr. M. Moberg.

The confusion about the possible relation between smoking and cervical neoplasia and between smoking and HPV infection indicates that smoking might be a weak factor to impact the development of cervical neoplasia and the infection of HPV. It is difficult to control the investigative settings of epidemiological studies to get consistent results. Seemingly, cigarette smoking at the most is a minor cofactor of some specific types of HPV in certain populations.

Hormonal contraception

It has been shown that long-term use of hormonal contraceptives tends to increase the risk of cervical neoplasia (Ylitalo et al., 1999; Hildesheim et al., 2001; Reeves, 1985; Vessey et al., 1989) or increase the HPV infection (Chang, 1989). By contrast, women using barrier contraceptives and spermicidal foams or jellies seem to be at lower risk for this neoplasm (Celentano et al., 1987). However, hormonal contraceptive reduces the risk of cervical neoplasm (Pike et al., 2000). Like smoking, hormonal contraception might be another weak cofactor of HPV infection in the development of cervical neoplasia.

Inheritance

Genetic factors are an attractive field although so far no specific oncogenes or tumor suppressor genes have been found. Significant family clustering of Swedish cases among biological, but not adoptive, relatives of patients with cervical tumors, which provided an epidemiological evidence of a genetic predisposition to cervical carcinoma has been reported (Magnusson et al., 1999). Several other articles with similar conclusion were also published (Magnusson, 2000; Maciag et al., 2000; Hemminki et al., 1999; Hemminki et al., 2001). They explained that genetic susceptibility to HPV infection appears to be important in determining the individual risk to develop this virally induced cancer.

OTHER RISK FACTOR

Lack of vitamins and carotenoids (Slattery et al., 1990; Potischman and Brinton, 1996) has also been linked to CIN II and invasive cervical cancer. The age at viral exposure is known to influences the risk of several other cancer forms, such as

Hepatitis B induced liver cancer (Coursaget et al., 1987) and Epstein stain virus induced Burkett's lymphoma (Magrath et al., 1992). Whether the association seen between age of first intercourse and risk of cervical cancer is an artifact of mere risk of HPV exposure or if it increases the risk between HPV-exposed remains to be shown. Other infectious agent such as- Chlamydia trochomatis and herpes simplex virus (HSV) 2 are suggested to have co factorial role in some case of cervical cancer but the association are weak and inconsistently found (Lehtinen et al., 2002; Smith et al., 2002). Proposed explanation includes enhanced access to basal membrane and increased proliferation due to inflammation.

It has been pointed out that the association between HPV and cervical cancer is higher than the association between smoking and lung cancer and only the association between the chronic carrier state of hepatitis B infection and liver cancer is stronger (Franco, 1995).

Signs and Symptoms

Symptoms for women with HPV in the absence of cancer range from no pain or symptoms to pain and irritation due to inflammation and the development of warts. Visible manifestations of HPV infections are anogenital warts located on the vulva, vagina and cervix (Keller et al., 1995). In detected cases, emotional distress often occurs (Kreitler et al., 1996). Pre-cancerous changes of the cervix are typically found to be relatively asymptomatic. Symptoms usually occur if abnormal cervical cells metastasize into surrounding cellular areas, causing abnormal vaginal bleeding and/or discharge and painful intercourse. In advanced cancerous stages, women report pelvic pain, heavy vaginal bleeding and edema (Women's Cancer Network [WCN], 1999).

Current treatments for HPV-induced cancers of the uterine cervix

Treatment options for HPV positive uterine cervical tumors vary with the stage of disease at diagnosis, the health of the patient, and desire for bearing children, but in all cases involves surgical removal of the malignant tumor (Moore, 2006) as early stages tumors are curable by surgical intervention alone. Procedures such as the Loop Electrosurgical Excision Procedure (LEEP) and Trachelectomy are conservative treatments that allow a patient to bear children in the future (Schlaerth and Abu-

Rustum, 2006). In more advanced stages of cervical cancer, hysterectomy is the treatment of protocol, and in the most advanced stages, radical surgery called pelvic exenteration, which includes removal of all pelvic organs (bladder, rectum, cervix, and vagina), is performed (Moore, 2006).

In more advanced cases as well as in cases of recurrent cervical cancers may require the use of radiation and/or chemotherapy in addition to surgical treatment. A variety of chemotherapeutic drugs can be used to treat cervical cancer. The standard first line treatment for invasive cervical cancer is usually cisplatin (Platinol) and 5-fluorouracil (5-FU, Adrucil, Efudex) used in combination and in addition to radiation. Recurrent and late stage cervical cancers are often treated with a combination of platinum, bleomycin, methotrexate, and 5-FU (DuPont and Monk, 2006). Chemotherapy is administered intravenously, through injection, or in pill form. Side effects often accompany treatment and may be severe, they can include nausea, vomiting, diarrhea, and leukopenia (low white blood cell count). Most of these side effects are a result of drug toxicity to healthy tissues such as skin, hair follicles, and epithelial cells that line the digestive tract (Stewart and Viswanathan, 2006). Patient tumors can develop resistance to these therapies as well (Muggia, 2004). Hence, there is the need to develop alternate, safer, less toxic, and more specific therapies for UCC.

CHEMOPREVENTION

Some of the most successful currently used anticancer compounds are extract from plants or other natural products. These include the breakthrough class of drugs known as catechins that come from the tea plant (*Camellia sinensis*), and the vinca alkaloids (Vincristine, Vinblastine, Vinorelbine), which are derivatives from the periwinkle plant (*Catharanthus roseus*). Other examples of natural products include the antitumor antibiotics such as anthracyclines, dactinomycin, bleomycin, adriamycin, and mithramycin, which are all compounds derived from fungi. These compounds affect the growth of all rapidly dividing cancerous cells.

Millions of research dollars each year go to the discovery of new natural products with limited side effects that may be used as effective antitumor agents.

Some compounds that are currently being investigated are components of dietary plants (Wilson and Danishefsky, 2006; Tan et al., 2006). Examples of such compounds are resveratrol, indole-3-carbinol, quercetin and other catechin compounds, azaphilones, and flavonoids. Many of these potential drugs are antioxidants and fall into a class of chemical compounds called polyphenols. It is believe that the chemopreventive activity of catechin (green tea) is due to the antioxidative and possible anti-proliferative properties of catechins (Bokuchava and Skobeleva, 1980). One of the principal goals of cancer chemotherapeutic or chemopreventive agents is the elimination of damaged or malignant cells through cell cycle inhibition or induction of programmed cell death (apoptosis), leaving normal cells unaffected (Srivastava and Gupta 2006).

The nutritional etiology of cervical neoplasia includes low dietary intake of vitamin C, carotenoids, vitamin E, and folate. For example, Nagata et al. (Nagata et al., 1999) reported a case-control study that suggested the role of plasma carotene in preventing cervical cancer in Japan. Many epidemiological and analytical studies have been conducted to investigate the relationship between antioxidant nutrients and cervical cancer (Mackerras et al., 1999; Shannon et al., 2002; Wideroff et al, 1998; Head, 1998). Today researchers are engaged all around the world to investigate beneficial effect of Green tea polyphenol especially EGCG green tea by exploring it as an anticancer agent.

Epigallocatechin Gallate (EGCG)

Apart from water, tea and coffee are the most widely consumed beverages worldwide (Islam et al., 2000; Roy et al., 2009; Siddiqui et al., 2009). Both contain chemical substances having antioxidant properties that may impact on human health. These substances include caffeine and polyphenols. Tea beverage is made from processed leaves of the plant *Camellia sinensis* a evergreen herb of *Theaceae* family (J). Based on the Ch'a Ching (Classic of Tea), tea was discovered in 2723BC by Emperor Shen Nung of China and was used exclusively for medicinal purposes before the Tang Dynasty (618 - 906AD). Tea was consumed as soup with onions, ginger or orange peel and salt. In the Song Dynasty (906- 1279AD), traditional tea

leaves were powdered to produce a bright green and low astringency frothy drink, which is known as Mattcha in Japan today. It was not until the Ming Dynasty (1369 - 1644AD) that tea leaves were brewed in hot water. This coincided with the arrival of westerners in China. Today, various tea-brewing techniques are used across cultures (Harbowy and Balentine, 1997).

In general, there are three types of manufactured tea: black tea, oolong tea and green tea. The type of tea manufactured depends on the degree of oxidation of leaves. Oxidation is an exogenous process: i.e. the natural browning of leaves catalysed by enzymes within the tea leaf (Harbowy and Balentine, 1997; Unno and Takeo, 1995; Unno et al., 1996).

Green tea extracts have been used in traditional Chinese medicine for centuries to treat and prevent chronic disease (Liao, 2001) but conventional medicine practitioners have only recently begun to explore the health-promoting benefits of green tea derivatives (Cabrera et al., 2006). In present study, focus is on the potential antioxidative effects of (-)-epigallocatechin-3-gallate (EGCG), which is the most abundant polyphenolic compound found in green tea (making up more than 40% of the total polyphenolic mixture (Jung et al., 2001). Because EGCG acts against cancer through a variety of mechanisms, its potential for use in human cancer prevention and treatment seems very promising. The growing number of in vivo and in vitro research studies on this topic reflects this potential. However, to date, relatively few large-scale epidemiological studies in western populations and randomized, controlled intervention trials have been conducted.

EGCG is colorless, astringent, water-soluble, and readily oxidizable (Graham, 1992). Its catechol structure also makes EGCG a strong chelator of metal ions (Guo et al., 1996). EGCG can bind the transition metal ions, prevent formation of hydroxyl radicals, and thus inhibit exogenous ROS-potentiated tumor invasion (Zhang et al., 2000). The oxygen scavenging effects of EGCG are superior to those of ascorbic acid (vitamin C) and tocopherol (vitamin E) with respect to some active oxygen radicals (Zhao et al., 1989).

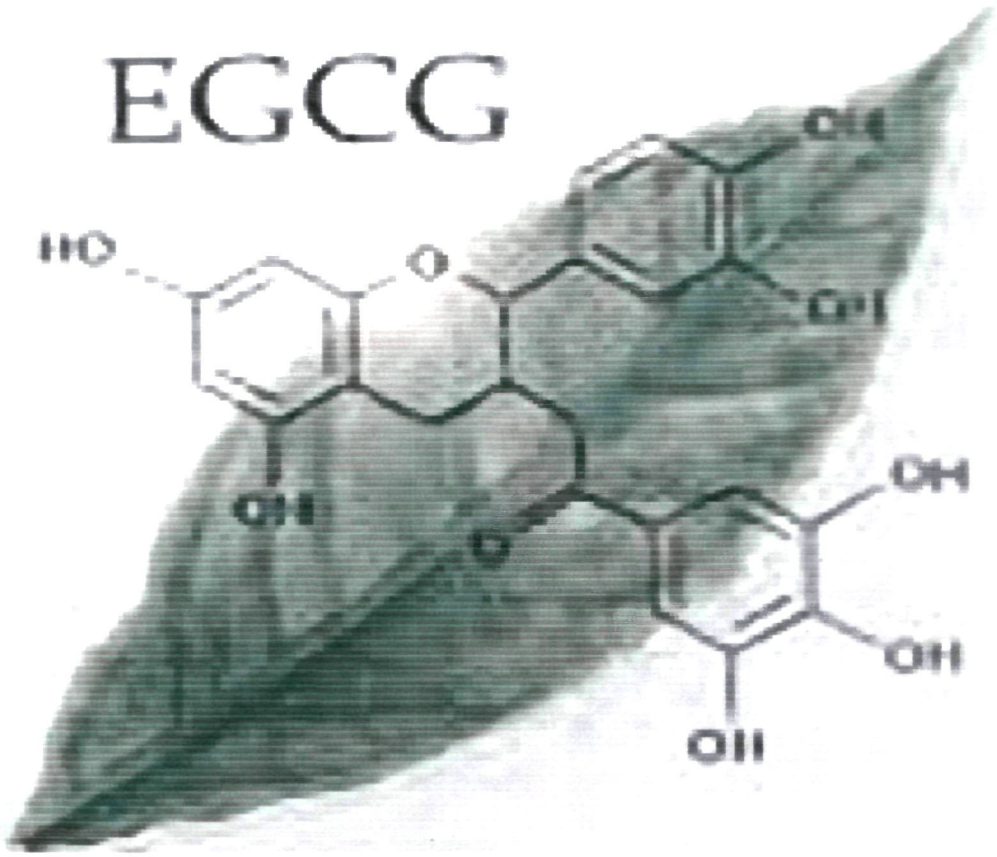

Figure 6: Structure of Epigallocatechin-3-gallate

Thus, EGCG could inhibit tumor cell invasion by scavenging oxygen radicals. It has high affinity for the lipid bilayers of the cell membrane, and can easily enter the nuclei of cancer cells (Okabe et al., 1997). EGCG, because of its polyphenolic structure, have been shown to exhibit antioxidant properties, free radical scavenging and chelation abilities (Castillo et al., 2002; Kimura et al., 2002; Young et al., 2002; Yang et al., 2002, Zhang et al., 2000).

The vicinal dihydrogen or trihydrogen structure contributes to these antioxidant activities. These compounds have also been reported to exert anti-inflammatory actions and modulate immune functions. They exert a cholesterol-lowering effect by enhancing reverse cholesterol transport and bile acid excretion, and decreasing the intestinal absorption of dietary cholesterol (Abe et al., 2000; Chugh et al., 2003; Maron et al., 2003). Anti-carcinogenic properties of EGCG associated with cytotoxicity to cancer cells have also been suggested (Ahmad et al., 2000; Krul et al., 2001; Kubo et al., 2002). In addition, various epidemiological studies have observed an inverse relationship between flavonoid consumption and coronary heart disease and stroke (Beretz et al., 1982; Hakim et al., 2003; Kris-Etherton et al., 2002a; Kris-Etherton 2002b).

One of the most frequently studied mechanisms of EGCG is its role as an antioxidant. Cancer development may be associated with oxidative damage to DNA, lipids, and proteins. Oxidative damage to cells may be caused by a number of factors, including UV light, carcinogens, and free radicals (Rietveld and Wiseman, 2003). Oxidative damage to DNA is an important source of gene mutations that modify gene expression and cellular regulation (Rietveld and Wiseman, 2003). Oxidative damage in cells can be assessed indirectly by measuring the byproducts of oxidative damage, such as oxidized derivatives of phosphatidylcholine in lipid damage and 8-hydroxyguanosine in DNA damage (Frei and Higdon, 2003; Rietveld and Wiseman 2003). EGCG has been found to reduce significantly the plasma levels of biomarkers for oxidative damage to both lipids and DNA (Rietveld and Wiseman 2003). The effect of EGCG on oxidative protein damage also has been studied, and results are mixed. In rats, EGCG was found to suppress oxidative modification of muscle proteins, however controlled studies in humans showed no effect of EGCG on biomarkers for oxidative protein damage (Frei and Higdon, 2003). Furthermore,

antioxidant molecules including EGCG, can alter the redox potential of the cell, thereby activating p53 and promoting apoptosis (Chung et al., 2003). In one in vitro study, cervical cancer cells exposed to 35 μM of EGCG were arrested at the G1 phase of the cell cycle, whereas those exposed to 100 μM of EGCG underwent apoptosis, suggesting that low concentrations of EGCG promote cell cycle arrest whereas high concentrations trigger apoptosis (Ahn et al., 2003).

Mechanism of EGCG against tumor progression and development

Urokinase, a hydrolase implicated in tumor cell invasion (Huang et al., 2000) facilitates degradation of the basement membrane and extracellular matrix. Urokinase is over expressed in breast, ovarian, and prostate malignancies and has clearly been demonstrated to play an essential role in metastasis formation (Gohji et al., 1997). Inhibition of urokinase-type plasminogen activator (uPA) activity can reduce tumor size or even cause complete remission of tumours in mice (Jankun et al., 1997a). EGCG directly impairs the activity of urokinase, interfering with its enzymatic activity and thus its role in the degradation of extracellular matrix (Jankun et al., 1997b).

EGCG-mediated inhibition of matrix metalloproteinase

MMP-2 and MMP-9, the MMP's most frequently over-expressed in cancer and in activated endothelial cells, are instrumental in degrading the basement membrane and facilitating cell invasion (Liotta et al., 1980). In experimental systems, cellular invasion is reduced by the presence of endogenous tissue inhibitors of metalloproteinases (TIMPs) and synthetic inhibitors of MMPs (Li et al., 2001). Some synthetic MMP inhibitors are currently being used in clinical trials for cancer therapy. Unfortunately, initial results have been disappointing due to undesirable side-effects, particularly musculoskeletal pain. Reports show that the concentration of EGCG that effectively inhibits MMP- 2 and MMP-9 in vitro is orders of magnitude lower than that reported for inhibition of urokinase activity (1/500) (Gabrisa et al. 2001). Because EGCG can form complexes with proteins and metal ions, the inhibition of MMP activities by EGCG may be partially related to its ability to chelate zinc, which is essential for MMP enzymatic activity (Maeda-Yamamoto et al., 1999).

Objectives of the present study

It's well known that not much study has been carried out on the role of EGCG- a natural antioxidant on cervical cancer monocytes in comparison to its respective cell lines i.e. HeLa cell lines. Thus, in the present study, the main objectives were to probe the programmed cell death induced by EGCG in cervical cancer monocytes and its biopsies and in turn, compare the efficacy of EGCG in cervical cancer monocytes with those in HeLa cell lines. The study involves immunological investigations, caspase-3, 8 and 9 assays, PARP-cleavage, FITC-Annexin assay, growth kinetics, cell cycle and cell viability assays, evaluation of protein expressions of Bcl-2 and TNF-alpha, IL-1 and IL-6 assays, etc. It is hoped that the results of the present study may probably help in the better understanding of the management of patients with cervical cancers.

EXPERIMENTAL

Materials:

N-acetyl-cysteine (NAC), H_2O_2, Agarose, Bovine serum albumin, Coomassie Brilliant Blue G-250 and R-250, p-nitrophenyl phosphate, anti-human IgG alkaline phosphatase conjugate, Tween-20, TEMED (N,N,N'N'-Tetramethylethylinediamine), dithiothreiotol (DTT), Phenylmethylsulphonyl fluoride (PMSF), Sodium azide, Ethidium bromide, Cumene hydroperoxide were from Sigma Chemical Company, U.S.A. Caspase 3 and PARP were from Bioscience, USA. Caspase-9 was from Bioivision, USA. Bcl-2, rabbit anti human antibody, was from Santa Cruz, USA. Fetal calf serum, antibacterial and anti fungal antibiotics were from Intergen, NY, U. S. A. Chloroform and Isoamyl alcohol were from Qualigens, India. Reduced glutathione, Glutathione reductase and reduced β-nicotinamide adenine dinucleotide phosphate were from HiMedia Chemical Company, India.

EGCG a Green tea polyphenol was obtained from Sigma Chemical Company, USA. RPMI-1640 medium were from HiMedia, India. MTT cell viability assay kit was from R & D Systems; U.S.A. 12-wells tissue culture plates were obtained from Techno Plastic Products (TPP), Switzerland. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were from NUNC, Denmark. All other chemicals were of the highest analytical grade available.

Study subjects:

Venous blood was obtained from healthy nonsmoking adult volunteers with no history of any disease. Also, blood from patients with cervical cancer was obtained from the patients admitted to J. N. Medical College Hospital of A.M.U., Aligarh. The diagnosis of cervical cancer was based on Pap smear and later confirmed by pathological examination of biopsies. Serum was separated and stored at –20°C until required.

Methods

1. Determination of protein concentration:

Protein was estimated by the methods of Lowry et al. (1951) and Bradford (1976).

This method is based on strong binding of the dye Coomassie Brilliant Blue G-250, in acidic medium, to protein hydrophobically, and at positively charged groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue color develops (λmax-595 nm).

Preparation of dye:

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1.0 litre and filtered through Whatman No. 1 filter paper to remove undissolved particles.

Procedure:

To 1.0 ml of solution containing 10-100 ug protein was added 5.0 ml of dye solution. The contents were mixed thoroughly by vortexing. The absorbance was read at 595 nm after 5 minutes against a reagent blank.

Polyacrylamide gel electrophoresis of proteins:

Polyacrylamide gel electrophoresis was performed under denaturing conditions as described by Laemmli (1970).

Reagents:

(i) Acrylamide-bisacrylamide (30:0.8)

A stock solution of 30% acrylamide containing 0.8% bisacrylamide was prepared by dissolving 30 gm of acrylamide and 0.8 gm of bisacrylamide in a total volume of 100 ml. The solution was stored at 4°C in an amber coloured bottle.

(ii) Resolving gel buffer

A stock solution was prepared by dissolving 36.3 gm Tris base in 48 ml of 1N HCl. The contents were mixed, pH adjusted to 8.8 and the final volume brought to 100 ml with distilled water.

(A) Protein estimation by the Lowry (Folin-Ciocalteau) method:

Protein estimation by this method involves complexing of the protein's peptide bonds with Cu2+ under alkaline conditions (Lowry et al., 1951). The resultant Cu+ appears to catalyze the oxidation of tyrosine and tryptophan residues by reducing phosphomolybdotungstate anions in the Folin's reagent (a mixture of sodium tungstate, molybdate and phosphate), added subsequently. This reaction develops a blue color due to the formation of heteropolymolybdenum blue, which can be quantified by its absorbance at 660 nm.

Reagents:

(i) Folin-Ciocalteau reagent

The reagent was diluted 1:4 with distilled water before use.

(ii) Alkaline copper reagent

The components of alkaline copper reagent were prepared as follows:

(a) 2% sodium carbonate in 100 mM NaOH.

(b) 0.5% copper sulphate in 1% sodium potassium tartarate.

The working reagent was prepared fresh before use by mixing the two components in the ratio 50:1, respectively.

Procedure

To 1.0 ml of protein sample was added 5.0 ml of freshly prepared alkaline copper reagent. After thorough mixing, the reaction mixture was allowed to stand at room temperature for 10 minutes, followed by the addition of 1.0 ml of 1:4 times diluted Folin-Ciocalteau reagent. The contents were mixed immediately. The reaction was allowed to proceed for 30 minutes at room temperature and each tube was subsequently monitored at 660 nm. The protein content of the unknown sample was determined by using bovine serum albumin to construct a standard calibration curve.

(B) Protein estimation by the Bradford method:

(iii) Stacking gel buffer

6.05 gm Tris was dissolved in 40 ml distilled water, pH adjusted to 6.8 with 1 N HCl and the final volume adjusted to 100 ml with distilled water.

(iv) Electrode buffer

3.03 gm Tris, 14.4 gm glycine and 1.0 gm SDS were dissolved in distilled water, pH adjusted to 8.3 and the final volume made up to 1.0 litre with distilled water.

(v) Sample buffer

a. 6.0 gm of Tris was dissolved in 80 ml distilled water and pH adjusted to 6.8 with phosphoric acid. The final volume was brought to 100 ml with distilled water.
b. 1.0 mg of bromophenol blue and 12.5 ml of glycerol were added to 12.5 ml of the above solution. B-mercaptoethanol was added just before use.

Recipe for 12.5% Native Gel

Reagents	12.5%
Acrylamide-bisacrylamide (30:0.8)	4.1 ml
Resolving gel buffer	2.5 ml
10% SDS	150 µl
10% Ammonium persulphate (APS)	50 µl
TEMED	12 µl

The final volume was raised to 10 ml with distilled water.

Procedure:

The glass plates (18 cm x 16 cm) were soaked in chromic acid and thoroughly washed with tap water followed by a final rinse with distilled water and ethanol. The plates were dried and sealed with 1% agarose using 1.5 mm thick spacers. The reagents were mixed and poured between the glass plates, a well-forming comb was inserted immediately and the gel was left to polymerize at room temperature. After ensuring complete polymerization, the protein samples (20–100 μg) in one-fourth volume of sample buffer were electrophoresed at 80 volts at room temperature. The gels were stained using 0.25% Coomassie Brilliant Blue R-250 or with silver stain reagent.

Preparation of whole cell lysate:

Principle:

Keratins are insoluble proteins. Therefore they are obtained by solubilizing with detergents like SDS or urea. Therefore, a SDS-lysis buffer is used which has EDTA and EGTA, which chelates the calcium ions, and SDS denatures the proteins. The cell suspension was subsequently sonicated, to cause cell disruption by shear forces by high-pressure sound waves, shears the DNA that is then pelleted down by centrifugation. Therefore the supernatant will have only the proteins of the cells.

Materials:

- 1x PBS
- SDS lysis buffer
- 20mM Tris pH 7.2
- 5mM EDTA pH 8
- 5mM EGTA pH 8
- 0.4% SDS

Procedure:

1. The adherent cells in the dish are washed twice with 1X PBS.

2. 300 µL of SDS lysis buffer was added to the cells.
3. The adherent cells are scraped off with a rubber policeman.
4. The cell suspension was transferred into an eppendorf tube and incubated on ice for 45min.
5. The cell suspension then centrifuged at 10,000 rpm for 10 min at 4°C.
6. The supernatant was taken in fresh tubes and was stored.

Estimation of Proteins by Peterson Method

Principle:

Peterson method is a modified Lowry's method. The principle behind this method is that the, peptide nitrogen reacts with cupric ions under alkaline conditions and there is subsequent reduction of the Folin-Ciocalteau phosphomolybdic- phosphotungtic acid to heteromolybdenum blue by the copper catalysed oxidation of aromatic acids. Tartrate enhances the reduction of the Folin's reagent by cuprous ions.

Reagents:

- Bovine Serum Albumin (BSA) (1mg/ml) stock solution.
- Folin-ciocalteau reagent (1:5 ratio)
- CTC solution:
  - CTC reagent : (20%Na-bicarbonate+0.2%copper sulphate+0.4% sodium-potassium tartrate)
  - 0.8N NaOH
  - 10%SDS
  - Distilled water

  (Each should be in ratio of 1:1:1:1)

Procedure:

1. Gradients of the standard BSA between 5µL and 25µL were taken and 10µL of samples were taken.
2. The volume of the samples and of the standard BSA was made up to 1ml with distilled water.
3. To all the tubes, 1ml of CTC solution was added.
4. The mixture was mixed well and was incubated for 10 min at room temperature.
5. 0.5mL of Folin - ciocalteau reagent was added to the mixture.
6. The mixture was mixed well and incubated for 30 min at room temperature in dark.
7. The absorbance was measured at 750nm.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Principle:

SDS-Page is the most widely used method for analyzing protein mixtures qualitatively. SDS is an anionic detergent. The mercaptoethanol in the sample buffer reduces any disulphide bridges present that are holding together the protein tertiary structure, and the SDS strongly binds to, and denatures, the protein. The original native charge is therefore lost completely. The sample buffer also contains an ionisable tracking dye, bromophenol blue (BPB), that allows the electrophoretic run to be monitored, and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the buffer to the bottom when injected into the loading well.

A stacking gel is poured on top of the resolving gel. The purpose of the stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. Once the proteins reach the separating gel as a result of the small pore size, the negatively charged protein-SDS complexes now continue to move towards the anode, and, because they have the same charge per unit length, they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the protein separate, owing to the molecular sieving properties of the gel.

The bromophenol blue dye is totally unretarded as it is a small molecule and therefore indicates the electrophoresis front.

After the electrophoretic run, the gel is stained with the Coomassie brilliant blue (CBB) staining solution, where the Coomassie dye binds non-specifically to the aromatic amino acids of the proteins. The acetic acid and methanol mixture fixes the proteins onto the gel so that they will not get washed whilst they are being stained. The excess stain is then eluted using the same acetic acid – methanol – water mixture in the absence of the dye.

Reagents:

- **30% Acryl amide- Bisacrylamide solution**
  - [Acryl amide – 29.2g
  - Bis-acrylamide – 0.8g]
- 1.5M Tris buffer- pH 8.8
- 1M Tris buffer – pH 6.8
- TEMED
- 10% Ammonium persulphate(APS)
- 10% SDS
- **Sample Buffer:**
  - 0.5M Tris pH 6.8 — 1.25mL
  - Distilled water — 3.55mL
  - Glycerol — 2.5mL
  - 10% SDS — 2mL
  - Bromophenol blue(0.5% W/W) — 0.2mL
- **5X Tank buffer**:

- Tris base (0.1M) 1.51g
- Glycine (1.25M) 9.4g
- 10% SDS 5mL
- The mixture was then made up to 100mL with distilled water.

- **Staining Buffer:**

  0.25% (w/v) CBB in methanol: water: acetic acid (45: 45:10 by volume) mixture.

- Molecular weight marker (Page ruler Unstained Protein Ladder - Fermentas-Cat No: SM0661)

- **Resolving gel mix (for 10% gel):**

30% Acyrlamide solution	3.33 mL
1.5 M tris pH8.8	0.9 mL
Distilled water	3.6 mL
TEMED	25 µL
10% APS	40 µL
10% SDS	100 µL

- **Stacking gel mix:**

30% Acyrlamide solution	750 µL
1M tris pH6.8	1.25 mL
Distilled water	3mL
TEMED	12.5 µL
10% APS	20 µL
10% SDS	50 µL

Procedure:

1. The apparatus was fixed and checked for leakage
2. 5 mL of the resolving gel mix was poured which is followed by stacking gel

3. The comb was placed immediately
4. When gel has polymerized, the comb was removed and the apparatus was set in the electrophoresis apparatus.
5. The tank was filled with the electrode buffer and the molecular weight marker (10μL) and the samples were loaded.
6. Electrophoresis was carried out at 200 V till the dye front reaches the end of the gel.
7. The gel was then removed and stained using Coomassie brilliant blue staining solution for 2 hours in a rocker.
8. The gel was then destained using the destaining solution.

Western Blotting

Principle:

Western blotting is an analytical method used to detect the specific protein present in the cell lysate. The proteins are resolved on a SDS-Polyacrylamide gel and are then electrotransferred on to the Polyvinylidine Fluoride (PVDF) membrane. PVDF membrane has non-specific affinity for amino-acids. Protein binding is based on the hydrophobic and charged interactions between the membrane and the proteins. After electroblotting, staining the membrane with the Ponceau staining solution checks the efficiency of transfer.

The membrane is then probed with the mouse anti-GFP antibody (primary antibody), after having it blocked with Bovine Serum Albumin (BSA), which binds specifically to the Green Fluorescent Proteins (if present on the membrane). The membrane is then probed with a secondary antibody (anti-mouse), which binds specifically to an epitope of the primary antibody. The secondary antibody is conjugated with the Horse-Radish Peroxidase (HRP) enzyme. Subsequently, on the addition of acridinium based ECL plus substrate, the enzyme catalyses the conversion of the chromogenic substrate into a product (acridinium esters), which produces luminescent signals. These signals are

captured in an X-ray film, which is then developed to visualize the signals. Bands will appear as dark regions on the developed film.

Reagents:

- **Transfer buffer**

o	Tris base (0.02M)	9.1 g
o	Glycine (0.2M)	43.2g
o	Methanol	600mL
o	10% SDS	3mL

The volume is made up to 3L with distilled water.

- Ponceau staining solution
  - 0.1% Ponceau S in 1% acetic acid
- 10X Tris buffered saline (TBS):

o	1M Tris pH 8	50mL
o	5M sodium chloride	50mL

The volume was made upto 500 mL with distilled water.

- TBST
  - 0.1% Tween 20 in 1X TBS.
- Blocking solution
  - 3% BSA in 1X TBS
- Antibody dilutions

The antibodies are diluted with appropriate amounts of 0.5% BSA in 1X TBS.

- ECL plus Western Blot Detection System (Amersham, Cat. No: RPN2132).

Procedure:

1. The protein samples were resolved using 10% SDS-PAGE and were

transferred to the gel transfer cassette.

2. PVDF membrane was pre-activated with 100% methanol.
3. The membrane was then washed with water and subsequently saturated with transfer buffer.
4. The membrane was placed on top of the gel in such a way that there is no air-bubble in between the gel and the membrane.
5. The cassette was fixed and was run at 100 V for 1 hour.
6. The membrane was washed with distilled water.
7. The membrane was then removed and stained with Ponceau staining solution.
8. The membrane was incubated with blocking solution for 1 hour in a rocker at room temperature.
9. The membrane was then incubated with the primary antibody (Mouse anti-GFP antibody) for 1 hour in a rocker at room temperature.
10. The membrane was then washed with TBST four times with an interval of 10 min each to remove the excess unbound antibodies.
11. The membrane was incubated with secondary antibody (anti-mouse antibody) for 1 hour at room temperature in a rocker.
12. The membrane was then washed with TBST four times with an interval of 10 min each to remove the excess unbound antibodies.
13. The membrane was given a final wash with 1X TBS for 10 min in a rocker at room temperature.
14. The blots were then exposed to Lumigen detection reagent - ECL plus reagent mixture (40:1 ratio) in dark and were incubated for 4 min at room temperature.
15. The signals were then captured in X-ray films and they were then developed.

Hela Cell Line:

HeLa cell lines of cervical cancer were brought from National Center for Cell Sciences, Pune, India. Cells were maintained in CO_2 incubator maintained at 5% CO_2 using RPMI 1640 Growth medium. Gentamycin and fetal calf serum (10%) were used in culturing. Cells were harvested after fifth day and aliquots of the stock were kept in liquid nitrogen until use. The viability of the stock remained >99% at 1 year. Before use, aliquots were defrosted, followed by equilibration at 37°C for 45 minutes.

Preparation of EGCG Solution:

Epigallocatechin 3-gallate (EGCG) was purchased from Sigma, USA. The EGCG was dissolved in distilled water, after that EGCG solution was passed through the membrane (0.22 μm) filtered for in vitro uses.

Preparation of RPMI-1640 medium:

Dehydrated RPMI-1640 medium of one unit vial (16.3 gm) (HiMedia, India), was suspended in 950 ml of tissue culture-grade water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade water to remove all traces of powder and added to the above solution. 3.7 gm sodium bicarbonate was added to the medium and stirred until dissolved. The final volume was brought to 1000 ml with tissue culture grade water. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron using positive pressure rather than vacuum to minimize the loss of carbon dioxide, and stored at 4°C until use.

Treatment with EGCG and viability assay:

The effect of EGCG (0–80 μg/ml) on the viability of HeLa cells was assessed by using MTT Cell Viability Assay Kit (R & D Systems) according to the manufacture's instructions provided.

Reagents supplied in the kit:

Component	Quantity	Storage conditions
MTT reagent	25 ml	2 – 8°C
Detergent reagent	250 ml	18 – 24°C

Assay procedure:

Adherent cells were gently scraped with RPMI-1640 medium. After this, 5 x 10^3 cells/well were seeded in 96-well flat-bottomed plates and allowed to attach overnight.. Cells were incubated in RPMI-1640 with 2% autologous serum containing EGCG (0, 20, 40 and 80 µg/ml) for 48 hours at 37°C, 10% CO_2. After 48 hours, 10 µl of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well and incubation was continued for an additional two hours. When a purple precipitate was clearly visible under the microscope, 100 µl of detergent reagent was added to all wells, including control wells and incubated for two hours in the dark at 20°C. After incubation, the precipitate was solubilized and the absorbance of the resulting solution was measured at 630 nm using a microplate reader. Control cells were treated exactly the same except that no EGCG was added to the wells. The percentage of viable cells was calculated by the formula as described by (Islam et al., 2000) and the results were expressed as "Viable cells (% of control cells)".

$$\text{Viable (\% of control cells)} = \frac{\text{Absorbance value of control cells}}{\text{Absorbance value of treated cells}} \times 100$$

Trypan blue exclusion assay for Cell viability:

The exponentially growing Hela cells were seeded onto 12-well, flat-bottomed plates at a density of 5×10^3/mL and allowed to attach overnight. The cells were treated with the EGCG of varying concentration (0-80 µg/ml) for 48hr at 37°C in a CO_2 chamber (5%). The cells were collected by trypsinization and counted by a hemocytometer (Shanghai, China) under light microscope using the Trypan blue dye exclusion method for viability. The cells taking up Trypan blue (dead cells), and cells excluding the dye (viable cells) were counted. Percentage of viable cells was calculated by the following formula:

$$\% \text{ Cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained plus unstained)}} \times 100$$

Growth Kinetic Assay:

Exponentially growing HeLa cells were seeded at 0.1 x 10^6 cells/petri dish of 35 mm diameter for growth kinetics studies. Cells were treated with varying concentration of EGCG (0-80 µg/ml) at 37°C for continuous exposure for 24 and 48hrs. Total cell number of untreated cells at time of treatment was taken as initial value (N_0). Cells were harvested at every 24 hrs time interval up to 48 hrs by trypsinization and counted using hemocytometer. For determining the proliferation (N_t/N_0), where, N_0 is the total number of cells at treatment and N_t is the total number of cells at time t. Both floating and attached cells were counted and fixed in 80% chilled ethanol for cell cycle analysis.

Cell cycle analysis:

Progression of cells through different phases of cell cycle was also followed along with studies on cellular growth kinetics. Cells were washed with PBS by centrifugation at 600g for 5 min, fixed in 80% chilled ethanol and store at 4°C till measurement. Flow cytometric measurement of cellular DNA content were performed with ethanol fixed

cells using the intercalating DNA fluorochrome, propidium iodide (PI) as described earlier (Zolzer et al., 1995). Briefly, the cells (0.5-1 million) were washed with PBS after removing ethanol and treated with of RNase A (200µg/ml) for 30 min at 37°C. Subsequently cells were stained with PI (25µg/ml) for 15min at room temperature. Measurement were made with an argon laser-based Flowcytometer (FACS-Calibur Becton Dickinson San Jose, CA, USA) using the Argon laser (488nm) for excitation. Distribution of cells in different phases of cell cycle was calculated from the frequency distribution of DNA content by using the Mod fit Programme (Variety Software, CA, USA).

Annexin V binding:

Apoptotic cells were detected by the labeling of externalized phosphotidyl serine using annexin V-FITC in unfixed cells (Verma and Mazumder., 1995). Following treatments cells were harvested and aliquots of 10^5 cells resuspended in 100µl binding buffer (10mM HEPES/NaOH, PH 7.4, 10mM NaCl, 2.5mM $CaCl_2$) and 5µl Annexin V-FITC and 10µl PI (50µg/ml) were added. After 15 min at room temperature 400µl of binding buffer were added to each sample and analyzed by flow cytometry. The percentage of Annexin V-positive and negative cells were estimated by applying appropriate gates and using regional statistics analysis facility provided in the cells Quest Software (Becton Dickenson, San Jose, CA, USA).

Preparation of PBMC and treatment of monocytes culture

Peripheral blood mononuclear cells (PBMCs) from blood of healthy donors and cervical cancer patients attending O. P. D. of J. N. Medical College and Hospitals, AMU, Aligarh were isolated by density gradient sedimentation on Ficoll-Paque separation medium as described by us earlier (Mesiter and Anderson, 1983; Hasan et al., 2006). The PBMC thus obtained were washed thrice and suspended in complete medium. By cytostaining, PBMC were comprised of 90% non-adherant lymphocytes and 10% adherent monocytes. Thereafter, PBMCs ($5x10^6$ cells/well) were added in 12-well tissue culture plates (Costar Corp. Cambridge, MA) in complete RPMI-1640 medium, and were subsequently incubated at 37°C, 5% CO_2 for 1-2 hrs for adherence, washed to remove non-adherant

cells and rested for overnight in RPMI-1640 medium having 2% autologous serum. Then, the adherent monocytes were co-cultured for 24 hrs with varying doses of EGCG (0-25 μg/ml, dissolved in RPMI-1640 media). Cultures devoid of EGCG served as control. Also, some cultures were pre-treated with Z-VAD-FMK, Z-IETD-FMK and Z-LEHD-FMK, which are known inhibitors of capase-3, caspase-8 and caspase-9 respectively. Following 24 hrs, the above cells were lysed for 30 min at 4°C in 0.5 ml of protein lysis buffer, which was prepared using MLB buffer (50 mM 3-(N-Morpholino)-propanesulfonic acid (MOPS), pH 7.0, 250 mM NaCl, 5.0 mM EDTA, 0.1% NP-40 (a detergent), and 1.0 mM DTT) supplemented with protease inhibitors (1.0 mM PMSF, 5.0 μg/ml leupeptin, 10 mM NaF, 5.0 mM Na-pyrophosphate, 1.0 mM Na-orthovanadate, and 20 mM β-glycerophosphate). Thereafter, the suspension was centrifuged at 20,000 rpm for 15 minutes at 4°C and supernatants were stored at –20°C. Protein concentration was determined and the culture supernatants were stored at –20°C until use.

Caspase activity assay:

Caspase-3, caspase-8 and caspase-9 related protease activity in cell lysates was determined as described elsewhere (Green and Reed, 1998; Ashkenazi and Dixit, 1998; Stennicke and Salvesen, 1998). The substrates employed were Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA) (Biomol.), Ac-Ile-Glu-Thr-Asp-pNA (IETD-pNA) and Ac-Leu-Glu-His-Asp-pNA (LEHD-pNA) (Upstate Biotechnology, USA) for caspases-3, 8 and 9 respectively. Caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) were from R & D Systems Europe (Abingdon, UK). Briefly, $1x10^6$ cells in 12-well plates were incubated with RPMI-1640 medium containing EGCG (5 μg/ml) for 24 h. Thereafter, cells were lyzed in 80 μl of ice-cold lysis buffer as described above. Twenty microliters of the supernatant was added to 100 μl assay buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol, pH 7.4. Ten microliters of respective caspase substrates were then added in the reaction system to final concentration of 0.2 mM. The cleavage of the substrate was monitored spectrophotometrically at 405nm and the activities were calculated according to the instruction of the manufacturer.

In order to determine the effects of specific caspase inhibitors on EGCG-induced apoptosis, Z-DEVD-FMK, Z-IETD-FMK and Z-LEHD-FMK were dissolved in DMSO as 20 mM stock solution and added to the culture medium to a final concentration of 50 μM as described earlier (Liu et al., 2002). After 2 hr pre-incubation, EGCG (5 ug/ml) was added and incubated for 24 h. The changes of EGCG-induced apoptosis in the presence of these inhibitors were determined as described.

Cytomorphological and histomorphological effects of EGCG on cervical cancer biopsies:

In the present study, biopsy pieces were taken directly from untreated cervical cancer patient (n=9) admitted for the first time and showing frank fungating growth. The Institutional Committee approved the study utilizing biopsies and blood collection. Biopsies from subjects showing differentiated keratinising and necrotising lesions were excluded from the study. Biopsy pieces were washed with buffered normal saline to remove debris, transferred to RPMI-1640 culture medium, bisected into 2 halves of 4-5 mm size to serve as test and control. The samples in 15-ml Falcon tissue culture tubes were subjected to treatment without and with EGCG (5 μg/ml) and cultured at 37°C, 5% CO_2 for 48 hrs. Thereafter, samples were processed for cytocentrifuged smear, and further processed for paraffin sections and staining.

Glutathione peroxidase (GPx) assay:

The activity of glutathione peroxidase (GPx) was measured as described elsewhere (Mohandas et al., 1984; Mates et al., 1999; Hasan et al., 2006). The oxidized glutathione (GSSG) produced during GPx reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was regarded as the rate of GSSG formation during the GPx reaction. Cancerous cell cultures as described above were co-cultured for 24 hours with or without 10 mM NAC and 0–80 μg/ml EGCG. Thereafter, cells were gently scraped with lysis buffer containing protease inhibitors (50 mM Tris/HCl, pH 7.4; 1 mM EDTA; 500 mM PMSF). The cell suspension was homogenized with a sonicator on ice and centrifuged at 10,000 rpm for 10 minutes. Protein concentrations of supernatants were determined by the method of Bradford with

BSA as the standard, and were subjected to GPx activity determination. The reaction mixture (1.0 ml) containing 50 mM potassium phosphate (pH 7.0), 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 unit/ml glutathione reductase, 1.5 mM cumene hydroperoxide, and 20–100 µl of samples were incubated at 25°C for 5 minutes. The reaction was initiated by the addition of cumene hydroperoxide. The kinetic change was spectrophotometrically recorded at 340 nm (37°C) for 3 minutes. GPx activity was calculated after subtraction of the blank value, as µmol of NADPH oxidized/minute/mg protein (U/mg protein).

Statistical analysis:

Results were analyzed by paired t-test and the data expressed as mean ± SEM of six experiments unless otherwise specified. $P<0.05$ was considered statistically significant.

RESULTS

The present study involves investigations for the effect of EGCG – a green tea polyphenol as well as a natural antioxidant on two cervical cancer model systems, which are: **(a)** monocytes and biopsies from cervical cancer patients and **(b)** cervical cancer cell lines i.e. HeLa cell lines. Interesting data were generated, where, in summary, the model system involving monocytes and biopsies from cervical cancer patients showed high magnitude anti-inflammatory as well as apoptotic effect of EGCG (0-100 μg/ml), while on the other hand, EGCG (0-100 μg/ml) exerted high magnitude anti-inflammatory but low apoptotic effect on HeLa cell lines. The data obtained are discussed below under separate subheadings of the two model systems undertaken in the study.

(A). STUDIES ON THE EFFECT OF ANTI-OXIDANTS IN CERVICAL CANCER:

As the active role-played by reactive oxygen species (ROS) in the cervical cancer is well established, thus an attempt was made in the present study to probe natural compounds having antioxidant properties in arresting ROS in cancer cells. Various workers on cervical cancer HeLa cell lines have extensively carried out such a study, but no or little work has been done on cervical cancer monocytes. Thus, comparative efficacy of natural antioxidants was carried out on cervical cancer monocytes versus HeLa cell lines.

(i). Effect of EGCG on the GPx Activity of Healthy Control Human Monocytes:

Prior to any study, an attempt was made first to evaluate the effect of EGCG on the GPx activity of healthy control human monocytes. Thus, normal healthy monocytes were co-cultured separately for 24 hours with varying doses of EGCG as described in methods. As evident from Figs. 7, in comparison to untreated cells, none of the doses of EGCG (0-50 μg/ml showed any effect on the GPx activity. In fact, the GPx activities for untreated and treated normal healthy monocytes at various doses of EGCG were nearly the same (Figs. 7; n=3; $p<0.001$).

(ii). Dose Response Effect of EGCG on GPx Activity in 24 hr Cultures of Cervical Cancer Monocytes and HeLa Cell Lines:

Thereafter, dose response effects of EGCG (0-50 μg/ml) on GPx activity in 24 hr culture supernatants of cervical cancer monocytes as well as HeLa cell lines were determined. In comparison to healthy control untreated monocytes in Fig. 7, the untreated cervical cancer monocytes exhibited an appreciable suppression by around 2.14-folds in the GPx activity (Fig. 8; n=3; $p<0.001$). However, as evident from Fig. 8, co-culturing of cervical cancer monocytes with varying doses of EGCG exhibited appreciable amelioration in the GPx activity. The EGCG-mediated amelioration in the GPx activity was found to be of the order of 1.58-folds ($p<0.001$), 1.8-folds ($p<0.001$), 1.9-folds ($p<0.001$), 1.97-folds ($p<0.001$), 2.02-folds ($p<0.001$) and 2.01-folds ($p<0.001$) with 5, 10, 20, 30, 40 and 50 μg/ml of EGCG respectively (Fig. 8).

Next, an attempt was also made to probe the effect of varying doses of EGCG on the GPx activity in HeLa cell lines. When compared to healthy control untreated monocytes as illustrated in Fig. 7, the HeLa cell lines exhibited an appreciable suppression by around 1.98-folds in the GPx activity (Fig. 9; n=3; $p<0.001$). On the contrary, co-culturing of HeLa cell lines with varying doses of EGCG exhibited appreciable amelioration in the GPx activity. Computational analysis of the data in Fig 9, revealed that the EGCG-mediated amelioration in GPx activity was of the order of 1.27-folds ($p<0.001$), 1.52-folds ($p<0.001$), 1.66-folds ($p<0.001$), 1.76-folds ($p<0.001$), 1.79-folds ($p<0.001$) and 1.79-folds ($p<0.001$) with 5,10, 20, 30, 40 and 50 μg/ml of EGCG respectively (Fig. 9).

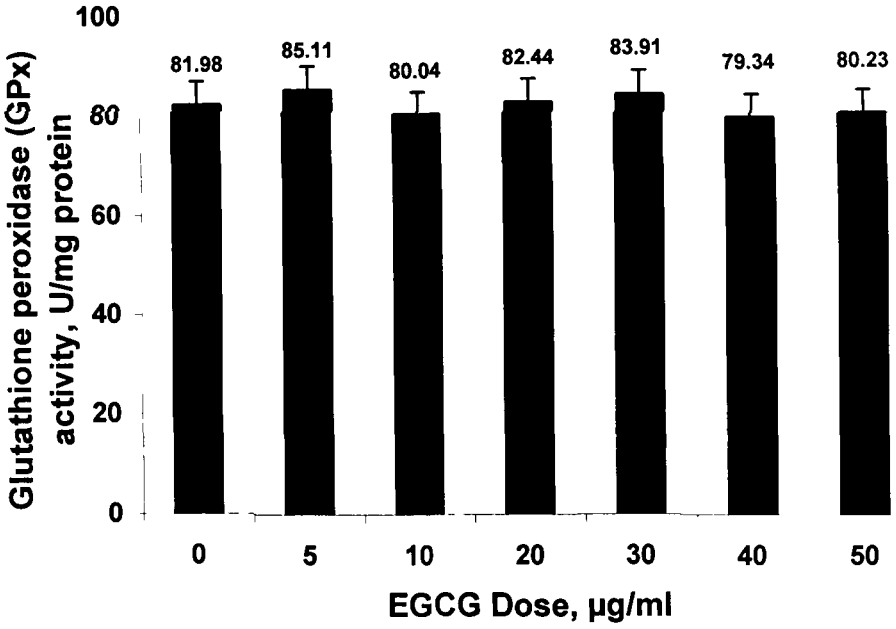

FIGURE 7: Effect of EGCG on Healthy control human monocytes: Dose response effect of EGCG (0-50 µg/ml) on 24 hr culture supernatants of normal healthy monocytes. Data are mean (±S.E.) of 3 experiments. (*$p<0.001$*).

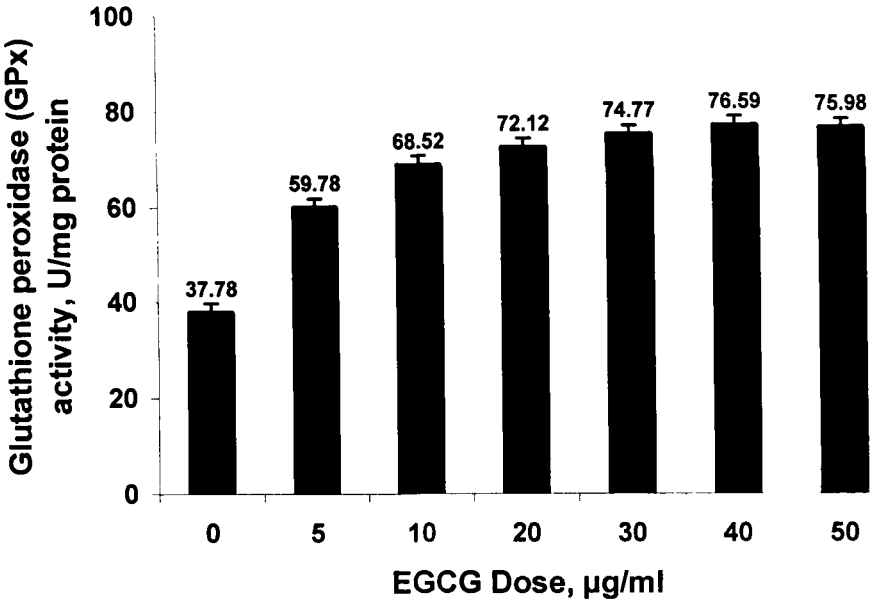

FIGURE 8: Effect of EGCG on Cervical cancer monocytes: Dose response effect of EGCG (0-50 µg/ml) on 24 hr culture supernatants of cervical cancer monocytes. Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

(iii). Effect of NAC on the GPx Activity of Healthy Control Human Monocytes and HeLa Cell Lines:

Next, was a known antioxidant i.e. N-Acetyl Cysteine (NAC) that was employed in the present study to determine its effect on the GPx activity on healthy control human monocytes and HeLa cell lines. Normal healthy monocytes as well as HeLa cell lines were co-cultured separately for 24 hours with varying doses of NAC as described in methods. As evident from Figs. 10, in comparison to untreated cells, none of the doses of NAC (0-10 mM) showed any effect on the GPx activity. The GPx activities for untreated and treated normal healthy monocytes at various doses of NAC were nearly the same (Figs. 10; n=3; $p<0.001$).

(iv). Dose Response Effect of N-Acetyl Cysteine (NAC) on GPx Activity in 24 hr Cultures of Cervical Cancer Monocytes and HeLa Cell Lines:

Dose response effects of NAC (0-10 mM) on GPx activity in 24 hr culture supernatants of cervical cancer monocytes as well as HeLa cell lines were determined in the present study. In comparison to healthy control untreated monocytes in Fig. 7, the untreated cervical cancer monocytes exhibited an appreciable suppression by around 2.12-folds in the GPx activity (Fig. 11; n=3; $p<0.001$). However, as evident from Fig. 11, co-culturing of cervical cancer monocytes with varying doses of NAC exhibited appreciable amelioration in the GPx activity. The NAC-mediated amelioration in the GPx activity was found to be of the order of 1.15 -folds ($p<0.001$), 1.37-folds ($p<0.001$), 1.46-folds ($p<0.001$), 1.67-folds ($p<0.001$) and 1.83-folds ($p<0.001$) with 2, 4, 6, 8 and 10 mM of NAC respectively (Fig. 11).

Thereafter, an attempt was also made to probe the effect of NAC (0-10 mM) on GPx activity in HeLa cell lines. Co-culturing of HeLa cell lines with varying doses of NAC exhibited appreciable amelioration in the GPx activity. Computational analysis of the data in Fig 12, revealed that the NAC-mediated amelioration in GPx activity was of the order of 1.15 -folds ($p<0.001$), 1.34-folds ($p<0.001$), 1.47-folds ($p<0.001$), 1.55-folds ($p<0.001$) and 1.59-folds ($p<0.001$) with 2, 4, 6, 8 and 10 mM of NAC respectively (Fig. 12).

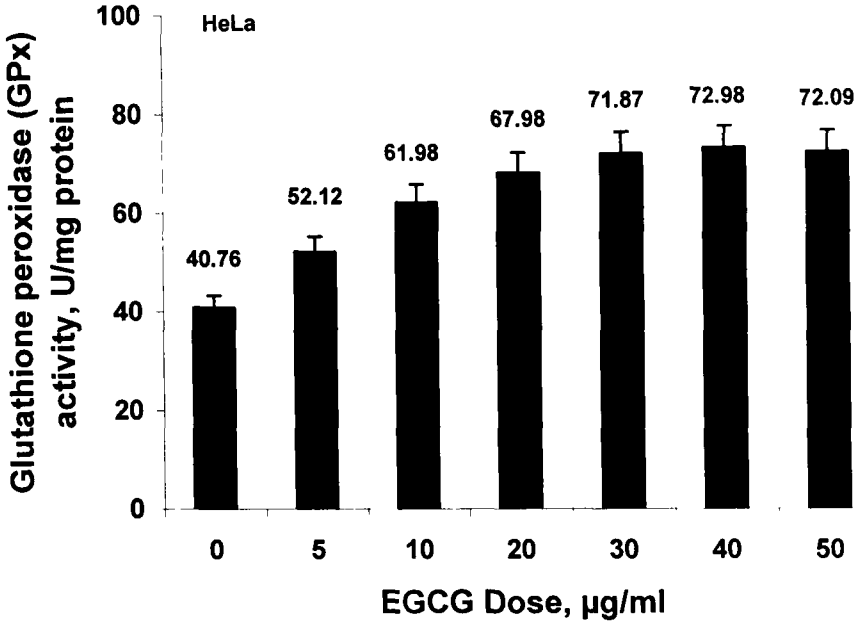

FIGURE 9: Effect of EGCG on HeLa cell lines: Dose response effect of EGCG (0-50 µg/ml) on 24 hr culture supernatants of HeLa cell lines. Data are mean (±S.E.) of 3 experiments. (p<0.001).

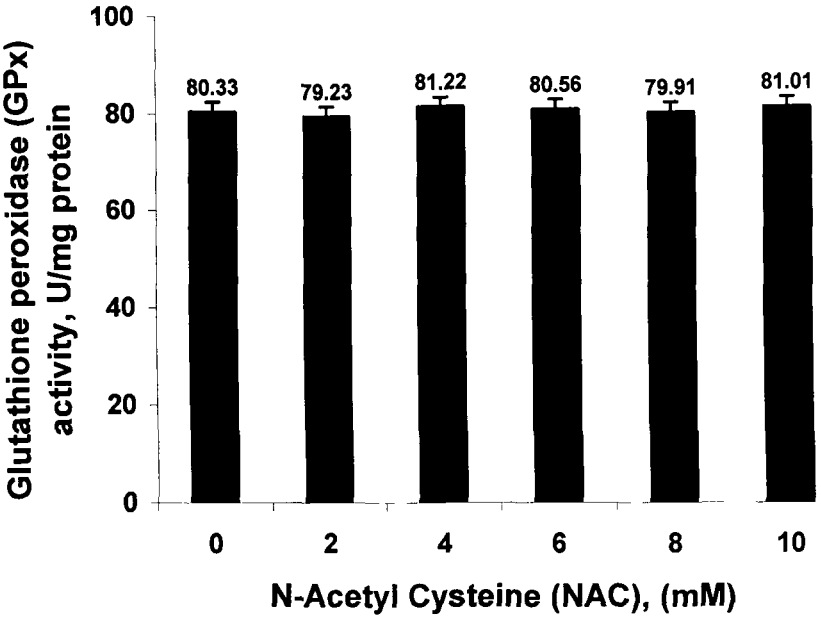

FIGURE 10: Effect of NAC on Healthy control human monocytes: Dose response effect of NAC (0-10 mM) on 24 hr culture supernatants of normal healthy monocytes. Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

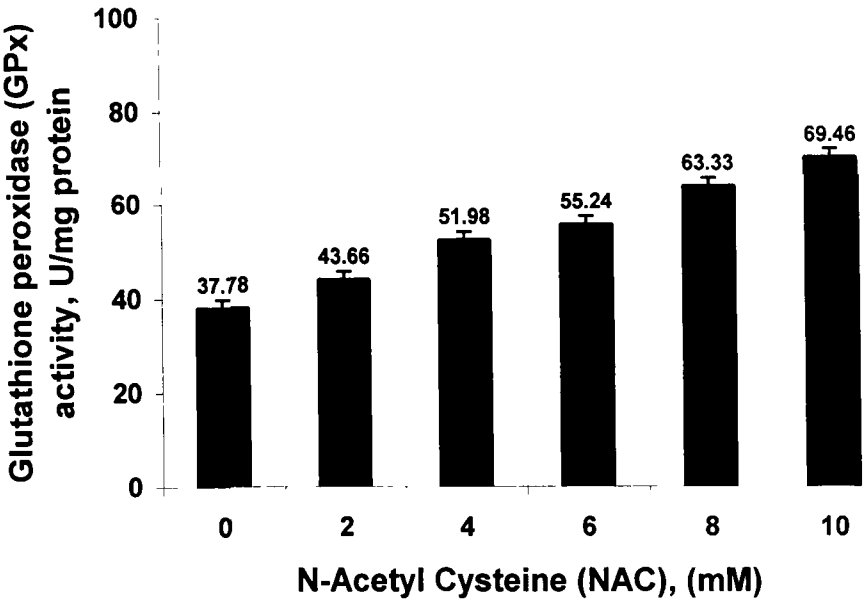

FIGURE 11: Effect of NAC on Cervical cancer monocytes: Dose response effect of NAC (0-10 mM) on 24 hr culture supernatants of cervical cancer monocytes. Data are mean (±S.E.) of 3 experiments. (p<0.001).

(v). Effect of Curcumin on the GPx Activity of Healthy Control Human Monocytes:

Next, an attempt was also made to evaluate the effect of Curcumin on the GPx activity of healthy control human monocytes. Normal healthy monocytes were co-cultured separately for 24 hours with varying doses of Curcumin as described in methods. As evident from Figs. 13, in comparison to untreated cells, none of the doses of Curcumin (0-50 μg/ml showed any effect on the GPx activity. GPx activities for untreated and treated normal healthy monocytes at various doses of Curcumin were nearly the same (Figs. 13; n=3; $p<0.001$).

(vi). Dose Response Effect of Curcumin on GPx Activity in 24 hr Cultures of Cervical Cancer Monocytes and HeLa Cell Lines:

Next, a dose response effect of Curcumin (0-50 μg/ml) on GPx activity in 24 hr culture supernatants of cervical cancer monocytes as well as HeLa cell lines were determined in the present study. In comparison to healthy control untreated monocytes in Fig. 7, the untreated cervical cancer monocytes exhibited an appreciable suppression by around 2.12-folds in the GPx activity (Fig. 14; n=3; $p<0.001$).

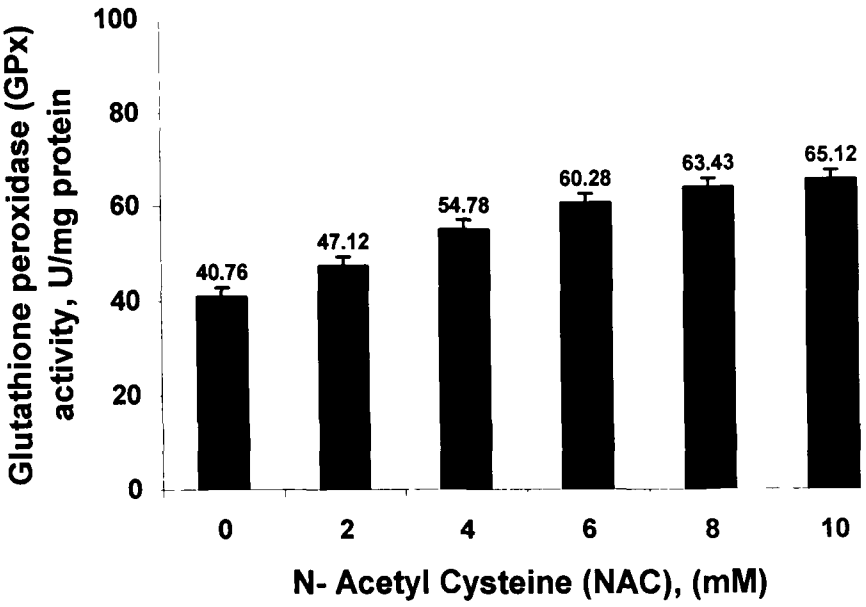

FIGURE 12: Effect of NAC on HeLa cell lines: Dose response effect of NAC (0-10 mM) on 24 hr culture supernatants of HeLa cell lines. Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

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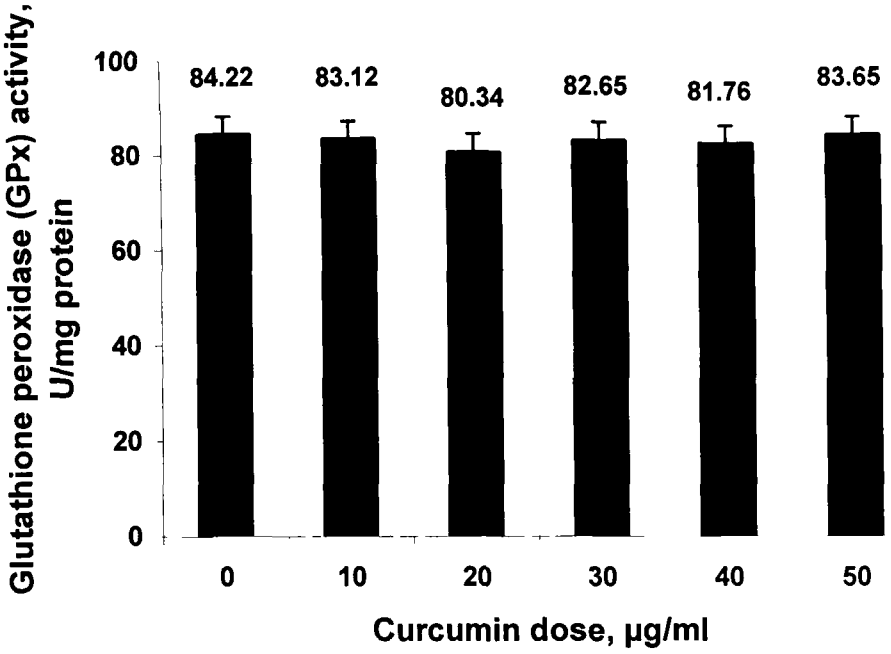


FIGURE 13: Effect of Curcumin on Healthy control human monocytes: Dose response effect of Curcumin (0-50 µg/ml) on 24 hr culture supernatants of normal healthy monocytes. Data are mean (±S.E.) of 3 experiments. (p<0.001).

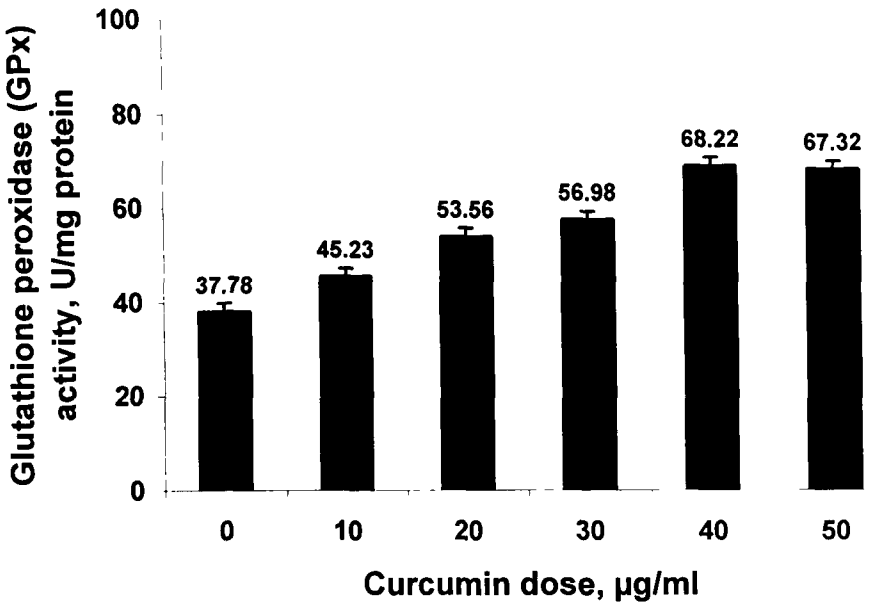


FIGURE 14: Effect of Curcumin on Cervical cancer monocytes: Dose response effect of Curcumin (0-50 µg/ml) on 24 hr culture supernatants of cervical cancer monocytes. Data are mean (±S.E.) of 3 experiments. (p<0.001).

However, as evident from Fig. 8, co-culturing of cervical cancer monocytes with varying doses of Turmeric exhibited appreciable amelioration in the GPx activity, where the amelioration was found to be of the order of 1.21 -folds (p<0.001), 1.41-folds (p<0.001), 1.50-folds (p<0.001), 1.80-folds (p<0.001) and 1.78-folds (p<0.001) with 10, 20, 30, 40 and 50 μg/ml of Curcumin respectively (Fig. 14).

Thereafter, effects of Curcumin (0-50 μg/ml) on the GPx activity in HeLa cell lines were also probed. Co-culturing of HeLa cell lines with varying doses of Curcumin exhibited appreciable amelioration in the GPx activity. Computational analysis of the data in Fig 15, revealed that the Curcumin-mediated amelioration in GPx activity was of the order of 1.22 -folds (p<0.001), 1.28-folds (p<0.001), 1.42-folds (p<0.001), 1.50-folds (p<0.001) and 1.61-folds (p<0.001) with 2, 4, 6, 8 and 10 mM of NAC respectively (Fig. 15).

(vii). Effect of H_2O_2 on the GPx Activity of Healthy Control Human Monocytes:

Next, an attempt was also made to probe the effect of H_2O_2 on the GPx activity of healthy control human monocytes. Thus, normal healthy monocytes were co-cultured separately for 24 hours with varying doses of H_2O_2 as described in methods. As evident from Fig. 16, in comparison to untreated cells, H_2O_2 was found to suppress the GPx activity linearly in a dose dependent manner. The H_2O_2 –mediated suppression was found to be of the order of 1.16 folds, 1.32-folds, 1.42-folds, 1.61-folds and1.66-folds with 2, 4, 6, 8 and 10 nM of H_2O_2 respectively (Fig. 16; n=3; p<0.001).

(viii). Dose Response Effect of H_2O_2 on GPx Activity in 24 hr Cultures of Cervical Cancer Monocytes and HeLa Cell Lines:

Thereafter, dose response effects of H_2O_2 (0-10 nM) on GPx activity in 24 hr culture supernatants of cervical cancer monocytes as well as HeLa cell lines were determined. In comparison to healthy control untreated monocytes in Fig. 7, the untreated cervical cancer monocytes exhibited an appreciable suppression by around 2.14-folds in the GPx activity (Fig. 17; n=3; p<0.001).

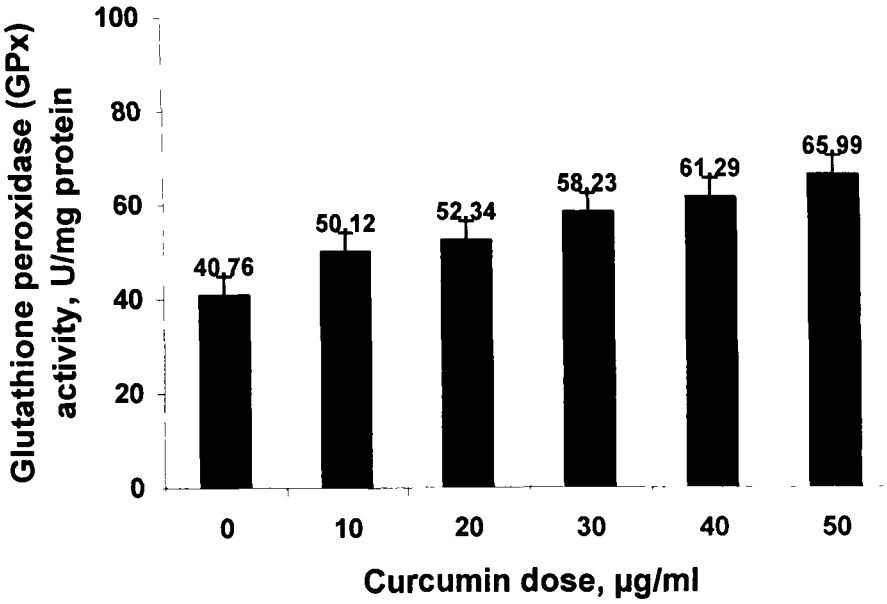


FIGURE 15: Effect of Curcumin on HeLa cell lines: Dose response effect of Curcumin (0-50 µg/ml) on 24 hr culture supernatants of HeLa cell lines. Data are mean (±S.E.) of 3 experiments. (p<0.001).

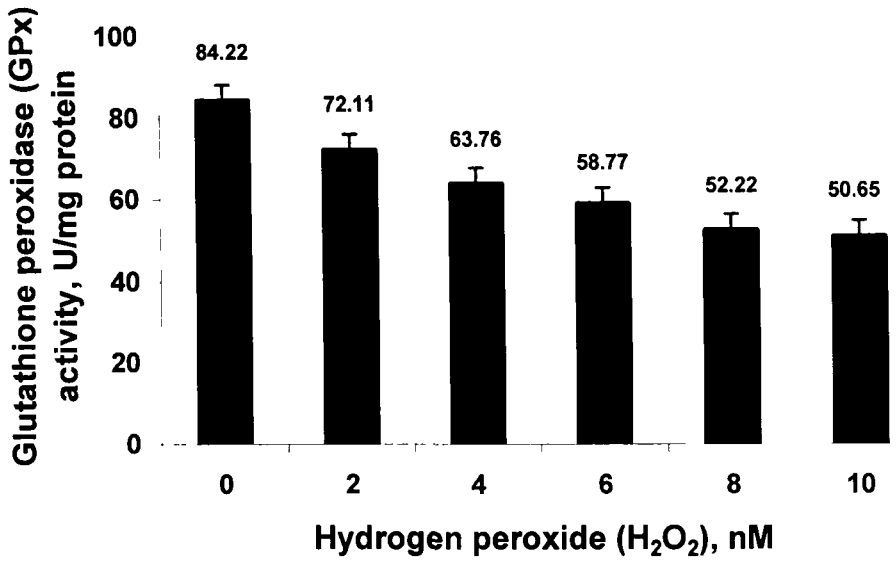


FIGURE 16: Effect of Hydrogen peroxide (H_2O_2) on Healthy control human monocytes: Dose response effect of H_2O_2 (0-10 nM) on 24 hr culture supernatants of normal healthy monocytes. Data are mean (±S.E.) of 3 experiments. (p<0.001).

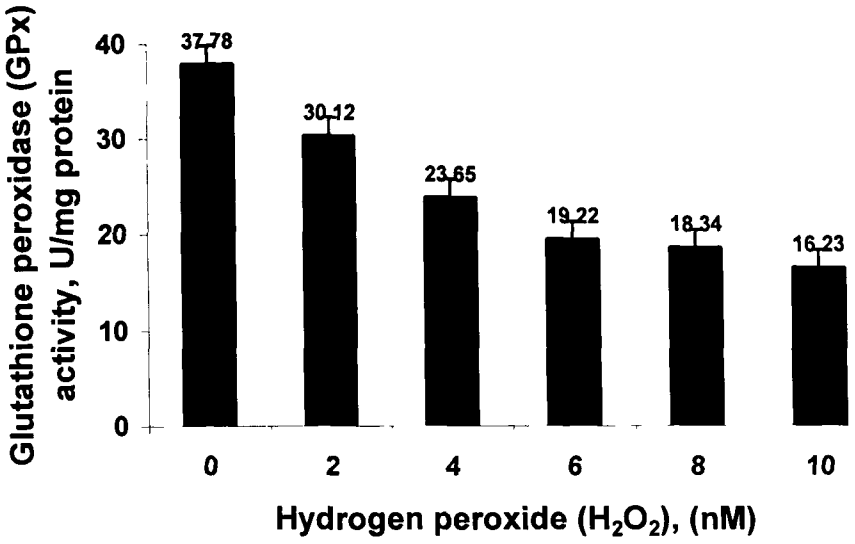


FIGURE 17: Effect of Hydrogen peroxide (H_2O_2) on Cervical Cancer Monocytes: Dose response effect of H_2O_2 (0-10 nM) on 24 hr culture supernatants of cervical cancer monocytes. Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

However, as evident from Fig. 17, co-culturing of cervical cancer monocytes with varying doses of H_2O_2 exhibited a further suppression in the GPx activity. The H_2O_2-mediated further suppression in the GPx activity was found to be of the order of 1.25-folds ($p<0.001$), 1.59-folds ($p<0.001$), 1.96-folds ($p<0.001$), 2.05-folds ($p<0.001$) and 2.32-folds ($p<0.001$) with 2, 4, 6, 8 and 10 nM of H_2O_2 respectively (Fig. 17).

Next, an attempt was also made to probe the effect of varying doses of H_2O_2 on the GPx activity in HeLa cell lines. When compared to healthy control untreated monocytes as illustrated in Fig. 7, the HeLa cell lines exhibited an appreciable suppression by around 1.98-folds in the GPx activity (Fig. 18; n=3; $p<0.001$). On the contrary, co-culturing of HeLa cell lines with varying doses of H_2O_2 exhibited further suppression in the GPx activity. Computational analysis of the data in Fig 18, revealed that the H_2O_2-mediated further suppression in GPx activity was of the order of 1.19-folds ($p<0.001$), 1.53-folds ($p<0.001$), 2.2-folds ($p<0.001$), 2.27-folds ($p<0.001$) and 2.39 -folds ($p<0.001$) with 2, 4, 6, 8 and 10 nM of H_2O_2 respectively (Fig. 18).

(B). Model System - Healthy Monocytes: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus EGCG (20 µg/ml), NAC (10 mM) and Curcumin (20 µg/ml) on the GPx activities in healthy control human monocytes.

After observing the role of hydrogen peroxide (H_2O_2) and EGCG- a green tea polyphenol as well as a natural antioxidant as a positive and negative regulator respectively of GPx activity, an attempt was also made to probe the competitive effect of the above said positive regulator versus negative regulator present in the same cell culture. Thus, normal healthy monocytes were co-cultured separately for 24 hours with 10nM of H_2O_2 along with 20 µg/ml of EGCG. As evident from Fig. 19, addition of 10nM of H_2O_2 showed a down-regulation in the GPx activity by ~1.66-folds (n=3; $p<0.001$) in comparison to untreated healthy monocytes. However, healthy monocytes cultures receiving H_2O_2 along with EGCG showed interesting results. EGCG here was found to ameliorate by ~1.42-folds (n=3; $p<0.001$) the H_2O_2-mediated suppressed GPx activity.

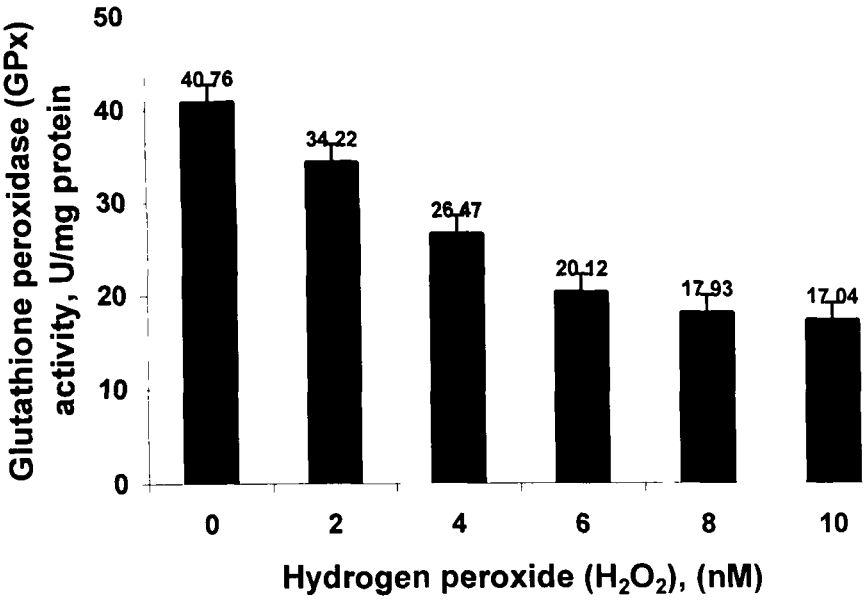


FIGURE 18: Effect of Hydrogen peroxide (H_2O_2) on HeLa Cell Lines: Dose response effect of H_2O_2 (0-10 nM) on 24 hr culture supernatants of HeLa cell lines. Data are mean (±S.E.) of 3 experiments. (p<0.001).

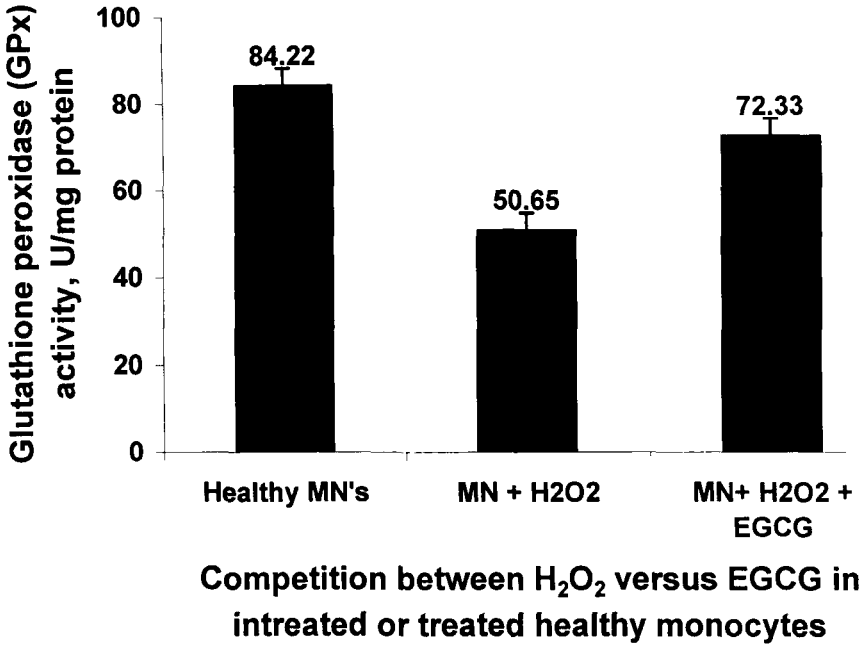


FIGURE 19: Healthy Control Monocytes: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus EGCG (20 μg/ml) on GPx activity in healthy control human monocytes: Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

Next, after EGCG, an attempt was also made to probe the competitive effect H_2O_2 versus NAC and Curcumin on the GPx activity in healthy monocytes. Both, NAC and Curcumin are proven antioxidants. As evident from the data, 10 mM NAC and 20 µg/ml Curcumin were found to ameliorate by ~1.31-folds (Fig. 20; n=3; $p<0.001$) and ~ 1.38-folds (Fig. 21; n=3; $p<0.001$) respectively the suppressed GPx activity in H_2O_2 treated healthy monocytes.

Thus, analysis of the data in Fig 19-21, it was observed that the capability of ameliorating the suppressed GPx activity in healthy monocytes due to H_2O_2 treatment was found to be of the order of EGCG (~1.42-folds) > Curcumin (~1.38-folds) ≥ NAC (~1.31-folds).

(C). Model System - Cervical Cancer Monocytes: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus EGCG (20 µg/ml), NAC (10 mM) and Curcumin (20 µg/ml) on the GPx activities in cervical cancer monocytes.

Next, the model system for competitive probe was cervical cancer monocytes. Thus, cervical cancer monocytes were co-cultured separately for 24 hours with 10nM of H_2O_2 along with 20 µg/ml of EGCG. As evident from Fig. 22, a suppressed the GPx activity by ~2.22-folds (n=3; $p<0.001$) was observed in cervical cancer monocytes was observed in comparison to healthy control monocytes (Fig. 21). The suppressed GPx activity in untreated cervical cancer monocytes was further suppressed by ~ 2.32-folds (Fig. 22; n=3; $p<0.001$) by the addition of 10nM of H_2O_2. However, cervical cancer monocytes cultures receiving H_2O_2 along with EGCG showed amelioration by ~3.64-folds (Fig. 22; n=3; $p<0.001$) in the H_2O_2-mediated suppressed GPx activity.

Next, an attempt was also made to probe the competitive effect H_2O_2 versus NAC and Curcumin on the GPx activity in cervical cancer monocytes. As evident from the data, 10 mM NAC and 20 µg/ml Curcumin were found to ameliorate by ~3.09-folds (Fig. 23; n=3; $p<0.001$) and ~ 3.37-folds (Fig. 24; n=3; $p<0.001$) respectively the suppressed GPx activity in H_2O_2 treated cervical cancer monocytes.

Thus, analysis of the data in Fig 16-18, it was observed that the capability of ameliorating the suppressed GPx activity in cervical cancer monocytes due to H_2O_2 treatment was found to be of the order of EGCG (~3.64-folds) > Curcumin (~3.37-folds) ≥ NAC (~3.09-folds).

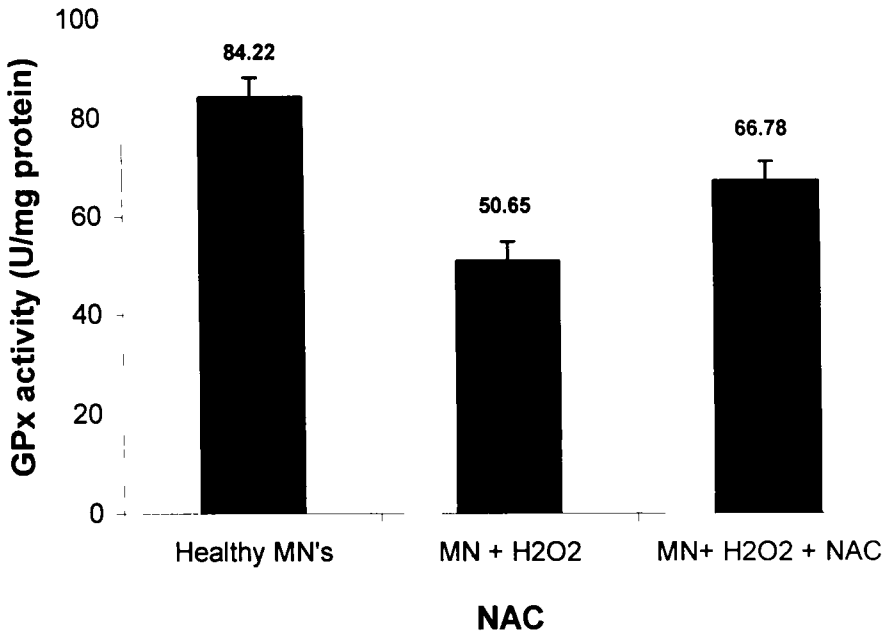


FIGURE 20: Healthy Control Monocytes: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus NAC (10 mM) on GPx activity in healthy control human monocytes: Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

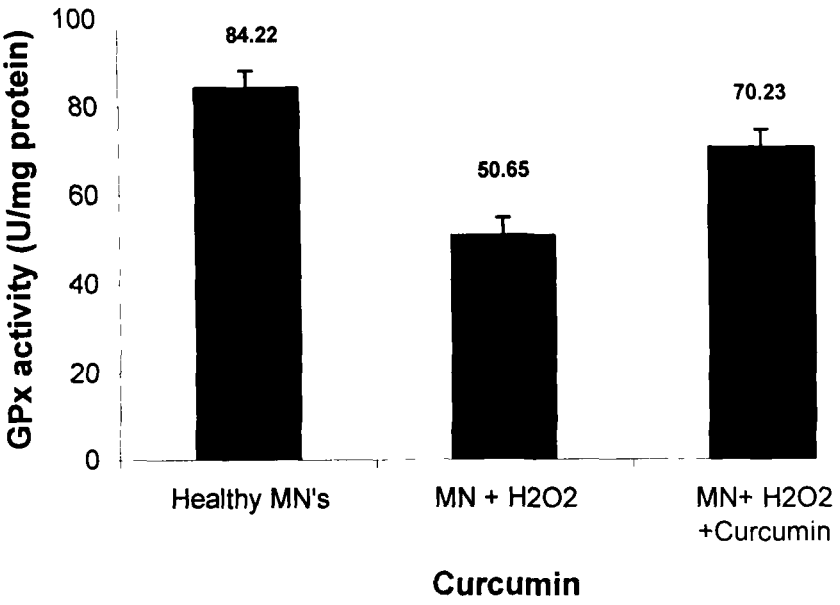


FIGURE 21: Healthy Control Monocytes: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus Curcumin (20 µg/ml) on GPx activity in healthy control human monocytes: Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

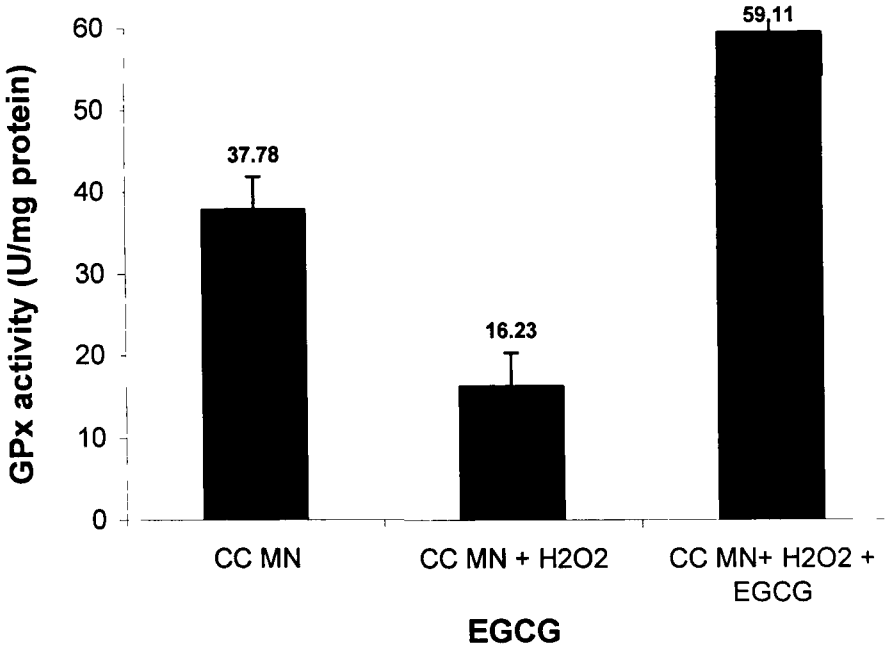


FIGURE 22: Cervical Cancer Monocytes: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus EGCG (20 μg/ml) on GPx activity in cervical cancer monocytes: Data are mean (±S.E.) of 3 experiments. (p<0.001).

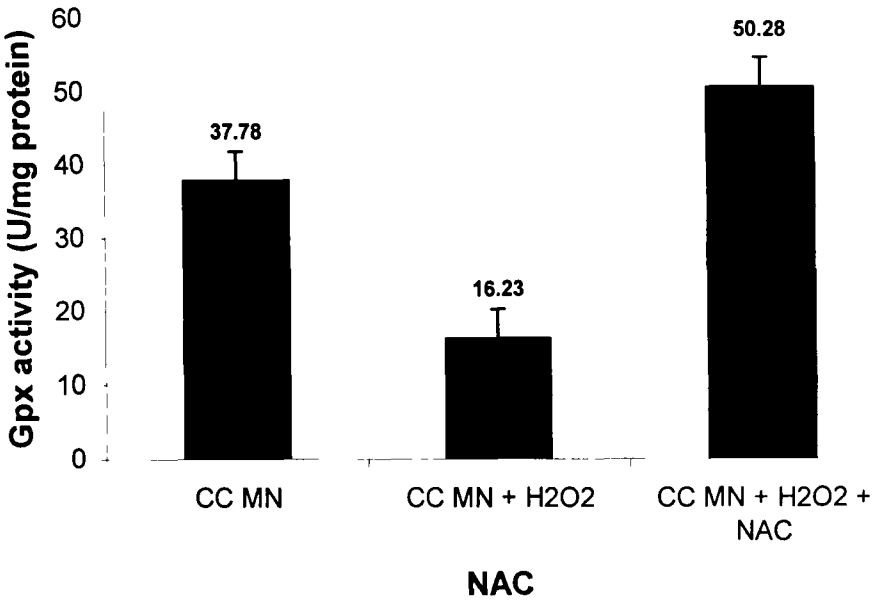


FIGURE 23: Cervical Cancer Monocytes: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus NAC (10 mM) on GPx activity in cervical cancer monocytes: Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

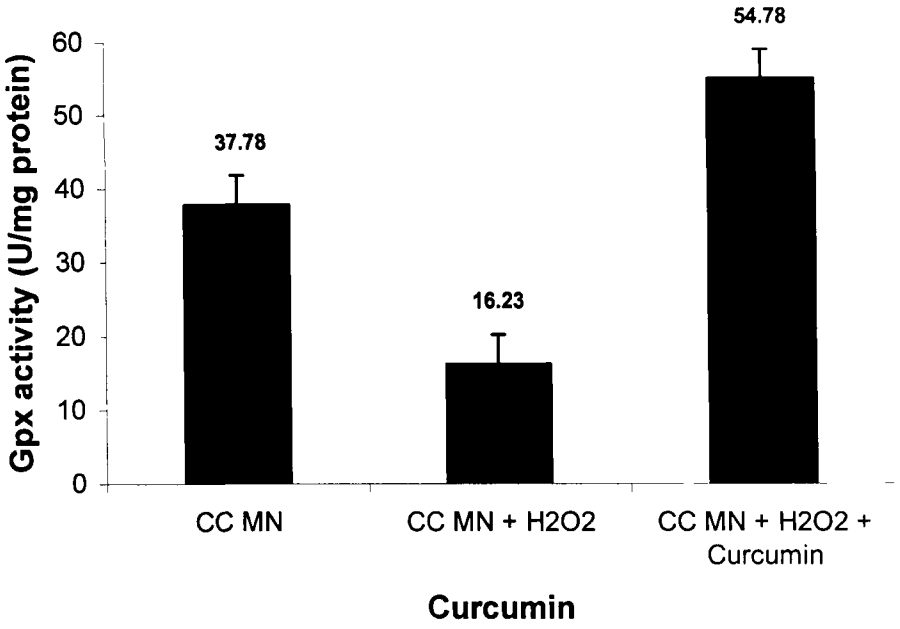


FIGURE 24: Cervical Cancer Monocytes: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus Curcumin (20 µg/ml) on GPx activity in cervical cancer monocytes: Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

(D). Model System – HeLa Cell Lines: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus EGCG (20 µg/ml), NAC (10 mM) and Curcumin (20 µg/ml) on the GPx activities in HeLa cell lines.

Thereafter, the next model system for competitive probe was HeLa cell lines. Thus, HeLa cell lines were co-cultured separately for 24 hours with 10nM of H_2O_2 along with 20 µg/ml of EGCG. As evident from Fig. 25, a suppressed the GPx activity by ~2.06-folds (n=3; $p<0.001$) was observed in HeLa cell lines was observed in comparison to healthy control monocytes (Fig. 21). The suppressed GPx activity in untreated cervical cancer monocytes was further suppressed by ~ 2.34-folds (Fig. 25; n=3; $p<0.001$) by the addition of 10nM of H_2O_2. However, HeLa cell line cultures receiving H_2O_2 along with EGCG showed amelioration by ~3.21-folds (Fig. 25; n=3; $p<0.001$) in the H_2O_2-mediated suppressed GPx activity.

Next, an attempt was also made to probe the competitive effect H_2O_2 versus NAC and Curcumin on the GPx activity in HeLa cell lines. As evident from the data, 10 mM NAC and 20 µg/ml Curcumin were found to ameliorate by ~2.95-folds (Fig. 26; n=3; $p<0.001$) and ~ 3.05-folds (Fig. 27; n=3; $p<0.001$) respectively the suppressed GPx activity in H_2O_2 treated HeLa cell lines.

Analysis of the data depicted in Fig 25-27, showed that the capability of ameliorating the suppressed GPx activity in HeLa cell lines due to H_2O_2 treatment was found to be of the order of EGCG (~3.21-folds) > Curcumin (~3.05-folds) ≥ NAC (~2.95-folds).

(i). Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined in 24 hr culture supernatants of cervical cancer monocytes (CC MN) and HeLa cell lines that were cultured for 24 hrs without and with either EGCG (20 µg/ml), NAC (10 mmol/l) and Curcumin (20 µg/ml). GPx activity was determined in culture supernatants at 24 h. Healthy human monocyte cultures served as controls. As evident from Fig. 28, cervical cancer monocytes and HeLa cell lines showed suppressed GPx activity in comparison to healthy control monocytes. However, co-culturing the above cancerous cells with EGCG, NAC or Curcumin resulted in amelioration of GPx activity. The order of amelioration of GPx activity was EGCG > NAC > Curcumin, and that, the amelioration capacity of EGCG was somewhat stronger in cervical cancer monocytes than in HeLa cell lines.

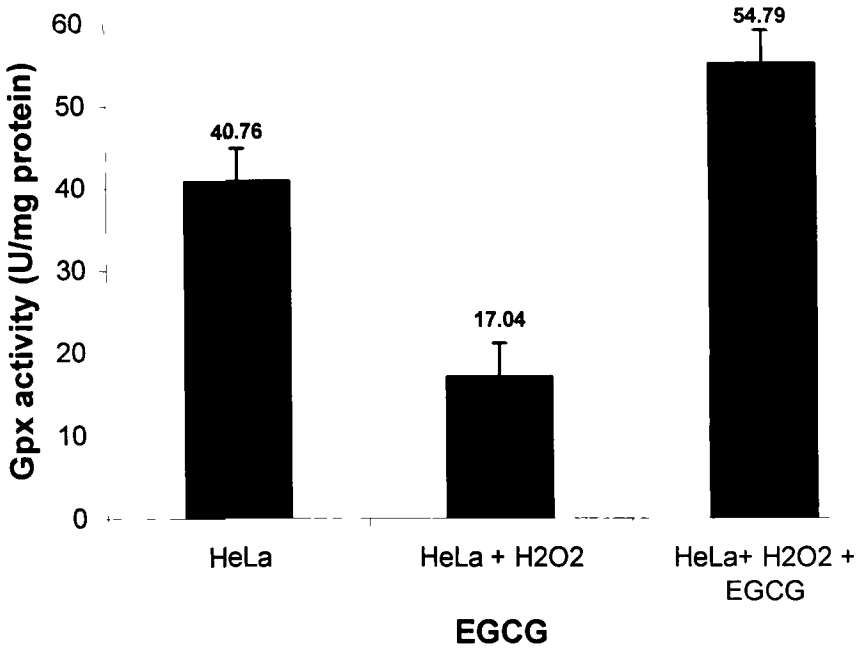


FIGURE 25: HeLa Cell Lines: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus EGCG (20 μg/ml) on GPx activity in Hela cell lines. Data are mean (±S.E.) of 3 experiments. (p<0.001).

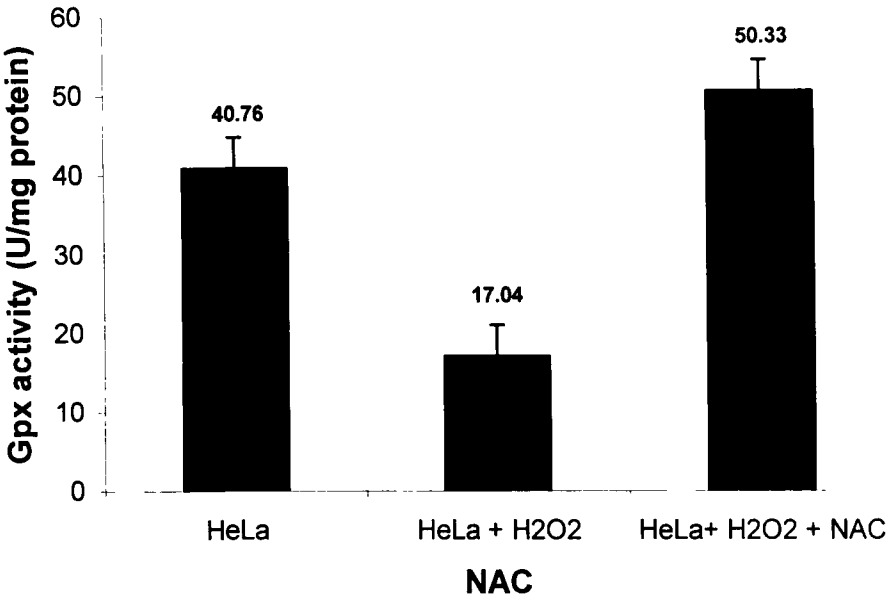


FIGURE 26: HeLa Cell Lines: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus NAC (10 mM) on GPx activity in HeLa cell lines. Data are mean (±S.E.) of 3 experiments. (p<0.001).

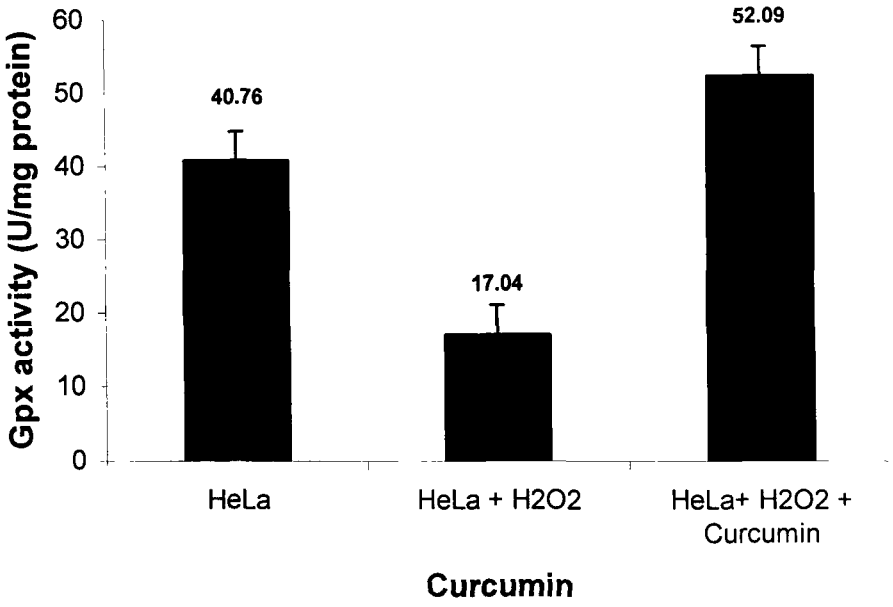


FIGURE 27: HeLa Cell Lines: Competitive Effect of Hydrogen peroxide (H_2O_2) (10 nM) versus Curcumin (20 μg/ml) on GPx activity in HeLa cell lines. Data are mean (±S.E.) of 3 experiments. ($p<0.001$).

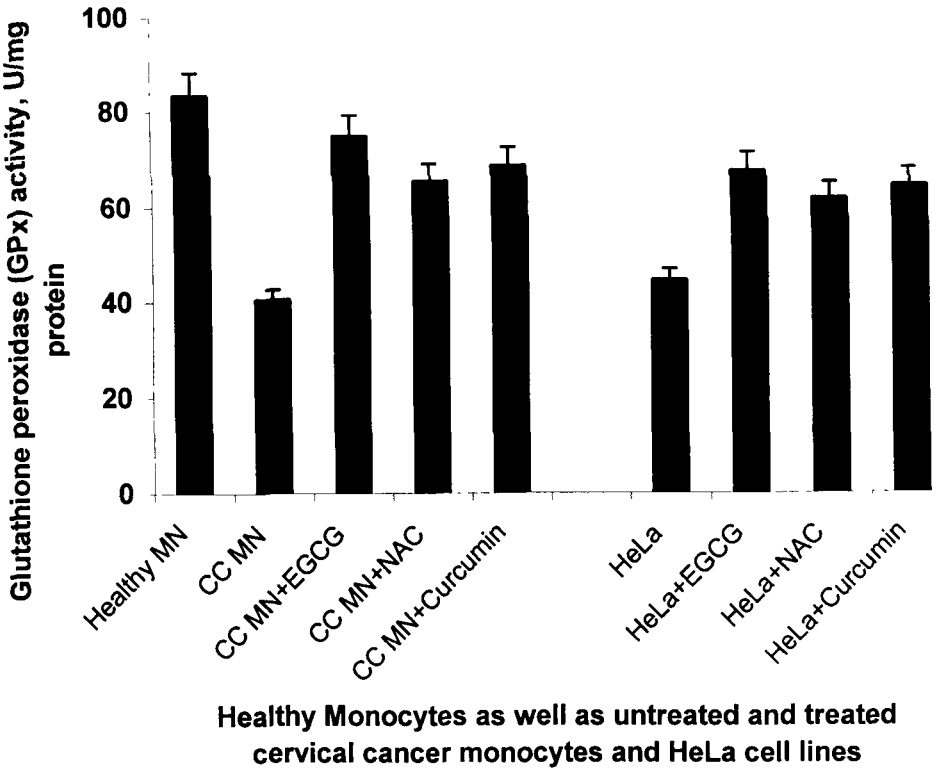


FIGURE 28: Determination of Glutathione peroxidase (GPx) activity. Cervical cancer monocytes (CC MN) and HeLa cell lines were cultured for 24 hrs without and with either EGCG (20 μg/ml), NAC (10 mmol/l) and Curcumin (20 μg/ml). GPx activity was determined in culture supernatants at 24 h. Healthy human monocyte cultures served as controls. Data are mean (±S.E.) of 5 experiments. ($p<0.001$ for all).

(ii). Caspase Assay: Effect of EGCG on Caspases-3, 8 and 9 in 24 hr culture supernatants of cervical cancer monocytes and HeLa cell lines.

Induction of EGCG-induced apoptosis was ascertained by measuring the activity of caspase-3, caspase-8 and caspase-9-like proteases in EGCG treated and untreated monocytes from cervical cancer patients as well as HeLa cell lines.

Monocyte and HeLa cell line cultures treated with EGCG and cultured for 24 hrs exhibited significantly higher activity of caspase-3 followed by caspase-8 and caspase-9 like proteases, thereby indicating that the activation of caspase-3 like proteases was associated with reduced cell survival and apoptotic death of EGCG-treated cervical cancer monocytes and HeLa cell lines (Figs. 41-46).

Cervical cancer monocytes and HeLa cell lines devoid of any EGCG treatment exhibited a caspase-3 activities to the order of only 5.88 pMol unit hydrolyzed/minute and 6.39 pMol unit hydrolyzed/minute respectively (Figs. 29 and 30; n=5; $p<0.001$ for both). On the contrary, co-culturing with 20 μg/ml of EGCG, the caspase-3 activities in cervical cancer monocytes and HeLa cell lines was found to be of the order of 134.96 pMol unit hydrolyzed/minute and 193.87 pMol unit hydrolyzed/minute respectively (Figs. 29 and 30; n=5; $p<0.001$ for both).

Thereafter, an attempt was made to assess the effect of EGCG on caspase-8. Cervical cancer monocytes and HeLa cell lines devoid of any EGCG treatment exhibited caspase-8 activities be of the order of only 2.93 pMol unit hydrolyzed/minute and 4.70 pMol unit hydrolyzed/minute respectively (Figs. 31 and 32; n=5; $p<0.001$ for both). On the contrary, co-culturing with 20 μg/ml of EGCG, the caspase-8 activities in cervical cancer monocytes and HeLa cell lines was found to be of the order of 109.24 pMol unit hydrolyzed/minute and 153.35 pMol unit hydrolyzed/minute respectively (Figs. 31 and 32; n=5; $p<0.001$ for both).

Next, an attempt was made to assess the effect of EGCG on caspase-9. Monocytes from patients with cervical cancer and HeLa cell lines devoid of any EGCG treatment exhibited caspase-9 activities to be of the order of only 2.62 pMol unit hydrolyzed/minute and 5.74 pMol unit hydrolyzed/minute respectively (Figs. 33 and 34; n=5; $p<0.001$ for both). On the contrary, co-culturing with 20 μg/ml of EGCG, the caspase-9 activities in cervical cancer monocytes and HeLa cell lines was found to be

of the order of 49.64 pMol unit hydrolyzed/minute and 69.62 pMol unit hydrolyzed/minute respectively (Figs. 33 and 34; n=5; $p<0.001$ for both).

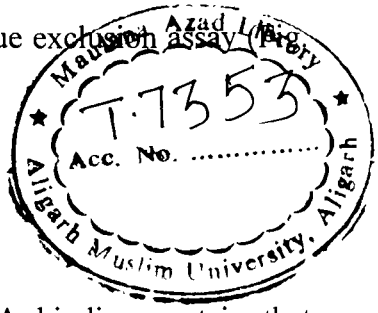
The above apoptotic effect of EGCG was further ascertained by the results obtained from cervical cancer monocytes and Hela cell lines that were pre-treated with cell permeable inhibitor Z-VAD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor) and Z-LEHD-FMK (caspase-9 inhibitor) followed by EGCG for 24 hrs (Figs 35-37; $p<0.001$ for all). In this case, the cell viability assay showed that the presence of Z-VAD-FMK inhibitor blocked the effect of EGCG on the viability of cancer monocytes.

(iii). Effect of varying doses of EGCG on the viability of cervical cancer monocytes

To probe that whether EGCG affects the viability of cervical cancer cells, monocytes from randomly selected cervical cancer patients (n=3) as well as HeLa cell lines (n=3) were treated with varying doses (0 –20 μg/ml) of EGCG and cultured for 24 and 48 hors respectively. Thereafter, MTT assay determined the cell viability. The cell viability data is expressed as percent mean viable cells compared to untreated cells (taken as 100% viable) at different doses of EGCG. Our results show that EGCG were potent inhibitor of cell proliferation and the cell viability was around 50.01%, 38.11%, 31.23% and 26.12% after 24 hrs of treatment with 5, 10, 15 and 20 μg/ml of EGCG respectively (Fig. 38). Based on this data ($p<0.001$ for all), EGCG was used at a concentration of 5 μg in subsequent experiments as this concentration produced high magnitude inhibition in cell proliferation when compared to controls. On the contrary, HeLa cell lines co-cultured with EGCG showed negligible or low magnitude effects when harvested after 24 hours.

Thereafter, attempts were also made to assess the time-dependent EGCG induced effects on both cervical cancer monocytes as well as HeLa cell lines i.e. after 48 hrs of culture. The cell viability of cervical cancer monocytes treated for 48 hrs with varying doses of EGCG was recorded to be around 41.23%, 29.23%, 24.33% and 21.39% with 5, 10, 15 and 20 μg/ml of EGCG respectively (Fig. 34; n=3; $p<0.001$ for all). After 48 hrs of EGCG treatment, HeLa cell lines showed appreciable effects. The cell

viability of HeLa cell lines treated for 48 hrs with varying doses of EGCG was found to be around 80.27%, 64.44%, 58.05% and 55.13% with 5, 10, 15 and 20 μg/ml of EGCG respectively (Fig. 39; n=3; $p<0.001$ for all). Thus, comparative computational analysis of the data depicted in Fig. 39, exhibited EGCG to reduce the viability 1.95 folds, 2.20 folds, 2.39 folds and 2.58 folds more effectively in cervical cancer monocytes in comparison to HeLa cell lines with 5, 10, 15 and 20 μg/ml of EGCG respectively. Similar observations were made with Trypan blue exclusion assay (Fig. 40).



(iv). Western Blot for PARP cleavage and Bcl-2 expression

It is well established that PARP, the zinc-dependent DNA binding protein that specifically recognizes DNA strand breaks, is cleaved by caspase-3, and the cleavage of PARP is regarded as a specific marker of apoptosis (Rukmini et al., 2004, Islam et al., 2000) To confirm the apoptotic effects of EGCG in cervical cancer monocytes, a western blot was performed to examine the PARP cleavage. We observed that in EGCG (20ug/ml) treated cells, the content of the intact form (113 kDa) of PARP was decreased and its 89 kDa cleaved fragment was increased (Fig. 41). No changes were observed when samples were blotted by anti-ß-actin (Fig. 41). Also, high dose of EGCG (80 μg/ml) affected the Bcl-2 expression in HeLa cell lines at both 24 hrs and 48hrs (Fig. 42), and that, this is in accordance to our results obtained for Cell Cycle Analysis and FITC-Annexin-V Assay depicted below.

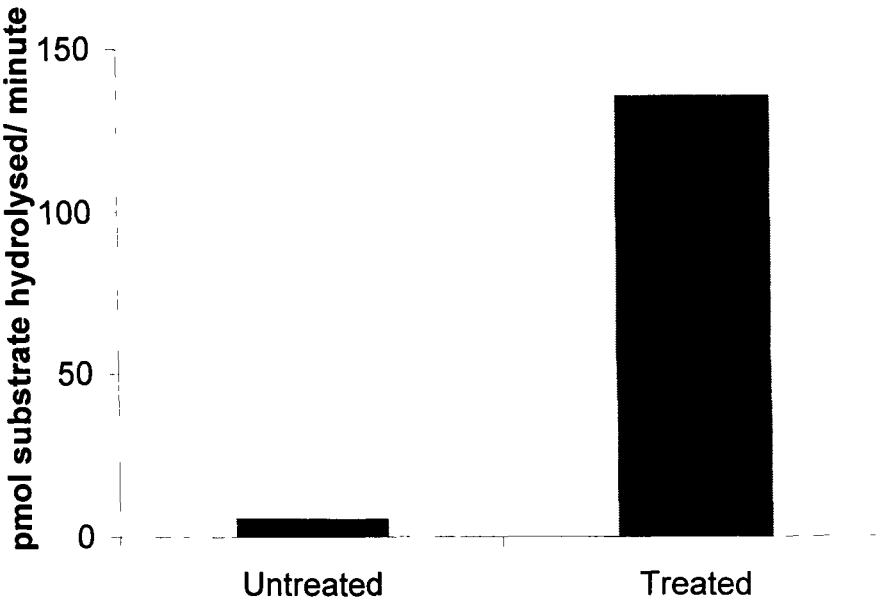


FIGURE-29: Caspase–3 activity assay: In 24 hr supernatant of monocyte culture from cervical cancer patient that were either treated with or without 20 µg/ml of EGCG ($p<0.001$). Data represents mean ± SEM of 10 experiments.

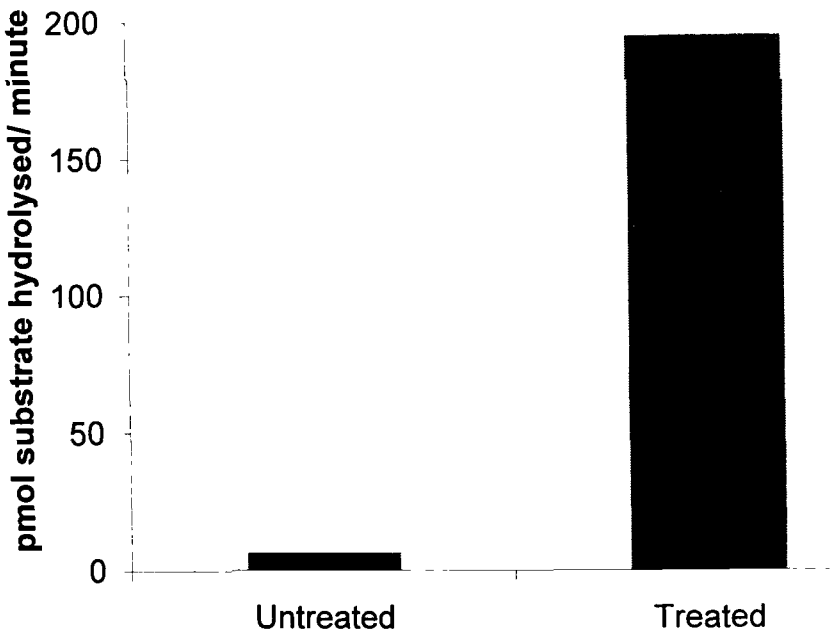


FIGURE-30: Caspase–3 activity assay: In 24 hr supernatant of HeLa cell lines culture that were either treated with or without 20 µg/ml of EGCG (p<0.001). Data represents mean ± SEM of 10 experiments.

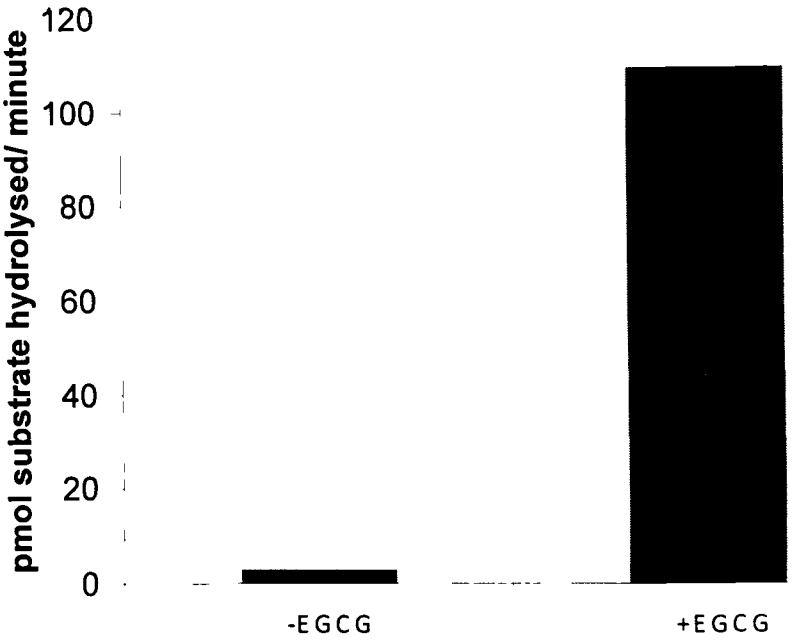


FIGURE-31: Caspase–8 activity assay: In 24 hr supernatant of monocyte culture from cervical cancer patient that were either treated with or without 20 μg/ml of EGCG (p<0.001). Data represents mean ± SEM of 10 experiments.

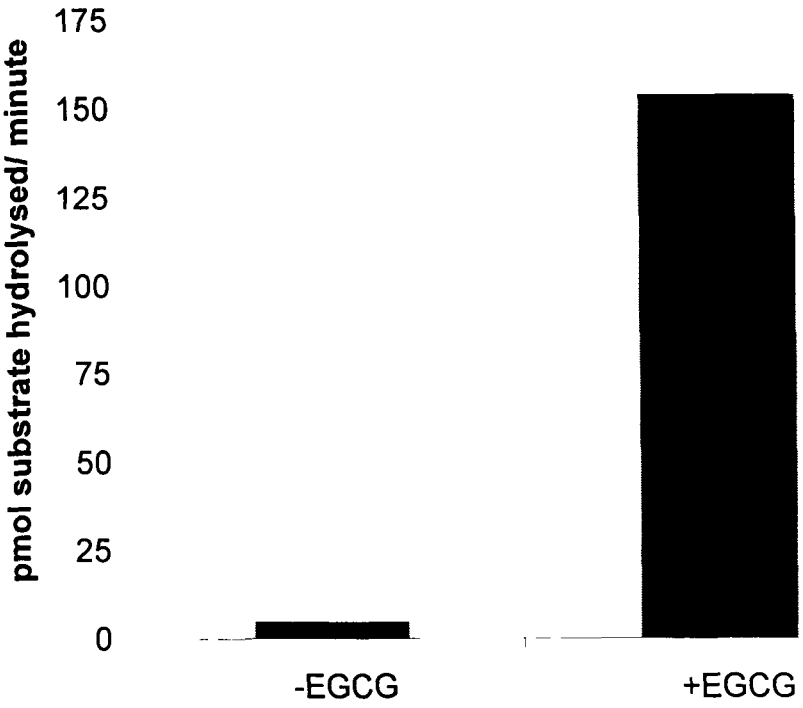


FIGURE-32: Caspase–8 activity assay: In 24 hr supernatant of HeLa cell lines culture that were either treated with or without 20 μg/ml of EGCG (p<0.001). Data represents mean ± SEM of 10 experiments.

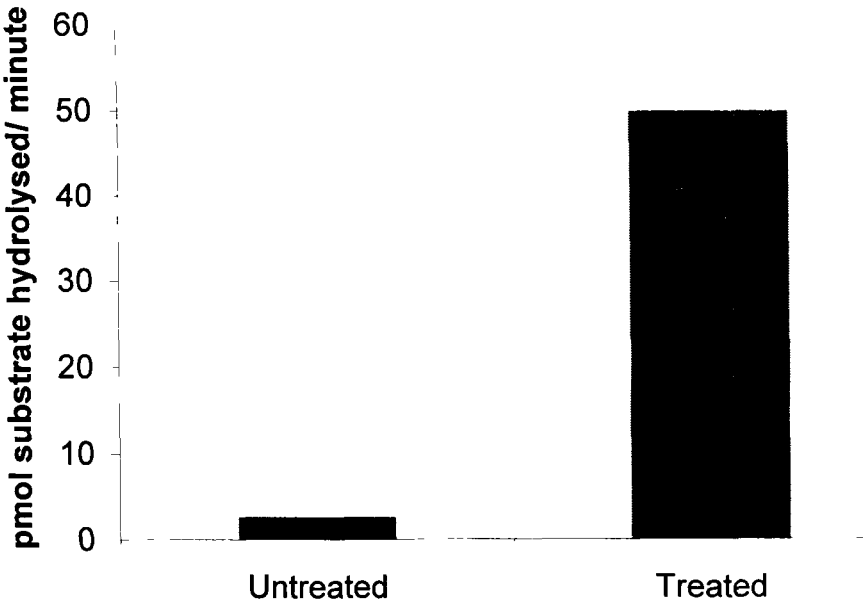


FIGURE-33: Caspase–9 activity assay: In 24 hr supernatant of monocyte culture from cervical cancer patient that were either treated with or without 20 μg/ml of EGCG ($p<0.001$). Data represents mean ± SEM of 10 experiments.

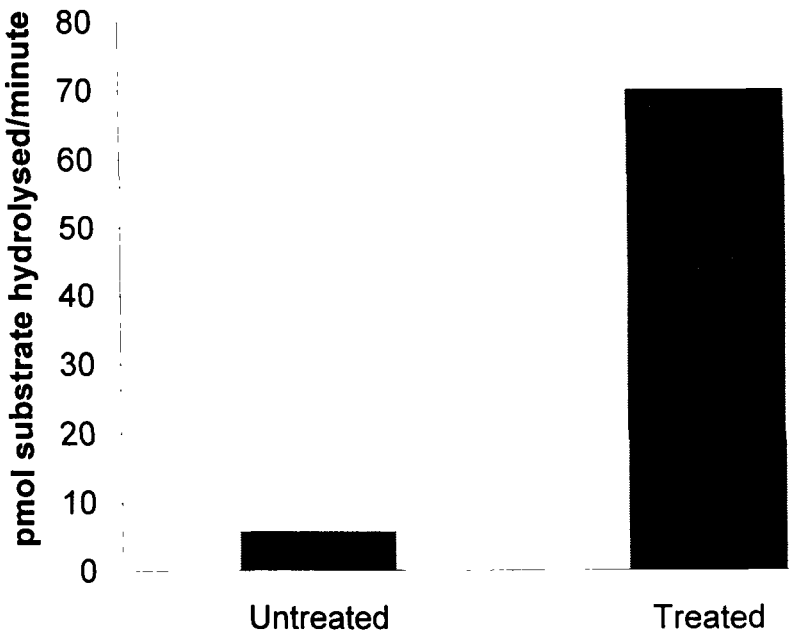


FIGURE 34: Caspase–9 activity assay: In 24 hr supernatant of HeLa cell lines culture that were either treated with or without 20 μg/ml of EGCG ($p<0.001$). Data represents mean ± SEM of 10 experiments.

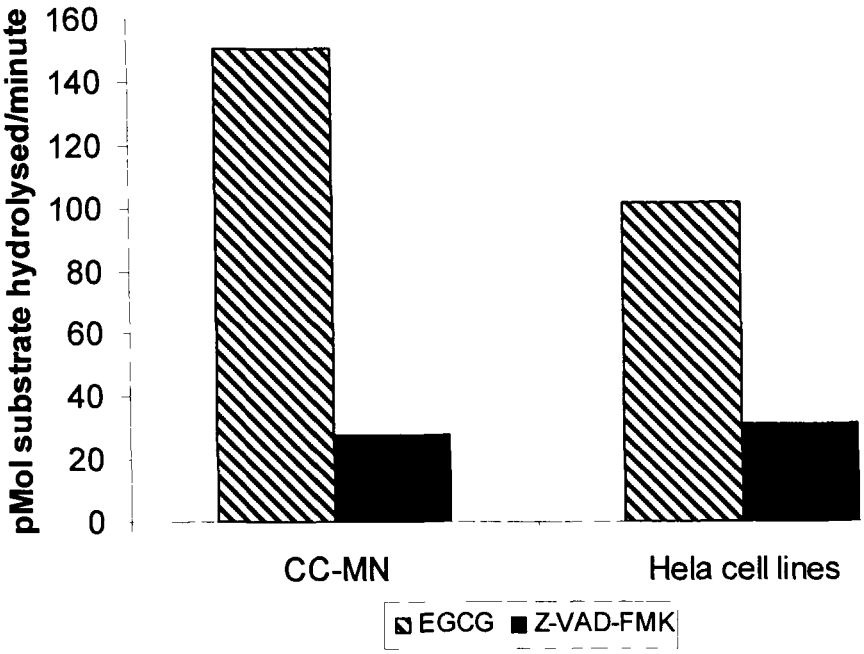


FIGURE-35: A comparative analysis of Caspase-3 expression in Cervical Cancer monocyte and HeLa cell lines treated with EGCG (lined bar) and an inhibitor of Caspase-3 (Black bar). Data represents mean ± SEM of 3 experiments

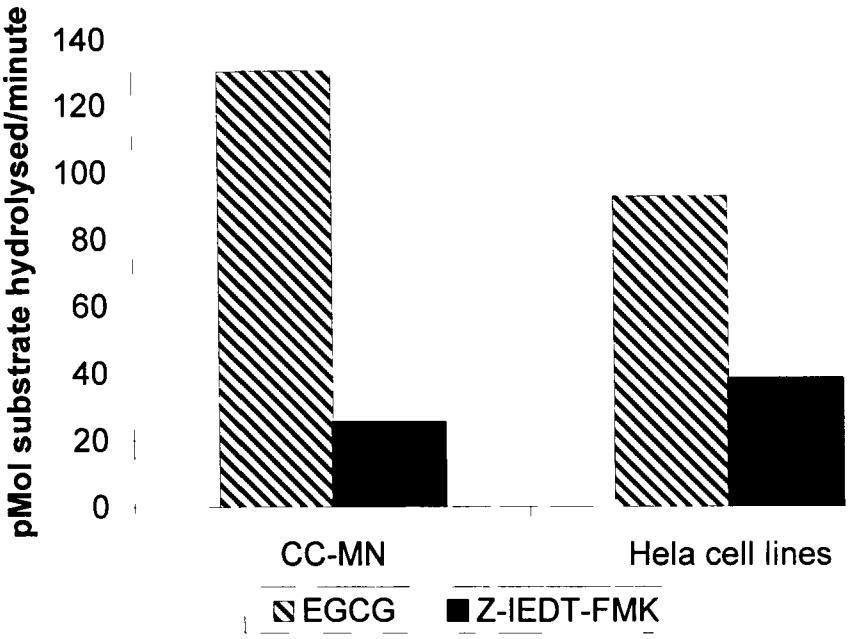


FIGURE-36: A comparative analysis of Caspase-8 expression in Cervical Cancer monocyte and HeLa cell lines treated with EGCG (lined bar) and an inhibitor of Caspase-8 (Black bar). Data represents mean ± SEM of 3 experiments.

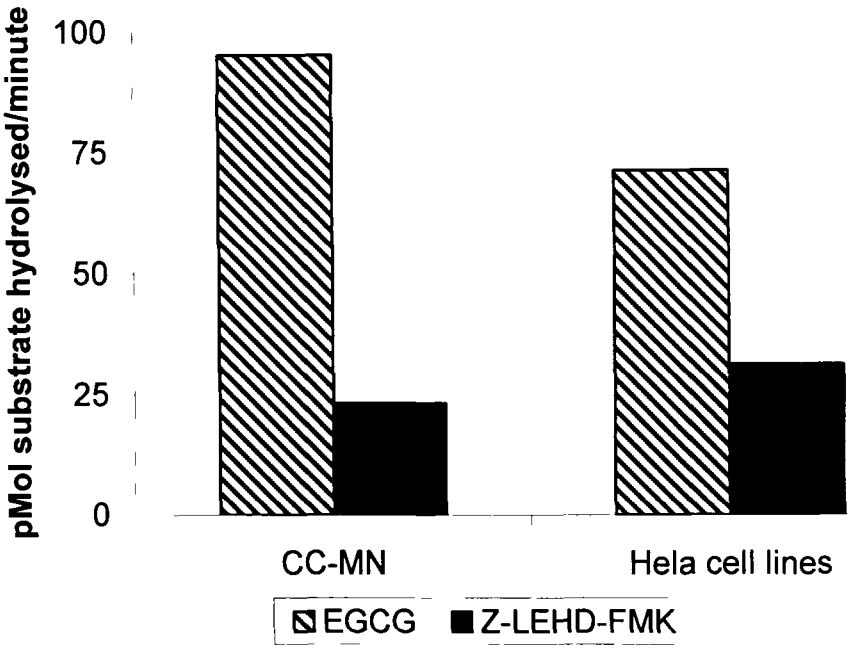


FIGURE-37: A comparative analysis of Caspase-9 expression in Cervical Cancer monocyte and HeLa cell lines treated with EGCG (lined bar) and an inhibitor of Caspase-9 (Black bar). Data represents mean ± SEM of 3 experiments.

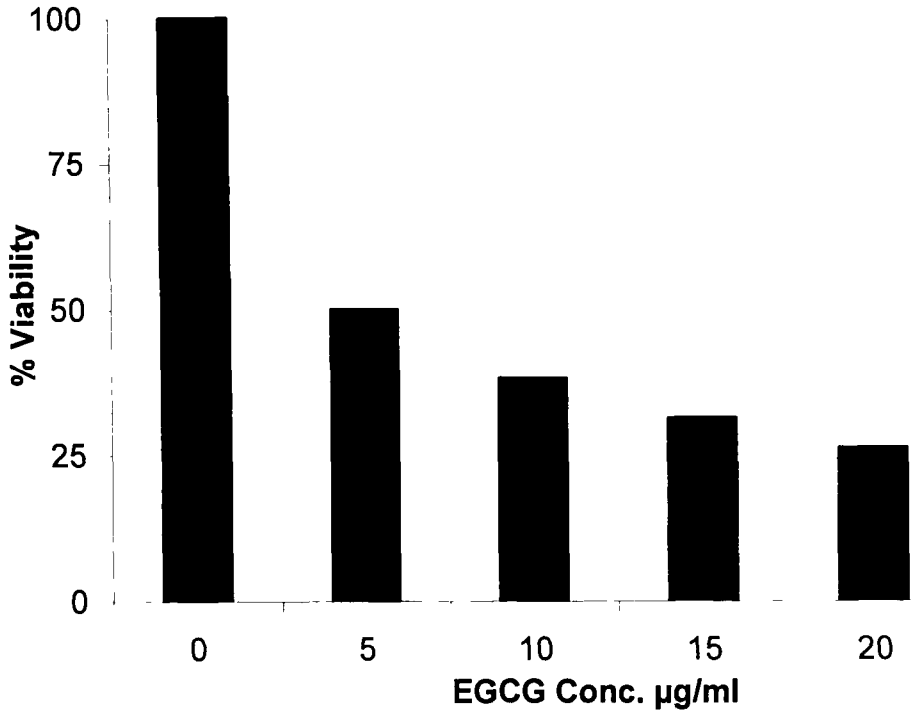


FIGURE 38: MTT cell viability assay: For dose response effect of EGCG (0-20 µg/ml) after 24 hr on cervical cancer monocytes (plz note: in HeLa cell line at 24 hr negligible effect of EGCG was observed). Data represents mean ± SEM of 3 experiments.

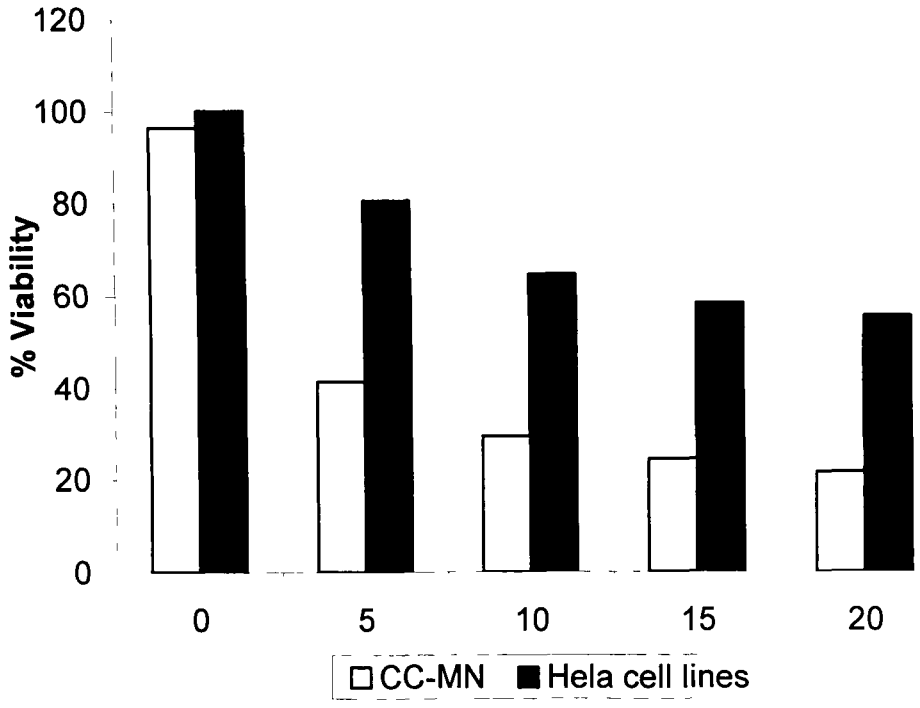


FIGURE 39: MTT cell viability assay: For dose response effect of EGCG (0-20 μg/ml) after 48 hr on cervical cancer monocytes (blue bar) and HeLa cell line (grey bar). Data represents the analysis of three independent experiments in duplicate, expressed as mean viable cells (± SEM) percentage of controls.

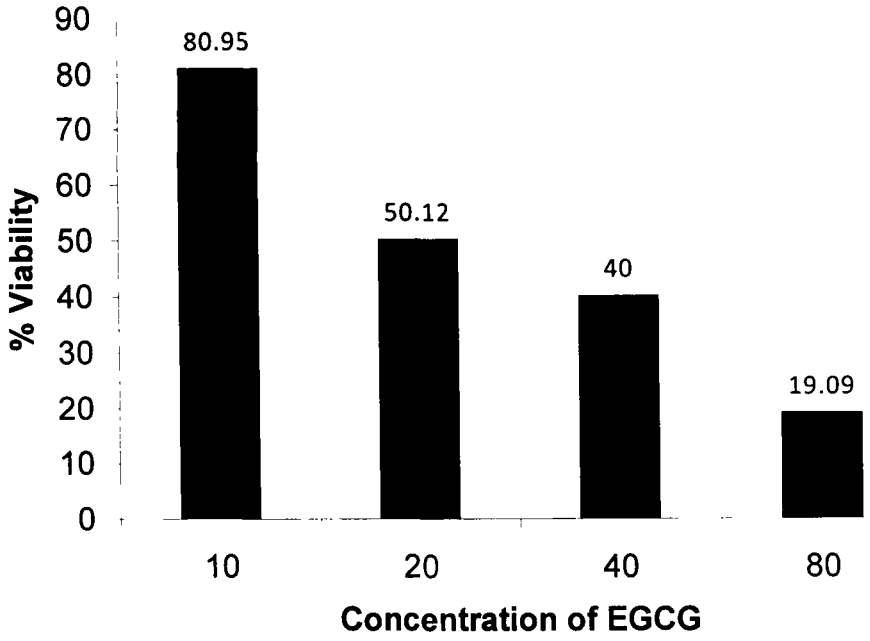


FIGURE-40: Trypan blue cell viability assay: Viability of HeLa cells following EGCG treatment. HeLa cells were incubated with EGCG (0-80 μg/ml 48hr) and the number of viable cells is counted by trypan blue exclusion assay. Cell viability is represented as percent viable cells, where the vehicle treated cells are regarded as 100%. Data represents the analysis of three independent experiments in duplicate, expressed as mean viable cells (± SEM) percentage of controls.

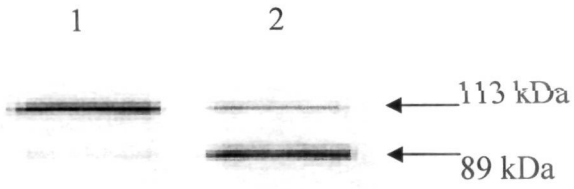


FIGURE 4: Immunoblot Analysis of PARP cleavage: the HeLa cell lysate was prepared following treatment and 50 µg of protein was subjected to 12% tris glycine gel electrophoresis, followed by immunoblot analysis and chemiluminescence detection. Lane#1 = untreated control cancer cells; Lane#2 = EGCG-treated cancer cells showing PARP cleavage (113 kDa i.e. upper row and 89 kD i.e. lower row).The data shown here are from a representative experiment repeated three times with similar results.

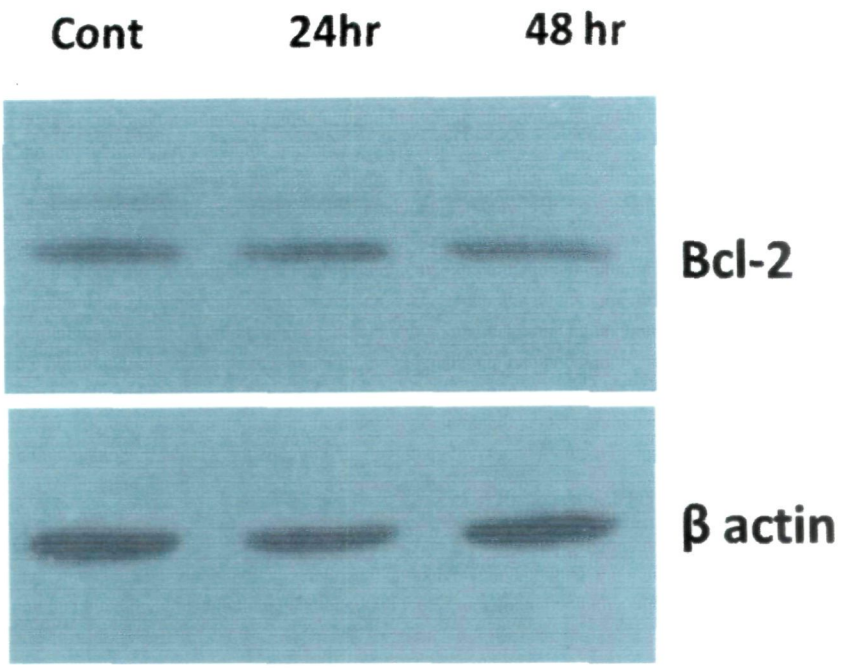


Figure 42: Western blot: Effect of EGCG on protein expression of bcl-2 in HeLa cell line. The cells were treated with Vehicle or EGCG (80 μg/ml for 24 and 48hr), and then harvested. Total cell lysate were prepared and 50ug protein was subjected to SDS-Page followed by Western blot analysis and chemiluminescent detection. Data shown below the blots represents fold modulation in protein expression normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

(v) Growth kinetic analysis

The growth kinetic analysis was carried out to investigate the antiproliferative effect of EGCG. In this study, HeLa cell lines were co-cultured with 0-80 μg/ml of EGCG for 24 and 48hrs. The growth analysis curves depicted in Fig. 43 exhibited a marginal decrease in proliferation rate (N_t/N_o) at 24 hrs. On the contrary, at 48hrs, this decrease in proliferation of cell became more significant / more appreciable in a dose-dependent manner (Fig. 43). Thus, the results obtained are in accordance to our observations made in the above cell viability assays at 48 hrs.

(vi). Cell Cycle Analysis and FITC-Annexin V Assay

As we all know that, cells proceeds from $G_1 - S - G_2$ but after G_2 cells undergo mitosis and cell proliferates. In this study we were keen to observe the effect of EGCG on these dividing cells. As evident from Fig. 44, it was observed that at 80 μg/ml of EGCG, there is cell arrest at G_2/M phase of cell cycle, the number of cell at this phase were significantly increases at 48hr which is correlated with the growth kinetics of cells at the same time point. This arrest may due to DNA damage or by some other reasons but DNA damage is the most evident cause of cell arrest at G_2/M phase. At 10 and 20 μg/ml concentration there was no significant changed was observed at all the time point (data not shown). The increase in sub G1 population of cells at 48 hrs by EGCG (40 and 80 μg/ml) treatment also indicates the apoptotic cell death, which is further confirmed by Annexin-V assay (Fig. 45).

(vii). Effect of EGCG on cytomorphological and histomorphological appearance of cervical carcinoma (biopsy) and HeLa cell lines

Cytomorphological investigation for the appearance of carcinoma cells co-cultured along with 20 μg/ml of EGCG (Fig.46) show better differentiation of nuclear and cellular outlines as well as increased eosinophilia of cytoplasm along with compact and condensed chromatin as compared to cells devoid of any EGCG (Fig. 47) (PAP X 400). Also, effects of EGCG on non-neoplastic ectocervical cytosmears was probed where enhanced keratanization process in comparison to control samples devoid of EGCG was observed, and that, no apoptotic cells were observed (data not shown).

Histomorphological data showed that in comparison to carcinoma cells devoid of any EGCG treatment (Fig. 48), co-culturing with EGCG (20 μg/ml) resulted in augmented

eosinophilia of the cytoplasm along with apoptotic body (arrow) having pyknotic nucleus and dense eosinophilic cytoplasm (Fig. 49) (H&E X 400). Similar observations were made in HeLa cell lines (Fig. 50).

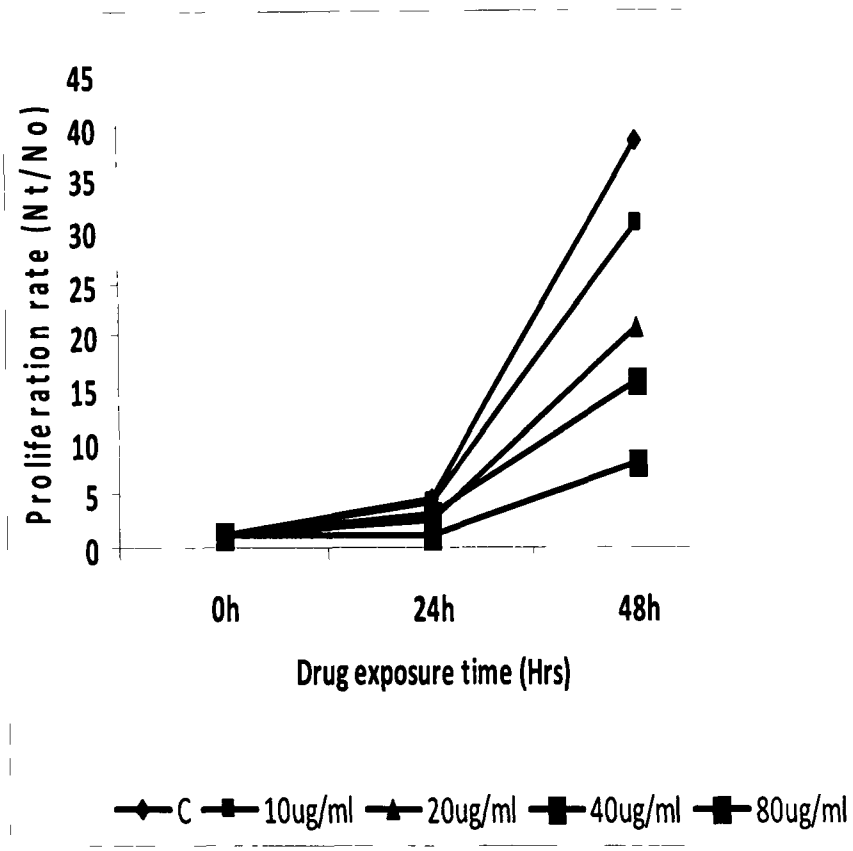


Figure 43: Proliferation Rate by Flow cytometer: HeLa cell were treated with Vehicle or varying concentrations of epigallocatechin gallate for 24 and 48 hr, and analysed by flow cytometry. The data shown here are from a representative experiment repeated three times with similar results. At 10 and 20 μg/ml effect are negligible (Data not shown).

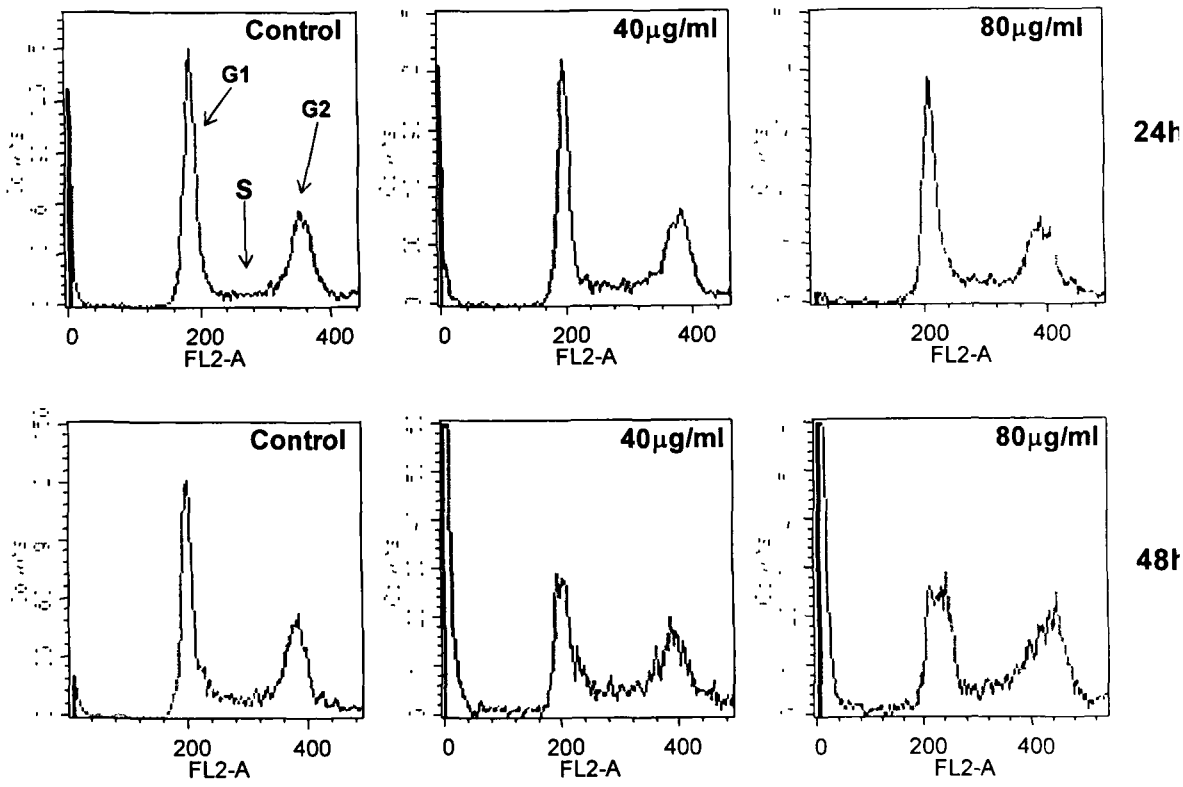


Figure 44: Cell cycle analysis by Flow cytometer: HeLa cell were treated with Vehicle or epigallocatechin gallate (40 & 80 μg/ml) for 24 and 48 hr, and analysed by flow cytometry. The data shown here are from a representative experiment repeated three times with similar results. At 10 and 20 μg/ml effect are negligible (Data not shown).

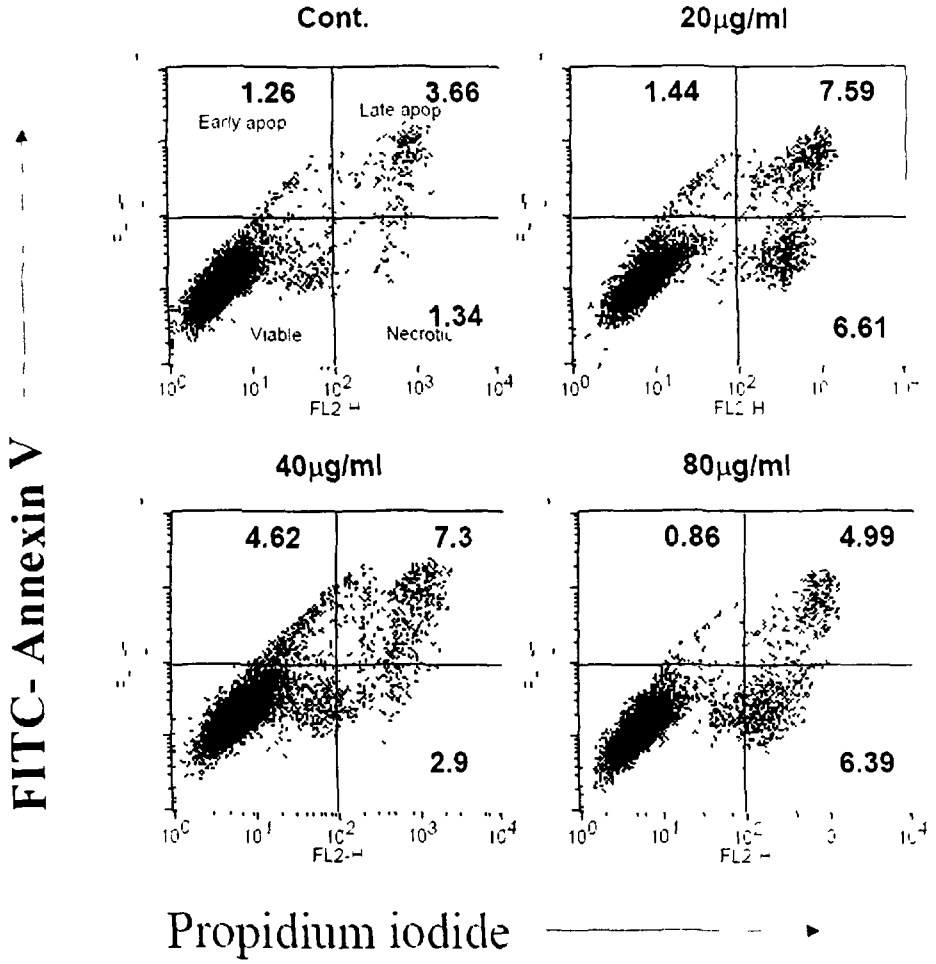


Figure-45: Apoptotic analysis by Annexin–V FITC: Dose and time dependent quantification of apoptosis in HeLa by flow cytometry. Cells were treated at the indicated dose and time with EGCG, labeled with Annexin-V FITC and PI by using an apoptosis kit followed by flow cytometry. At the concentration of 10 µg/ml, effects are negligible (Data not shown). The data shown here are from a representative experiment repeated three times with similar results.

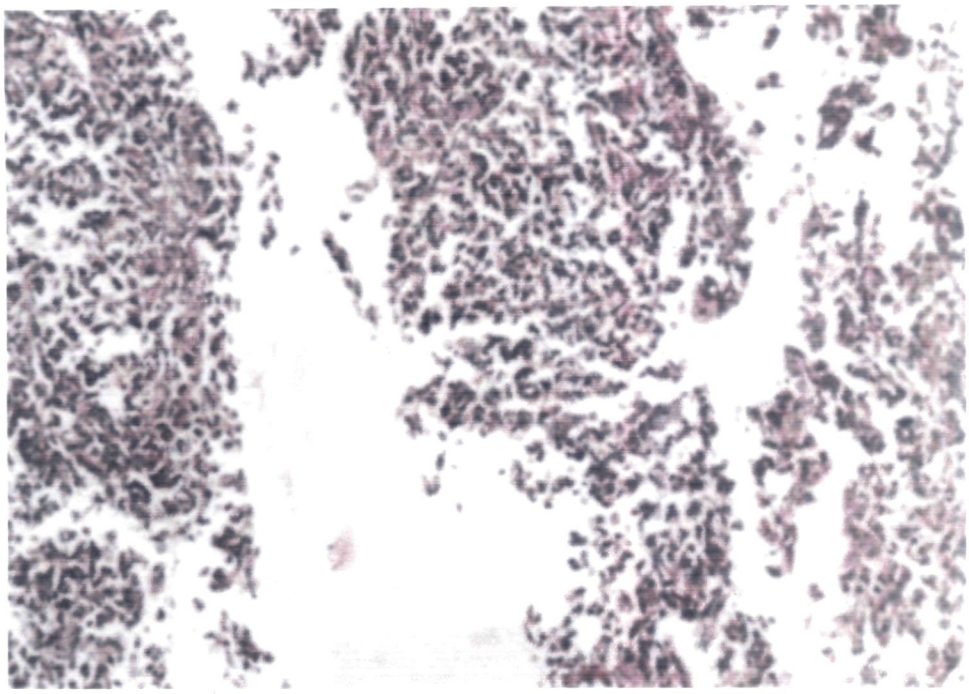


FIGURE 46: Effect of EGCG on cytomorphological investigation for the appearance of carcinoma cells co-cultured along with 20 μg/ml of EGCG.

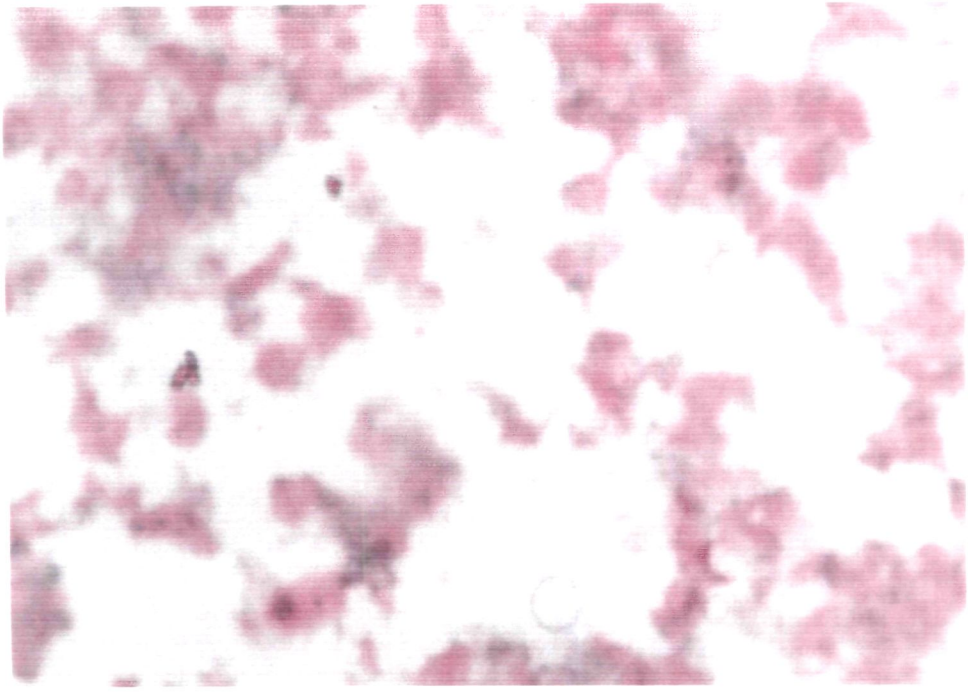


Figure 47: Effect of EGCG on cytomorphological appearance show better differentiation of nuclear and cellular outlines as well as increased eosinophilia of cytoplasm along with compact and condensed chromatin as compared to cells devoid of any EGCG (Fig. 46A) (PAP X 400).

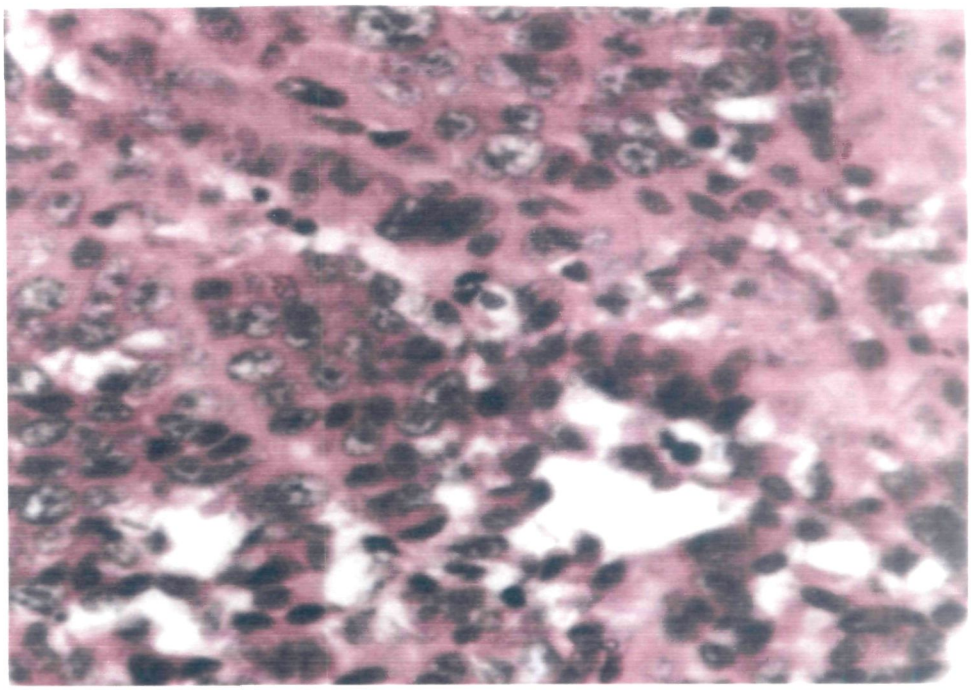


Figure 48: Histomorphological data showed that in comparison to carcinoma cells devoid of any EGCG treatment.

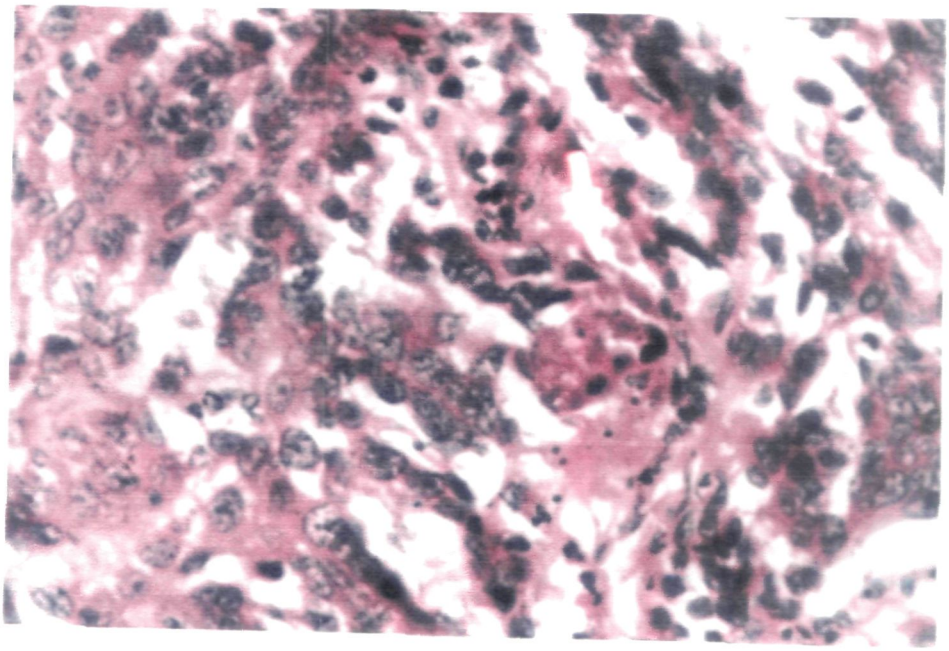


Figure 49: Histomorphological data showed that in comparison to carcinoma cells co-culturing with EGCG (20 μg/ml) resulted in augmented eosinophilia of the cytoplasm along with apoptotic body having pyknotic nucleus and dense eosinophilic cytoplasm (Fig. 47A) (H&E X 400).

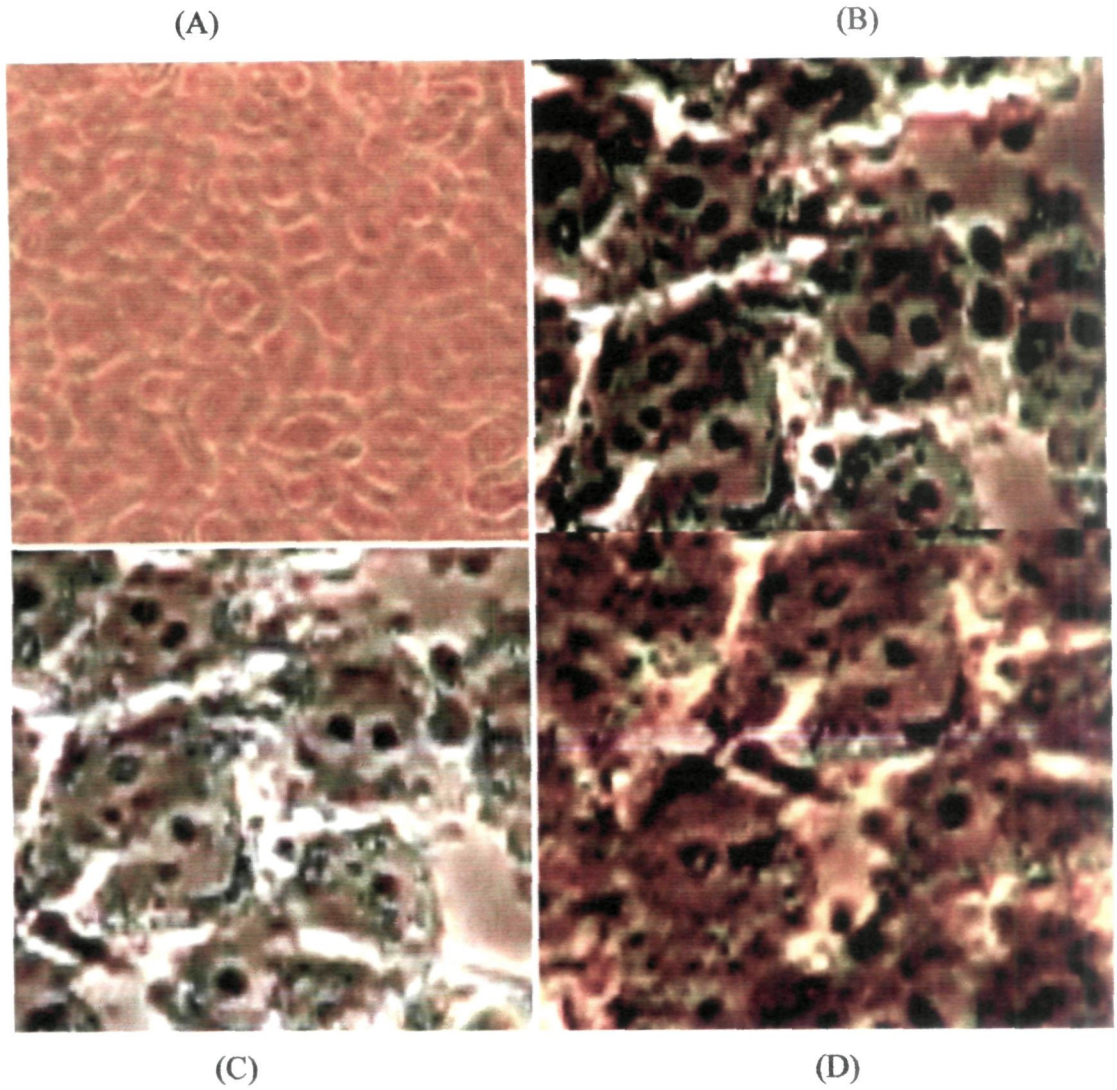


FIGURE 50: Morphological analysis of HeLa cell lines treated with EGCG (A) Monolayer of HeLa Cell line (B) Control at 48hr.(C&D)-Treated with 40 & 80 (µg/ml) EGCG at 48 hr, picture were taken by using a phase contrast microscope at 400X magnification. The data shown here are from a representative experiment repeated three times with similar results.

DISCUSSION

Globally, cervical cancer takes the lives of more than 250000 women each year, particularly in under-resourced areas of low-, middle-, and high-income countries. Options for cancer control and treatment have reached a point that there are interventions for control that could be adopted for virtually every resource and demographic situation (Cain et al., 2009). Women die despite the availability of attractive control options, which means that educating policy makers, women's health professionals, as well as women themselves, must become a major focus for ongoing control of this disease. The human right to life, to prevention of suffering, and to education are all key rights linked to improving the control of cervical cancer and saving the lives of women, particularly in resource-poor parts of the world (Cain et. al., 2009).

Emerging evidence demonstrates that targeting the tumor proteasome is a promising strategy for cancer therapy (Yang et al., 2009). Results about the clinical application of specific proteasome inhibitors and natural products with proteasome-inhibitory activity for cancer prevention or therapy have been reviewed (Yang et al., 2009). Bortezomib, the reversible proteasome inhibitor that first entered clinical trials, has been studied extensively as a single agent and in combination with glucocorticoids, cytotoxic agents, immunomodulatory drugs and radiation as treatment for multiple myeloma and other hematological malignancies. There is less evidence of bortezomib's efficacy in solid tumors. Novel irreversible proteasome inhibitors, NPI-0052 and carfilzomib, have also been developed and clinical trials are underway therapy (Yang et al., 2009). Natural products with proteasome-inhibitory effects, such as green tea polyphenol (-) epigallocatechin-3-gallate (EGCG), soy isoflavone genistein, and the spice turmeric compound curcumin, have been studied alone and in combination with traditional chemotherapy and radiotherapy against various cancers. There is also interest in developing these natural compounds as potential chemopreventive agents (Yang et al., 2009). Thus, in the present study, EGCG – a natural antioxidant was employed to explore the potential chemopreventive mechanism in cervical cancer.

The major constituents of green tea are the Flavan-3-ols (catechins) that include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC). These polyphenol compounds have antioxidative and anti-mutagenic properties. A variety of antioxidants and

chemopreventive agents are cytotoxic to cancer cells. Cellular growth inhibition by green tea has been established in many tumor cells where EGCG has been a prime candidate for mediating this effect. Recently, anti-proliferative and anti-cancer action of EGCG has been reported in cancer cell lines (Ji et al., 2006; Pasckha et al., 1998; Elatter and Virji, 2000; Jung et al., 2001; Ahn et al., 2003; Huh et al., 2004). A vast variety of naturally occurring substances are known to protect against experimental carcinogenesis. It is becoming increasingly evident that certain phytochemicals, particularly those included in our daily diet, may have important cancer chemopreventive properties (Sanaha et al., 1997). Some anti-inflammatory chemopreventive agents have been found to suppress growth and proliferation of transformed or malignant cells through induction of programmed cell death or apoptosis (Bellosillo et al., 1998).

Regarding EGCG, population-based and clinical studies indicate that the antioxidant properties of green tea may help prevent atherosclerosis, particularly coronary artery disease. It has been reported that 70% EGCG is a potent tool in nutritional arsenal not only as an antioxidant, but to address arterial inflammation. Highly sensitive C-reactive protein (hs-CRP) is a marker of arterial inflammation. Inflammation is also believed to play a role in heart disease; EGCG is a potent anti-inflammatory. According to Japanese research, green tea reduces the levels of LDL or 'bad' blood cholesterol, thereby reducing the risk of coronary heart disease. Research also indicates that tea polyphenols may reduce the activity of platelets, which are the clotting agents of the blood. This is good, because 'sticky' blood is more likely to form artery-blocking clots. Green tea has demonstrated an ability to lower total cholesterol and raise HDL ("good") cholesterol in both animals and people. This suggests that polyphenols in green tea may block the intestinal absorption of cholesterol and promote its excretion from the body. EGCG has been reported to inhibit lipid peroxidation, an oxidative process implicated in several pathologic conditions, including atherosclerosis. It has been suggested that EGCG and other tea catechins suppress tumor promotion by inhibiting the release of tumor necrosis factor-alpha, which is believed to stimulate tumor promotion and progression of initiated cells as well as premalignant cells Furthermore, EGCG was shown to reduce specific binding of both the 12-Otetradecanoylphorbol-13-acetate (TPA)-type and the okadaic acid-type tumor promoters (the two major classes of tumor-promoting agents) to their

receptors. An EGCG act against urokinase, an enzyme often found in large amounts in human cancers, inhibits ornithine decarboxylase (a rate-limiting enzyme closely associated with tumor promotion), and blocks type-1 5alpha-reductase (5AR). Inhibitors of 5AR may be effective in the treatment of 5 alpha dihydrotestosterone-dependent abnormalities, such as benign prostate hyperplasia, prostate cancer, and certain skin diseases. EGCG, inhibits tumor growth by inhibiting VEGF induction in human colon carcinoma cells. In the in vitro studies, (-)-epigallocatechin gallate (EGCG) inhibited Erk-1 and Erk-2 activation in a dose-dependent manner. However, other tea catechins such as (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC) did not affect Erk-1 or 2 activation at a concentration of 30 M. EGCG also inhibited the increase of VEGF expression and promoter activity induced by serum starvation. In the in vivo studies, athymic BALB/c nude mice were inoculated subcutaneously with HT29 cells and treated with daily intraperitoneal injections of EC (negative control) or EGCG at 1.5 mg day mouse starting 2 days after tumour cell inoculation. Treatment with EGCG inhibited tumor growth (58%), microvessel density (30%), and tumour cell proliferation (27%) and increased tumour cell apoptosis (1.9-fold) and endothelial cell apoptosis (3-fold) relative to the control condition ($P< 0.05$ for all comparisons). EGCG may exert at least part of its anticancer effect by inhibiting angiogenesis through blocking the induction of VEGF (Fatima, Z., 2008). Tea polyphenol EGCG inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines (Barnes and Cave, 2003). Hyper-methylation of CpG islands in the promoter regions is an important mechanism to silence the expression of many important genes in cancer. Treatment of human esophageal cancer KYSE 510 cells with 5-50 microM of EGCG for 12-144 h caused a concentration- and time-dependent reversal of hypermethylation of p16(INK4a), retinoic acid receptor beta (RARbeta), O(6)-methylguanine methyltransferase (MGMT), and human mutL homologue 1 (hMLH1) genes as determined by the appearance of the unmethylation-specific bands in PCR. This was accompanied by the expression of mRNA of these genes as determined by reverse transcription-PCR (Zeeshan, 2008) thereby demonstrating for the first time the inhibition of DNA methylation by a commonly consumed dietary constituent and in turn suggesting for the potential use of EGCG *for the prevention or reversal of related gene silencing in* the prevention of carcinogenesis. In addition to this EGCG activates endothelial nitric oxide synthase by a PI3K-, PKA-, and Akt-dependent pathway, and leads to

endothelial-dependent vasorelaxation, the EGCG-induced endothelium-dependent vasodilation is primarily based on rapid activation of eNOS. This suggests that tea catechins may reduce the risk of cardiovascular (Zeeshan, 2008). Interleukin-1beta (IL-1beta) induced inflammatory response in arthritic joints include the enhanced expression and activity of matrix metalloproteinases (MMPs) and their matrix degrading activity contribute to the irreversible loss of cartilage and may also be associated with sustained chronic inflammation. EGCG inhibited the IL-1 beta-induced mRNA and protein expression of MMP-1, and MMP-13 in human chondrocytes. a differential dose-dependent effect of EGCG on the expression and activity of MMPs and on the activities of transcription factors NF-kappaB and AP-1 and provide insights into the molecular basis of the reported anti- inflammatory effects of EGCG (Zeeshan, 2008). It's well established that fruits and vegetables contain components like polyphenols, terpenes, alkaloids, and phenolics that may provide substantial health benefits beyond basic nutrition. Research over the last decade has shown that several micronutrients in fruits and vegetables reduce cancer (Aggarwal and Shishodia, 2006). The active components of dietary phytochemicals that most often appear to be protective against cancer are curcumin, genistein, resveratrol, diallyl sulfide, *S*-allyl cysteine, allicin, lycopene, capsaicin, diosgenin, 6-gingerol, ellagic acid, ursolic acid, silymarin, anethol, catechins, eugenol, isoeugenol, dithiolthiones, isothiocyanates, indole-3-carbinol, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, Vitamin C, d-limonene, lutein, folic acid, beta carotene, selenium, Vitamin E, flavonoids, and dietary fiber. These dietary agents are believed to suppress the inflammatory processes that lead to transformation, hyperproliferation, and initiation of carcinogenesis. Their inhibitory influences may ultimately suppress the final steps of carcinogenesis as well, namely angiogenesis and metastasis (Aggarwal and Shishodia, 2006).

Various workers on cervical cancer HeLa cell lines have extensively carried out studies in multiple directions, but no or little work has been done on cervical cancer monocytes. Thus, the present study involves investigations on the effect of EGCG – a green tea polyphenol as well as a natural antioxidant on two cervical cancer model systems, which are: **(a)** monocytes and biopsies from cervical cancer patients and **(b)** cervical cancer cell lines i.e. HeLa cell lines. As the active role-played by reactive oxygen species (ROS) in the cervical cancer is well established,

thus an attempt was made in the present study to probe natural compounds having antioxidant properties in arresting ROS in cancer cells. Thus, comparative efficacy of natural antioxidants was carried out on cervical cancer monocytes versus HeLa cell lines.

The most striking finding of the present study is the model system involving monocytes and biopsies from cervical cancer patients showed high magnitude anti-inflammatory as well as apoptotic effect of EGCG (0-100 μg/ml), while on the other hand, EGCG (0-100 μg/ml) exerted high magnitude anti-inflammatory but low apoptotic effect on HeLa cell lines. Glutathione directly reacts with ROS, and GPx catalyzes the removal of hydrogen peroxide (Yokoigawa et al., 2005). Decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms (Ottaviano et al., 2006). Co-culturing of cancerous (cervical) cells with NAC, turmeric or EGCG seems to induce apoptosis or acted as anti-inflammatory. Furthermore, apart from the above, a decline in GPx activity was observed in cervical cancer monocytes and cervical HeLa cells that were untreated or treated with H_2O_2 thereby correlating with earlier reports that substantial amounts of ROS are being generated in cancerous cells due to cellular activation (Islam et al., 2004). Enhancement / amelioration of GPx activity in cervical cancer monocytes and cervical HeLa cell cultures after addition of NAC, a precursor of in vivo antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Surprisingly, here augmented amelioration in GPx activity was observed when EGCG was co-cultured instead of NAC, indicating EGCG to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in cancerous cell. When compared to healthy monocytes, cervical cancer monocytes as well as HeLa cell lines exhibited an appreciable EGCG-mediated suppression by around 2.14-folds and 1.98-folds respectively in the GPx activity. On the contrary, upon co-culturing of cervical cancer monocytes and HeLa cell lines with varying doses of EGCG, an appreciable amelioration in the GPx activity was recorded. Interestingly, EGCG was found to possess a higher potential to ameliorate the GPx activity in cervical cancer monocytes than in HeLa cell lines. Similar were the observations recorded with NAC and turmeric. Furthermore, the present study shows enhanced GPx activity by EGCG, which correlated inversely with the downregulation of TNF-alpha, IL-1 and IL-6 protein expressions and ROS in cervical cancerous cells. As suggested by (Aggarwal

and Shishodia, 2006) as well as by the present study, since, currently available modern medicines for treating cancers are very expensive, toxic, and less effective in treating the disease, thus, one must investigate further in detail the agents derived from natural sources, described traditionally, for the prevention and treatment of cancer and disease. Voluminous clinical trials are needed to validate the usefulness of these agents either alone or in combination with existing therapy.

Although the chemopreventive effect of EGCG has been extensively studied by a number of workers in cancerous cell lines (Barnes and Cave, 2003; Ahn et al., 2003; Wu et al., 2009) but its apoptotic effect and mechanism in biopsies and monocytes of cervical cancer patients still remains to be properly understood. Thus, in the present study, we show the mechanism of EGCG mediated apoptosis in monocytes of cervical cancer patients which involved activation of caspase-3, the executor of apoptosis and cleavage of PARP - a specific marker of apoptosis as well as suppression of IL-6. EGCG was highly effective in inhibiting the viability of cervical cancer monocytes by PARP cleavage in response to activation of caspase-3. This resulted in the loss of normal PARP function which irreversibly commits the cell to die (Bellosillo et al., 1998; Rukmini et al., 2004; Islam et al., 2000; Islam et al., 2002).

Apoptosis is one of the most investigated areas in carcinogenesis because of its wide-ranging implications and possible role in therapeutic interventions (Khan et al., 2006; Nihal et al., 2005). Caspases are recognized as key molecules in apoptotic response and are reflective of the protein alterations as consequence of apoptotic changes in cell. Apoptosis occurs via two major different activation pathways (Singh et al., 2002; Raff, 1998). One pathway involves changes in mitochondrial transmembrane potential, leading to the release of cytochrome c. Cytochrome c then binds the apoptosis activating factor 1 and procaspase-9, resulting in the activation of caspase-9 by proteolytic cleavage. The other pathway starts with death receptor ligation or Fas/FasL interaction, followed by oligomerization of the receptor, recruitment of Fas-associated death domain protein (FADD), and activation of caspase-8 (Gastman, 2001). Both caspase-9 and caspase-8 are defined as initiator caspases and can in turn activate caspase-3, the executor of apoptosis (Green, 1998; Green and Reed, 1998). Also, cross communication exists between the two pathways,

as caspase-8 may activate caspase-9 via Bid, a member of Bcl-2 family (Ashkenazi and Dixit, 1998).

Our present study demonstrated the activities of caspase-3, caspase-8 and caspase-9 in cervical cancer monocytes were activated by EGCG, indicating that both death receptor-related apoptotic pathway and the mitochondria-related pathway were activated. On the contrary, suppression or reduction in cell viability was inhibited by inhibitors of caspase-3, 8 and 9 namely Z-VAD-FMK, Z-IETD-FMK and Z-LEHD-FMK respectively, which specifically blocked PARP cleavage.

We further observed that either specific caspase-8 or caspase-3 inhibitor blocked the EGCG-induced apoptosis by an appreciably high magnitude, whereas the caspase-9 inhibitor only partially inhibited it. This implies that apoptosis induced by EGCG in cervical cancer monocytes were initiated by a pathway involving the activation of caspase-8, leading to the activation of caspase-3 and the cleavage of PARP. The activation of caspase-9 is probably a secondary consequence of the activation of caspase-8 through the cross communication between the two apoptotic pathways (Islam et al., 2004; Stennicke, and Salvesen, 1998; Stennicke et al., 1998).

Cytosmears from non-neoplastic ectocervical biopsy cultures with EGCG also showed only keratinization promoting effects of EGCG and no apoptotic cells could be seen. On the contrary, cytosmears from cervical cancer biopsy cultured with EGCG showed distinguished nuclear condensation and rounded contours with deep eosinophilia of cell cytoplasm, thereby indicating for EGCG-induced apoptotic. Histopathological sections from EGCG treated test samples also showed evidences of apoptosis, showing one apoptotic cell per 2-3 high power fields to more than one dispersed apoptotic cells in a single high power field. Occasionally, apoptotic cells were seen in groups implying foci of apoptotic changes.

Next, we also report suppression of high levels of IL-6 in cultures of cervical cancer monocytes by EGCG to be dose-dependent. In accordance with its potential role in cancer progression, various types of cancer show elevated level of IL-6 (Orth et al., 1996) and that, the strong ability of malignant cells to escape immune surveillance is a well known phenomenon. The anti-tumor immune response has been

reported to be regulated by several factors, which includes cytokines produced by tumor and other cells of tumor stroma. It seems likely that the local cytokine microenvironment, acting on tumor cell or on the adjacent cells, can either block or facilitate tumor growth, and that proinflammatory cytokines strongly influence the immunologic state (Orth et al., 1996).

Treatment with EGCG increased the proportion of cells in the G_1 phase of the cell cycle and induced apoptosis. The results in the present study indicated EGCG to decrease the anti-apoptotic Bcl-2 protein expression, and activation of caspase-3, 8 and 9, suggesting that EGCG induces apoptosis via a both cellular as well as mitochondrial pathway, although the major pathway was cellular. Reports indicate that treatment with EGCG inhibited phosphorylation of the EGFR, signal transducer and activator of transcription3 (Stat3), and extracellular regulated kinase (ERK) proteins and also inhibited basal and transforming growth factor- α -stimulated c-fos and cyclin D1 promoter activity. EGCG at 0.1 μg/ml (a concentration found in serum after oral administration) markedly enhanced the growth-inhibitory effects of 5-fluorouracil. Taken together, the findings of the present study as well as by other workers (Liao et al., 2004; Schuller et al., 2004; Lu et al., 2006) provide insights into molecular mechanisms of growth inhibition by EGCG. Furthermore, reports also indicate that EGCG inhibited growth and induced apoptosis in human prostate, lung, colon, and gastric carcinoma and human leukemia cancer cell lines (Yang et al., 2005; Ju et al., 2005; Siddiqui et al., 2006; Roomi et al., 2005). Studies with EGCG in some cell types indicated that it could inhibit signaling pathways related to the activation of growth factor receptors. HNSCCs often display up-regulation of TGF- α/EGFR signal transduction pathways, (Stat3 lies downstream of the TGF- α/EGFR signaling pathway, and Stat3 is strongly implicated in the growth of these carcinomas and in protecting them from apoptosis.

The observations made in the present study demonstrate that EGCG, a major component of green tea, possesses cervical cancer cell growth inhibitory properties. It is likely that EGCG inhibits cancer cell growth through many different regulatory pathways, along with apoptosis and cell cycle arrest. The results of the present study are consistent with EGCG inhibiting cell growth and inducing apoptosis in cervical cancer biopsies and HeLa cell lines. The cytotoxic properties of EGCG seemed to be more effective against cervical cancer monocytes than HeLa cells. At this time, there

is no explanation for these differences and more experiments are needed for further analysis. The induction of apoptotic cell death by EGCG was accompanied by characteristic morphological and structural changes as well as internucleosomal DNA degradation. PARP-cleavage and FITC-Annexin assays in the present study clearly confirmed apoptosis induction in EGCG-treated cervical cancer cells. As per the manual supplied by the manufacturer of FITC-Annexin assay kit, apoptosis is a normal physiologic process, which occurs during embryonic development as well as in maintenance of tissue homeostasis. Certain morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA, characterize the apoptotic program. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD) to allow to identify early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC Annexin V and PI negative; cells that are in early apoptosis are FITC Annexin V positive and PI negative; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. This assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway because in either case, the dead cells will stain with both FITC Annexin V and PI. However, when apoptosis is measured over time, cells can be often tracked from FITC Annexin V and PI negative (viable, or no measurable apoptosis), to FITC Annexin V positive

and PI negative (early apoptosis, membrane integrity is present) and finally to FITC Annexin V and PI positive (end stage apoptosis and death). The movement of cells through these three stages suggests apoptosis. In contrast, a single observation indicating that cells are both FITC Annexin V and PI positive, in of itself, reveals less information about the process by which the cells underwent their demise. Apoptosis is a tightly regulated process which involves changes in the expression of distinct genes. One group of genes regulating apoptosis are cytoplasmic aspartate specific cysteine proteases of the ICE/CED-3 family, better known as caspases (Klaus et al, 1998). Caspase-3, for example, inactivates poly (ADP-ribosyl) polymerase by proteolytically degrading the 116 kDa enzyme into an 85 kDa fragment (Duriez and Shah, 1997; Li and Darzynkiewicz, 2000; Nigata, 2000). When cervical cancer monocytes were treated with EGCG, the expression level of procaspase-3 was decreased (data not shown) indicating that the active form of caspase-3 was generated. It is therefore inferred that EGCG-induced apoptotic death in cervical cancer monocytes was mediated in part by activation of caspase-3.

In summary, EGCG, a natural antioxidant, is a potent inducer of apoptosis in monocytes, biopsies of cervical cancer patients as well as HeLa cell lines, and that, induction of EGCG-mediated apoptosis involved activation of caspase-3 / CPP32, caspase-8 and caspase-9 as well as IL-6 suppression. The apoptotic as well as anti-inflammatory / anti-proliferative effects of EGCG was more prominent in cervical cancer monocytes than in HeLa cell lines. Why EGCG is more effective in cervical cancer monocytes than in HeLa cell lines warrants further in-depth investigation. It is hoped that in view of the above aspects, whether EGCG can be promising candidate for the chemoprevention of cervical cancer might be an interesting topic for further investigation.

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