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Research Article

Silymarin Mediates Apoptosis Through Activation of JNK/ERK Signaling Pathway in Human Colon Carcinoma Cells in Response to (^{60}Co) Gamma Radiation

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Abstract

Colon cancer is the third most common neoplasm worldwide and one of the major causes of death. Thus, major objective of present investigation was to identify and assess a natural molecule available in dietary sources which can sensitize colon carcinoma cells following radiation exposure. HCT-15 and RKO cells were pretreated (-30min) with silymarin followed by radiation (2 and 2.5Gy) exposure and samples were collected, analyzed at different time intervals (0-48 hrs in relation to radiation and silymarin + radiation time point respectively). Expression of proteins related to ERK/ JNK and mode of cell death was measured using western blotting (γ-H2AX, PCNA, ERK/JNK). A significant activation of JNK was observed in both the cells after treatment with silymarin and radiation. In all the samples effect was higher than silymarin alone and radiation with respect to combination group. An increase in apoptosis with increase in DNA damage was found to be accompanied with decreased SOD2 levels (0-48 hr) by combination treated group. In present investigation, silymarin was found to be potent inducer of cell death through ERK/JNK pathway. Silymarin was found to sensitize cells to radiation at non-toxic concentration and sub lethal radiation dose. Silymarin is already being utilized in traditional system of medicine (homeopathy, ayurveda and Chinese system of medicine) and therefore it possesses potential utility in future for treatment of colon cancer.

Keywords: Ionizing Radiation (IR); Silymarin; JNK; ERK; PCNA

Introduction

Silymarin is an active constituent of plant milk thistle *(Silybum marianum).* It is available in different isoforms like, silybin, isosilybin, silychristin and silydianin. This plant has medicinal properties and has been used in traditional system of medicine. Various preparations of milk thistle, especially the seeds, have been used medicinally for over 2000 years [1]. It was taken as a tonic, demulcent, anti-depressant, and stimulant for milk production in nursing mothers. In homeopathy, a tincture of the fruits is used to treat bronchitis, cough, gallstones, hemorrhage, jaundice, peritonitis, uterine congestion, and varicose veins [2] Silymarin has been used as anti-viral, anti-inflammatory, immune-modulator, hepatoprotective [3], antioxidant agent etc. [4,5]. Silymarin is a powerful antioxidant [6], its anticancer properties have now begun to emerge. It is well known liver tonic, moreover it is safe, well tolerated and cause no serious side effects because no reduction in mortality was found, data are too limited to exclude a substantial benefit or harm of milk thistle on mortality and also to support recommending this herbal compound for the treatment of liver disease [7,8]. Colorectal cancer (CRC) is the disease which starts in the gland of epithelial lining of the colon. CRC is highly metastatic cancer of stage III and IV which is difficult to treat, various

modalities (Surgery is the only curative modality for localized colon cancer (stage I-III) [9]). Adjuvant chemotherapy is for resectable colon cancer 'Stage 0' and I [10] Neoadjuvant therapy for resectable metastatic disease [11]. Occupational exposures to chemotherapy may occur through inhalation, skin contact, skin absorption, ingestion or injection. Inhalation and skin contact/absorption are the most likely routes of exposure, but unintentional ingestion from hand to mouth contact and unintentional injection through a needle stick or sharps injury are also possible [12-14]. Several drugs like irinotecan, oxiplatin, and 5-FU can be used to treat colorectal cancers. Drugs like "Cyclophosphamide (carcinogenic in nature); Platinum-containing chemotherapy drugs include "cisplatin, oxiplatin, and carboplatin" cause adverse health effects such as carcinogenicity, teratogenicity, and other factors. For example, cisplatin is categorized at a group 2A carcinogen, meaning that there is inadequate evidence to designate it as a human carcinogen [15]. Natural molecule like curcumin, rutin, sulindac has been used as a dietary supplement prevents from developing colon cancer. Patients undergoing treatment with taxol or vincristine often suffer from peripheral neuropathy as a side effect [16]. Natural products served us well in combating cancer since last 40 years. They have reduced pain, suffering, and revolutionized medicine by enabling the transplantation of organs. The use of plant and their metabolites has aided in doubling of our life span in 20th centuary. Enediynes are among the strongest naturally produced anti-tumour compounds but are extremely toxic due to their action of causing apoptosis in normal cells as well as in tumour cells. They include calicheamicin, dynemicin A, esparamicin, kerdarcidin and neocarzinostatin. Recent development of new and multi targeted therapy is quite useful in elimination of resistance transformed cells also. Ionizing radiation plays important role in cellular stress, IR has ability to regulate multiple signaling pathways associated with cell survival or death. However it varies with respect to cell type and differentiation. Radiation with chemotherapeutic agents (5-Fluorouracil [17], paclitaxol) has enhanced radio sensitivity, by targeting Ras/MAPK pathway. MAP kinase pathway transduces signals from cell membrane to the nucleus and broadly divided into ERK, JNK and p38 pathways. Among these p38 and JNK strongly activated in response to stress and thereafter cell death. ERK is rapidly activated in transformed cells in response to radiation exposure. Transformed cells, which are sensitive to radiation, activate JNK/SAPK, but resistance cells failed to activate and survive. All together these molecular events can be targeted to enhance effectiveness of radiation [18].

In our previous studies silymarin with radiation combination has inhibited cell proliferation in HCT-15 and RKO cells, depolarized mitochondrial membrane potential, increase in reactive oxygen species (ROS) generation and DNA damage (comet assay) followed by increase in phosphorylation status of p53 [19]. Based on previous findings we had planned to analyze the expression of different cell signaling protein involved in apoptosis to understand radiation sensitization aspects in combination with silymarin and radiation. Silymarin targets JNK and induce apoptosis in time dependent manner in colon carcinoma cells. Silymarin and radiation combination has increased JNK activation in transformed cells. The current study was performed to elucidate mechanism involved in radiation sensitization by silymarin in combination with radiation in time dependent manner. Human colon carcinoma cell lines of large intestine HCT-15 and RKO cells was used during the experiments. Both cells were treated with silymarin before (-30min) radiation.

Materials and Methods

Chemicals of AR/cell culture grade *viz* bisacrylamide (N,N'-Methylenebisacrylamide), roswell park memorial institute (RPMI-1640), silymarin, glycine, TEMED (N,N,N',N'-Tetra methyl-ethylenediamine), sodium dodecyl sulphate (SDS), eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), sulfanilamide, phosphoric acid, ethylene diamine tetra acetic acid (EDTA), tris-base, penicillin, streptomycin, sodium phosphate dibasic anhydrous, hydrogen peroxide (H_2O_2), tween twenty, sodium pyruvate, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), ethanol, sodium hydroxide (NaOH), phenol red, ethylene glycol-bis(2-aminoethylether)-N,N,N,'N'-tetraacetic acid (EGTA), sodium orthovanadate, phenyl methane sulfonyl fluoride (PMSF), triton X-100, sodium fluoride, sodium deoxycholate, β-mercaptoethanol, bromophenol blue, ammonium persulphate, methanol were procured from sigma aldrich (St. Louis, MO, USA). Apart from these other AR grade chemicals used in the studies were procured from Sisco Research laboratory, India.

Cell cultures

Human colorectal adenocarcinoma (HCT-15) cells and RKO cells were obtained from National Centre for Cell Sciences, Pune,

India. HCT-15 and RKO cells were maintained at 37°C in humidified atmosphere of 5% CO_2 and 95% air in RPMI-1640 medium and Eagle's Minimal Essential Medium (EMEM) respectively. Both the media were supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), 100 units/ml of penicillin and 100 µg/ml of streptomycin. All experiments were performed on exponentially growing cells and were sub-cultured twice a week.

Preparation of silymarin solution

Silymarin was dissolved in (90% RPMI and 10% ethanol v/v in media) under aseptic conditions. Silymarin was prepared freshly and treatments of cells were performed as per indicated concentration(s).

Gamma irradiation of cells

Irradiation of cells were done using Bhabhatron- II Telecobalt unit (Bhabha Atomic Research Centre, Mumbai, India) ^{60}Co (dose rate 2.25-2.55Gy/min) at ambient temperature. Radiation dosimetry of unit was carried out by certified radiation safety officer in the institute and Baldwin Farmer secondary dosimeter and Fricke's chemical dosimeter methodologies were used for same [19].

Western blotting

Logarithmically growing HCT-15 and RKO cells (approx. 1x10^6) were treated with silymarin 20 *µg/ml (30 min pretreatment in case of silymarin alone group only) followed by radiation (2-2.5Gy) and after (0, 8, 24 and 48* hrs) incubation samples of all four groups (control, silymarin alone, radiation alone and silymarin+radiation) were processed for western blotting as described by [19]. Briefly following various treatment, cells were harvested and washed twice with ice cold PBS and they're after the cells were lysed in RIPA lysis buffer (1mM EGTA, 25mM sodium orthovanadate, 1% sodium deoxycholate, 1M Tris-Cl (7.4), 1.5M NaCl, 1%Triton X-100, 0.5M EDTA, 200mM PMSF, protease inhibitor, phosphatase inhibitor cocktail (Sigma Aldrich, USA), 0.5M sodium fluoride. After cell lysis supernatant was collected for western blotting and protein estimation. Protein concentration was measured by BCA (Sigma Aldrich, USA) reagent and loaded in equal quantity, (40-50 *µg protein/well) for separation on 10% and 12% SDS-polyacrylamide gel. Proteins were transferred into PVDF membrane (*Amersham, GE Healthcare, Germany) were blocked by using (4%) BSA (for phospho proteins) thereafter membranes were incubated overnight (at 4°C) with respective primary antibodies, SOD2 (1:1000), phospho ERK42/44 (1:1000), Total ERK (1:1000), anti-JNK (1:1000), phospho JNK (1:1000), PCNA (1:2000), Gamma H2AX (1:1000) and ß-actin (1:5000) all antibodies were purchased from sigma (St. Louis, MO, USA). Following washing membrane were incubated with secondary antibodies anti rabbit (1:2500-1:3000) and anti-mouse (1:2500) at room temperature for 3 hrs and after washing with TBST, the membrane was exposed on X-ray films, and protein bands were detected using the super signal West Pico chemiluminescent substrate (Thermo Scientific USA).

Determination of nitric oxide level

Nitric oxide, was measured as described by Gupta., *et al.* [20]. There were four groups control, silymarin alone (20 *µg), radiation alone (2 and 2.5Gy) and silymarin + radiation. Cells were treated with silymarin (-30 min pretreatment for silymarin alone only) and thereafter exposed with radiation. Cell culture supernatant (100 µl) was drawn from culture petri dish at 0* hr, 8 hr and 24 hr of RKO and HCT-15 cells (approx.1x10^6) and (100 *µl) of* sulphanilamide (1%) in 5% H_3PO4 and 100 *µl of 0.1% N-(1-napthyl) ethylenediamine dihydrochloride were added in* H_2O to each well of 96 well tissue culture micro titre plate. Respective O.D was measured immediately at 550 nm using ELISA reader (Biotech instruments USA).

Data analysis and statistical evaluations

Densitometric analysis (Western Blotting) was done by image J Software and all prism graph was plotted by using Graph PrismV Software. For nitrite assay, one-way analyses of variance (ANOVA) with Tukeys multiple comparison test were performed and p values were shown as significant (***$p < 0.001$).

Results

Silymarin and radiation decreased expression of SOD2

Superoxide Dismutases (SODs) are enzymes involved in converting toxic superoxide radical into molecular oxygen or hydrogen peroxide in biological systems. Initially we determined SOD-1 protein levels and activity in both the cell lines. Exponentially growing asynchronous cultures were harvested and total proteins were extracted. Equal amounts of total cellular proteins were separated by SDS–PAGE and immunoblotted for SOD1, SOD2, ERK, P38, JNK, PCNA and gamma H2AX and beta-actin protein

levels. SOD-1 protein levels (Figure 2A) were highest in RKO cells (~1.5fold) by (silymarin+2.5Gy) at 48 hrs as compare to control (~0.2fold); there was time dependent increase in expression of SOD1protein observed in RKO cells. Where as in HCT-15 cells (~1.0 fold) increase by (silymarin+2Gy) at 48 hrs (Figure 1A), which is comparatively, low as compared to RKO cells (Figure 2A). In order to determine if SOD1 overexpression affects other antioxidant enzymes or not, we evaluated the total protein extract prepared from exponential growing cultures to study the expression of SOD2 in both the cell lines (HCT-15 and RKO).

MnSOD is known to promote cell survival and inhibit apoptosis. Silymarin prevented expression of SOD2 in RKO cells (Figure 2B) between (24- 48 hr). Exposure of 2.5Gy radiation found to reduce levels of SOD2 with respect to control at 0 hr, however with time significant increase in its levels was observed. In case of combination of silymarin with radiation (8 hr (~0.5fold) and 24 hr (~0.4fold) the radiation induced changes in SOD2 was found to decreased expression of MnSOD as compare to control (~1.5fold) at 8 hr and 24 hr time point. Inactivated form of SOD2 results in ROS level Reactive oxygen species (ROS), are predominantly produced in the mitochondria as by-products of the electron transport chain, Elevated ROS levels in the mitochondria not only result in oxidative damage within the organelle, but also oxidize deoxyribonucleic acids in the nucleus, lipids in cellular membranes and cysteine residues in cellular proteins. Therefore, there was decrease in activity of SOD2 by combined treatment at '0'hr with respect to control, radiation and silymarin alone.

Results showed the maximum decrease in SOD2 expression at 48 hr by (silymarin+2Gy) (~0.2 fold) group in HCT-15 cells (Figure 1B) as compare to RKO cells (~1.0 fold) (silymarin+2.5 Gy) (Figure 2B). Whereas, expression level of silymarin alone treated group was (~0.8fold) and radiation alone (~0.7fold) 48 hr (Figure 1B) was found higher in HCT-15 cells. After 48 hr treatment combination group has completely abolished the expression of SOD2 with respect to silymarin alone and radiation alone in HCT-15 cells (Figure 1B). There was significant difference observed at 48 hrs of combination group as compare to other time points after treatment. Combination treatment showed elevated levels of SOD2 at 0 and 8 hr (~0.8 fold). The present findings reveal that depletion of SOD2 by combination group potentially induces cell death. Our data show that there are altered levels of enzyme expression may result in altered cellular response to oxidative stress.

These results indicate that RKO cells have high SOD1 activity compared to HCT-15 cells. And low expression of SOD2 in HCT-15 cells as compare to RKO cells. This means silymarin (-30min pretreatment followed by radiation (2Gy) has ability to downregulate the expression of SOD2 at longer time points and hence radiosensitizes HCT-15 cells. The profile of expression of SOD1 and SOD2 in both the cell lines indicates differentiated response of tumor cells depending on access to oxygen. Low level of SOD enzymes expression indicates decreased production of ROS. The present findings reveal that depletion of SOD2 by combination group potentially induces cell death. Our data show that there are altered levels of enzyme expression may result in altered cellular response to oxidative stress. Therefore, overexpression of SODs in cancers cells may set up a condition whereby exposure to ionizing radiation results in greater ROS generation which leads to cell death.

Silymarin and radiation enhance phosphorylation of ERK

Mitogen-activated protein kinases (MAPKs) have been implicated in regulation of apoptotic cell death in response to various stimuli. To investigate a potential involvement of MAPKs in apoptotic cell death induced by the combination treatment with radiation and silymarin we first examined changes in ERK after combination treatment by western blot. In the present investigation the combination group lead to dramatic increase in expression of total ERK compare to control (HCT-15 cells) (Figure 1C). However, phosphorylation of ERK levels (Figure 1D) remains constant at 0 hr, no significant change observed with respect to control, phosphorylated ERK1/2 expression was increased by combination group, but no activity observed in radiation alone treated group at 8hr. The ERK1/2 levels were found to be low at 24 hrs in all cases as compare to control, but after 24 hr, there was significant increase in nuclear phosphorylation of ERK1/2 stimulated by radiation (~1.3fold) and no effect by silymarin alone (~0.1fold), therefore significant changes observed by combination group at 48 hrs with respect to other time points. The observed changes related to alterations in phosphorylation of ERK MAPK known to be crucial in cell survival and cell cycle progression. The activation of ERK1/2 mediates cells death. The levels of total ERK in HCT-15 cells were found to vary with time and type of treatments. There were no significant changes in relation to total and phophorylated ERK were observed at 0 hr with respect to control. However, at 8 hr

both radiation (2Gy) and combination (20 *µg/ml+ 2Gy) treatment decreased the levels of total ERK with respect to control and same in case of 48* hrs observed. The maximum increase in phosphorylation of ERK by combination (silymarin +2Gy) group was observed 48 hrs with respects to silymarin alone.

Intracellular signaling of radiation induced apoptosis by silymarin

ERK pathway has been shown to play important role in cellular proliferation. This pathway is activated rapidly in cancer cells in response to radiation.We have investigated radiation induced intracellular signaling response by silymarin in RKO cells; we analyzed the phosphorylation of ERK ½ by western blotting. As shown in (Figure 2D), treatment with 20 µg/ml of silymarin (~0.9 fold) led to a strong activation of ERK at 0 hr with respect to radiation alone and (silymarin+2.5Gy) combination (~0.4 fold). Whereas in 8 hr combination group (silyamarin+ 2.5Gy) there was decrease (~0.3 fold) in phosphorylation ERK as compare to control (~1 fold). But significant decrease of ERK1/2 was observed at 8 hr, ERK1/2 expression was clearly decreased by silymarin+2Gy as compare to silymarin and radiation alone. Decrease in phosphorylation of ERK1/2 was dose dependent. At 24 hr low expression of ERK observed by silymarin and radiation alone as compare to radiation combination (where its expression was found to be little higher). The combination treatment with (20 µg/ml of silymarin+2.5Gy) which is known to have an apoptosis inductive effect in the RKO cells also showed significant activation of ERK as compare to silymarin and radiation alone only at 48hr. Inactivation by combination (20 µg/ml of silymarin+2.5Gy) was observed in 0 and 8 hours. Since inactivation of the ERK pathway was reported to be involved in the induction of apoptosis. It is likely that this upregulation of phosphorylation of ERK1/2 by silymarin and radiation combination contributed to induced apoptosis in RKO cells. In Figure 2C, of RKO cells, when cells were exposed to radiation and silymarin,the expression of total ERK was constant between (0-24 hrs) (~0.3 fold).

Silymarin increased radiation induced JNK (pTpY183/185) MAPK activation

c-Jun N-terminal kinases (JNKs) belong to the superfamily, of MAP kinases that are involved in the regulation of cell proliferation, differentiation and apoptosis. Analysis of pathways regulated by JNKs has shown that JNKs are indispensable for both cell proliferation and apoptosis. The members of JNK family are primary kinases responsible for N-terminal phosphorylation of c-Jun under different physiological conditions in different cell types. We wanted to determine whether activation of JNK is involved in combination treatment induced apoptotic cell death or not. Therefore, we measured total forms of JNK in HCT-15 cells, and after treatment with silymarin and radiation we found JNK levels were constant at 0 hr, no significant difference was there, whereas in case of 8 hr of silymarin and irradiated group there was slight increase in JNK expression but later after 8 hrs, pattern of JNK expression (24 and 48) was found nearby similar to 0 hr group (Figure 1E). Radiation induced phosphorylation by silymarin leads to rapid phosphorylation of JNK (Figure 1F). there was time dependent increase observed in phospho JNK levels after silymarin and radiation treatment. At 0 hr expression of phospho JNK was low in control and radiation, but at 8 hrs silymarin has enhanced activation of JNK and combination as compared to control. Increase in expression of phospho JNK was observed at 0-48 hr time points of combined treated group, higher expression of JNK observed at 48 hr (~1.0 fold) as compare to control (~0.2fold). Activation of JNK is important for sensitization point of view [21]. Here activation of JNK could be because of changes in intracellular redox status, DNA damage etc. which may lead to alteration in gene expression, cell death or altered cell proliferation. Moreover, the quantum of phosphorylation was found to be significantly higher with respect to radiation alone, which further suggests the sensitization efficacy of silymarin following IR exposure to HCT-15 cells.

Silymarin with radiation enhance phosphorylation of protein kinase (JNK/ SAPK)

C-jun-N-terminal kinase/stress activated protein kinase (JNK/ SAPK) belongs to family of MAPK, plays important role in cell proliferation and apoptosis. JNKs have been shown to be involved in stimulating apoptotic signaling. We provide evidence that SAPK/ JNK activity is required for radiation sensitization by silymarin induced apoptosis. The SAPK/JNK signaling pathway, which is activated in response to a variety of cytotoxic stresses, involves the sequential phosphorylation. Silymarin causes an early and persistent activation of SAPK/JNK, and sensitizes RKO cells in time dependent manner.

Changes in phosphorylation of SAPK/JNK begin at 0 hr h following treatment with silymarin and radiation and remain

activated till 48 hrs as compare to control (Figure 2F). The strong activation of JNK was observed at 0 hr (Figure 2F) by combination group (~4.1 fold) as compare to control (~0.1 fold), significant increase was observed at 8 hr and remain continued upto 48 hrs. JNK/SAPK was phosphorylated at early time point of incubation with 20µ g/ml of silymarin similar trend observed in total form of JNK at 0 hr time point. In contrast radiation treatment led slight reduction in phospho JNK level was detected at 8 hr as compare combination (20 µg/ml+2.5Gy). Significant increase on phosphorylation of JNK observed at 24 hrs and 48 hr, all treated group "silymarin alone and radiation with combination" showed similar activation. But no significant change was seen in expression of total JNK at 48 hrs (Figure 2E).

Higher levels of activation at 48 hrs by combined treatment (~5fold) in (Figure 2F) with respect to control conferred that JNK/SAPK pathway plays important role in radiation sensitization and required for radiation induced cell death by silymarin. Higher activation observed by combined treatment group at 8 hr as compare to radiation hence it is proved that silymarin is involved in upregulating and enhances phosphorylation of JNK at (pTpY$^{183/185}$) motif. Expression remains higher at 8 hr and 24 hrs of combination treated group, silymarin induced JNK activation was enhanced at 48 hrs. There was time dependent increase in JNK expression levels observed at 0, 8, 24 and 48 hr in all combination (silymarin+2.5Gy) treated groups with respect to control.

However, of combined treatment group, as compare to control and radiation alone, increase in expression of JNK enhances tumour growth and promotes apoptosis.

Silymarin enhance radiation induced expression of γ-H2AX in HCT-15 cells

Ionizing radiation induces DNA double strand breaks (DSBs) that trigger phosphorylation of the histone protein H2AX (γH2AX). H2AX, a member of the histone H2A family, is rapidly phosphorylated in response to ionizing radiation. Histone H2AX has been implicated in the maintenance of genomic stability in response to DNA double strand breaks (DSBs). It is phosphorylated at an evolutionary conserved (PI3KK) motif in the carboxyl terminus within seconds after exposure to ionizing radiation (IR). The generation of γ-H2AX was confirmed by western blotting. In time course experiment performed γ-H2AX signals (Figure 1G) were detected by radiation alone and silymarin+2Gy treated group at 0 hr with respect to control but very low expression observed by silymarin alone. But later after 8 hrs significant increase in γ-H2AX was observed by combination group as compare to silymarin and radiation alone. Silymarin and radiation have increased phosphorylation of γ-H2AX with respect to control; the most significant induction was detected at 48 hrs of combined treated group (~1.9fold) with respect to control (~0.1fold)/radiation (~0.1fold)/(~0.5fold) and silymarin alone (~0.6fold). Phosphorylation of γ-H_2AX at ser^{139} residue is rapid response to DNA damage, which is well established marker for DNA damage. HCT-15 cells treated with silymarin and radiation or combination showed changes in its phosphorylation. We observed changes in expression of Gamma H_2AX in time dependent manner. Time dependent increase in phosporylation of gamma H_2AX was observed by combined treatment group at all time point studied (0-48 hrs) as compare to control. This obtained result indicates that radiation combination group shows early response on HCT-15 cells to DNA double strand breaks (DSBs). The phosphorylation of γ-H_2AX is important factor at a site of DNA damage.

Silymarin enhance radiation induced expression of γ-H2AX in RKO cells

Gamma γ-H_2AX is a phosphorylated form of histone H_2AX; the phophorylation of histone variant H2AX on serine 139 is a rapid response detected within sec to min after exposure, it is a sensitive marker for double strand breaks (DSBs). Phosphorylation of γ-H_2AX after irradiation is triggered by DNA double strand breaks. We analyzed phosphorylation of γ-H_2AX after treatment with silymarin and exposure to 2.5 Gy of gamma radiation at different time points in RKO cells, as seen in (Figure 2G) γ-H_2AX is clearly detected within 0 hr of radiation, the levels of γ-H_2AX peaked at 48 hrs following exposure to radiation alone and combination. Following exposure to radiation induced by silymarin+2.5Gy group, γ-H_2AX was induced to levels (~2fold) higher than control in RKO cells. Radiation alone at 48 hrs showed significant increase in expression with respect to combination treated group. Silymarin alone has reduced or lowered γ-H_2AX expression as compare to radiation alone and combination (48 hrs). In the beginning hours (0-24 hr) of silymarin and radiation alone group showed slight increase in induction of γ-H_2AX phosphorylation, when cells were treated with silymarin (20 µg/ml) and radiation (2.5Gy), increase in gamma H_2AX phosphorylation observed at 48 hr which is comparatively higher than control.

Silymarin decreased radiation induced changes in PCNA (HCT-15 cells)

Proliferation cell nuclear antigen (PCNA) is known to be associated with proliferation of cells. PCNA is also known as cyclin (33kda protein) present in nucleoplasm of continually cycling cells throughout the cell cycle. Silymarin in combination with gamma radiation decreased proliferation of PCNA at 0 hr as compare to control, silymarin and radiation alone (Figure 1H). The significant increase in PCNA expression begins at 8 hrs by combined group; radiation alone lowers the expression of PCNA and found insignificant with respect to silymarin alone. But major difference was observed at 24 hrs that is silymarin enhance PCNA but no significant change observed at silymarin and radiation alone group. The significant increase observed at 24 and 48 hr of combined group with respect to silymarin and radiation alone in HCT-15 cells. The increase in expression of PCNA in tumour cell is due to growth factors that upregulate production of protein.

β-actin (42kda) was used as loading control in HCT-15 cells, actin is highly conserved proteins that are involved in cell motility structure and integrity and are expressed in eukaryotic cells, loading control was found to be equal in all samples.

Silymarin decreased radiation induced proliferation of PCNA (RKO cells)

Proliferating cell nuclear antigen also known as cyclin, PCNA 36kda protein is an auxillary protein of DNA polymerase δ is essential for DNA replication during S-phase, this protein is present in nucleoplasm of continually cycling cells throughout the cell cycle. This protein is most abundant during S-phase, and declines during G_2/M phase; increase in expression of PCNA in tumours is due to growth factors that upregulate the production of this protein.

Silymarin alone significantly increased PCNA at early time point 0 hr, inactivation of PCNA observed by radiation alone, following exposure to radiation silymarin has decreased expression of PCNA as compare to silymarin alone. Expression by combination was similar to control of 0hr. moving on 8 hr after exposure to IR upregulation of PCNA seen by combination; silymarin alone lowered the expression of PCNA as compare to radiation. Combination group completely abolished PCNA expression at 24 hrs compare to silymarin and radiation (Figure 2H).

Silymarin treatment completely decreased expression of PCNA at 48 hrs with respect to other time points. In combination treatment upregulation of PCNA was observed only at 8 hrs and thereafter it was found to be insignificant (at 24 and 48 hrs) with respect to control. The obtained results are in corroboration with cell cycle study as silymarin arrested cells in G2 phase of cell cycle [19] Proliferating cell nuclear antigen (PCNA) is found in nucleus, it is a co-factor of DNA polymerase delta having molecular weight of 33kda protein and useful marker to evaluate cell proliferation. PCNA is one of the downstream effects of the activation of MAPK/ERK ½ signaling and well correlated to the status of cell proliferation. β-actin (42kda) was used as loading control in RKO cells and found to be equal in all samples.

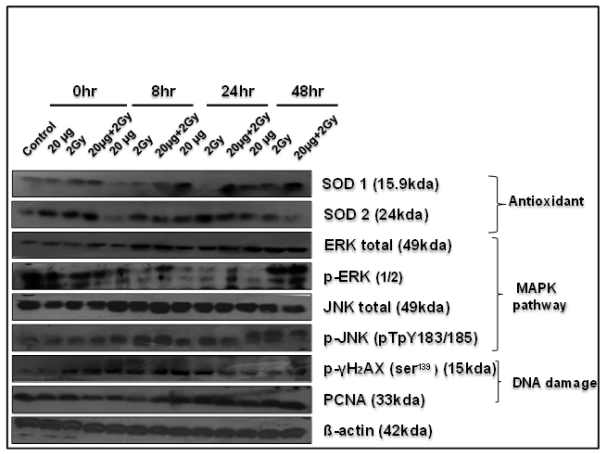

Figure 1: Western blot analysis of HCT15 cells (A) Role of antioxidant enzyme SOD1and (B) SOD2, phosphorylation of ERK and JNK: To assess role of stress response pathways in silymarin mediated radiation sensitization through activation of ERK and JNK phosphorylation status was measured. ERK protein, total ERK (C) and phospho ERK1/2 expression (D) were measured following various treatments and time intervals. to assess the response of Cell death associated proteins: phospho and total form of JNK(E-F) and γ-H2AX(G) protein upregulation and (H) Cyclin: PCNA, HCT-15 Cells were treated, harvested at different timepoint and samples were resolved on 10 or 12% SDS-PAGE, followed by transfer on PVDF membranes and blotted using specific antibodies, as described in methods section. After radiation in the presence of silymarin or absence of silymarin, the irradiated group with silymarin shows significantly higher expression of ERK, JNK, γ-H2AX and PCNA compared to the control group. β–actin was used as a loading control. Results were analyzed by using prism software. And densitometric analysis was done by imaje j software.

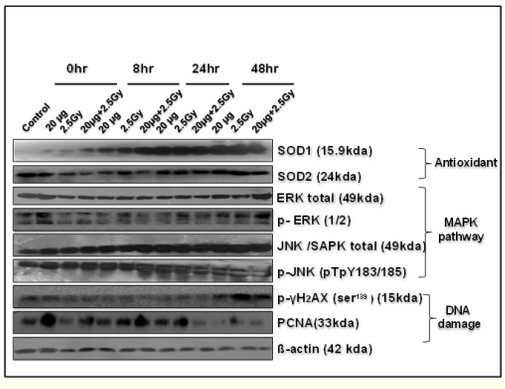

Figure 2: Western blot analysis of RKO cells (A) to assess the Role of antioxidant enzyme. SOD1 and SOD2 (B) protein expression was measured after various time points. RKO cells were treated, harvested (at different times) and samples were resolved on 10 or 12% SDS-PAGE, followed by transfer on PVDF membranes and blotted using specific antibodies, as described in methods section. β –actin was used as a loading control. To assess the role of MAPK pathway by silymarin mediated radiation sensitization. (C-F) Expression of phospho ERK, JNK along with their total form was measured, (G) association of DNA damage in cell death: γ-H2AX protein phosphorylation and (H) cyclin PCNA was analyzed by western blot after radiation in the presence of silymarin or absence of silymarin, the irradiated group with silymarin shows significantly higher expression of JNK and γ-H2AX and low expression of PCNA compared to the control group. β–actin was used as a loading control. p-ERK ½, pJNK, PCNA, and γ-H2AX are involved in silymarin induced radiation sensitization. Results was analysed by prism software.

Sensitization of IR induced nitric oxide level by silymarin

In HCT-15 cells (Figure 3) maximum increase in nitric oxide levels were detected at 24 hr time point as compare to control. However, the increase in levels of nitric oxide was found to be time dependent. Similarly, treatment of cells with silymarin showed increased levels of nitric oxide at in RKO cells (Figure 4) at 24 hrs as compared to 0 hr and 8 hr group. However, with respect to control no significant changes were observed in at 0 hr and 8 hr groups in RKO cells. Nitric oxide plays important role in cell signalling and at high levels it is known to form peroxinitrite which is deleterious for cells and induces cell death. The increase in levels of nitric oxide at later time points could be associated with cell death.

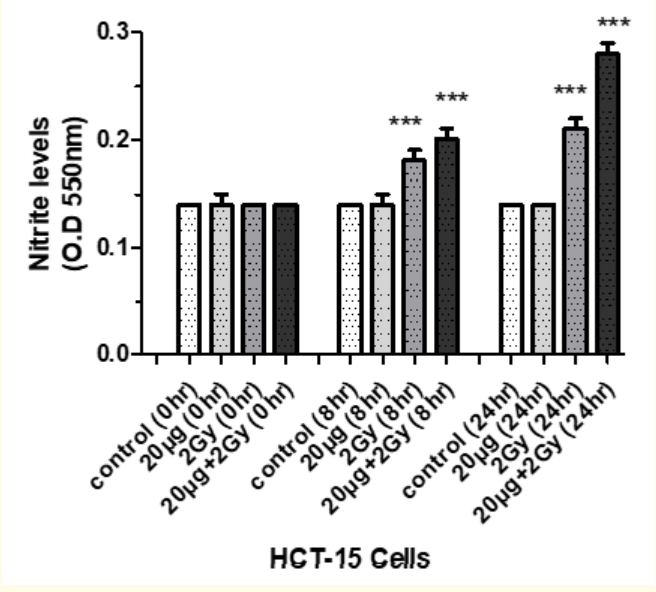

Figure 3: Effect of pretreatment of silymarin followed by radiation at different time interval on generation of Nitric Oxide (HCT-15 cells). after various treatments the generation of nitric oxide level was measured spectrophotometrically at 550nm. The experiment was repeated thrice and results have been expressed as mean ± S.D. and one way- ANOVA (Tukey's multiple comparison test) was performed. p value less than $p < 0.001$ considered as level of significance (*** $p < 0.001$).

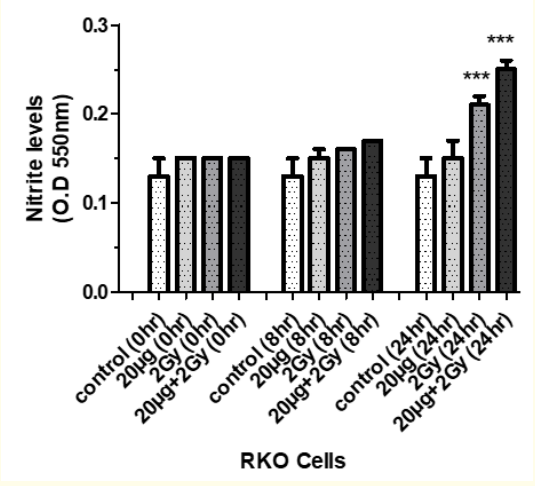

Figure 4: Effect of pre-treatment of cells with silymarin followed by radiation at different time interval on generation of Nitric Oxide by (RKO cells), after various treatments the generation of Nitric Oxide level was measured spectrophotometrically at 550nm. The experiment was repeated thrice and results have been expressed as mean ± S.D. and one way- ANOVA (Tukey's multiple comparison test) was performed, p value less than $p < 0.001$ considered as level of significance (*** $p < 0.001$).

Discussion

Ionizing radiation is known to cause damage to cellular bio molecules both directly by deposition of energy in them and indirectly by inducing ROS generation. Sudden burst in ROS can alter intracellular physiology in terms of redox status, gene expression and oxidative modification of biomolecules. These oxidative modifications in cellular biomolecules specifically in lipids, proteins, DNA etc. are very much dependent on amount of dose absorbed by cells, antioxidant defense systems, cell cycle phase etc. Intrinsic antioxidant defense system, cellular energy levels, expression of anti-apoptotic proteins etc., are known to support cells to cope with sudden changes in intracellular physiology or ROS levels.

MAPK pathway is known as mitogen activated protein kinase. Insulin, many growth factors and mitogens act through MAPK pathway. Mutation in these protein kinases leads to deficiency in human. Upon phosphorylation of serines and threonine of target protein substrate, members of MAPK regulate various processes such as: cell growth, cell differentiation, cell proliferation, gene expression, mitosis, cell motility, metabolism and apoptosis. The

overstimulation of these kinases (MAPK) leads to cancer. The targets of MAPK pathway are located in cellular compartments. This pathway is physically linked in signal transduction pathways from cytoplasm to the nucleus. ERK1/2 is activated by phosphorylation mediated by MAPKKK (a-raf, b-raf and c-raf). In certain cells their activation contributes to normal and aberrant growth in some cells they promote cell survival or differentiation. Their enzymatic activity is enhanced by dual phosphorylation on Thr and Tyr, by group of dual specificity protein kinases represented as MEK1 and MEK2. The upregulation of p53 activity by ERK activation during apoptosis depends on type of cell and tissues, external stimuli etc. The mechanism by which ERK1/2 promotes apoptosis is under investigation. It is reported that ERK activation increases cisplastin induced cell death in neuroblastoma cell lines [22], same report was suggested with osteosarcoma cells and mediated apoptosis and cell cycle arrest in p53 independent manner after treatment with ionizing radiation or etoposide [22] JNK was discovered because of its ability to phosphorylate N terminal transactivating domain of its transcription factor c-jun. Its activity was stimulated by cellular stress. This pathway is initiated by Rho family of GTPases, Rac1 and Cdc42. The substrates of JNK involves transcription factor of c-jun family. JNK pathway is involved in apoptosis; this was first studied by (Chen., *et al* in 1996).

ROS are produced as a result of the leakage of electron transport chain and their reaction with oxygen. Antioxidants play multiple roles in cancer initiation and progression. Free radicals and antioxidants affect cancer cells behavior in many ways, free radical species can increase proliferation, alter cell cycle, induce apoptosis/necrosis. Antioxidants inhibit cell growth in different ways. Activation of apoptosis in cancer cells is due to antioxidants inhibition depends on cell-to-cell type. The new proposed anti-cancer strategy involved for treatment of tumor is "oxidation therapy". For inducing cytotoxic stress there are two methods: (a) inducing the generation of ROS directly in solid tumours and (b) inhibiting antioxidant enzyme system of tumor cells [23,24]. Manganese-dependent superoxide dismutase (MnSOD) or SOD2 is a 24 kda protein and is modified for transport into the mitochondrian where it resides in the matrix. Decline in MnSOD activity leads to aging, cancer and asthma. MnSOD is essential for survival. Silymarin, a plant flavonoid, has been known to generate a wide variety of biochemical, anti-inflammatory, anti-angiogenic, anti-metastatic, antioxidant and pharmacological effects. In order to elucidate the molecular mechanism for the silymarin induced radiation sensitization, the human colon carcinoma cells were treated with silymarin and combined with radiation. The sensitivity of cells after gamma radiation exposure was found to be significantly enhanced by silymarin. In the present study, we investigated the potential of silymarin in modulating radiation effects on colon carcinoma cells. The increase in concentrations of silymarin was associated with increased cytotoxicity and therefore we have utilized sub-lethal concentration of silymarin (20 µg/ml) in combination with radiation for both HCT-15 and RKO cells respectively. The exposure of cells to IRs is known to reduce cell survival; however, it varies with cell to cell, radiation dose, dose rate etc. RKO cells were found to be more resistant to IR as compared to HCT-15 and therefore depending on radiation sensitivity the selected radiation dose for HCT-15 and RKO for combination studies were 2 and 2.5Gy.

In this study MAPKs have shown major oxidative stress signal transduction pathway in both the cells. In Addition, we have also studied level of nitric oxide in cancer cell lines after treating the cells with silymarin followed by radiation. After treatment with silymarin we found increase in level of nitric oxide as compare to control in both the cells Nitric oxide (NO) is known to cause DNA damage via generation of peroxinitrite (ONOO-) and N_2O_3 [25] Peroxinitrite oxidize and potentially cause strand breaks in DNA through attack on sugar phosphate backbone. N_2O_3 nitrosate amines to form N-nitrosamines then alkylate DNA. Nitrosation of primary amines leads to formation of diazonium ion and subsequent deamination and DNA crosslinks [26] Nitric oxide mediated DNA damage is to trigger p53 accumulation and induce apoptosis or cell death. This could be the one possible mechanism by which nitric oxide may induce death of tumour or transformed cells RKO and HCT-15. Silymarin with radiation significantly induced cell death in concentration and dose dependent manner. Therefore, enhance RNS and DNA damage sensor proteins and hereby induction of apoptosis might be one possible mode of action in relation to radiation sensitization of cells by silymarin.

Treatment of cells with silymarin followed by radiation exposure (combination), increased phosphorylation of gamma H2AX (Ser139) both in RKO and HCT-15 cells. The phosphorylation of gamma H2AX in chromatin located near double strand breaks. Gamma H2AX is substrate of phosphoinositide 3-kinase-related-protein kinases (PIKKs) of ATM-ATR-DNA-PK kinases.

The combination of silymarin and γ-radiation enhances radiation sensitization effects through activation of MAPK (JNK and ERK) pathway in RKO and HCT-15 cells (Human Colorectal Carcinoma). Furthur more, recent study indicates activation of JNK pathway which is important mediater of apoptosis [27] and might have played

important role in silymarin induced apoptosis of RKO cells. Here we report for the first time that silymarin treatment of RKO cells results in activation of JNK and induction of JNK dependent apoptosis. The expression of cell proliferation nuclear antigen (PCNA) protein (marker of cell proliferation) was found to decrease by combined treatment at 48hrs in RKO cells. However, in case of HCT-15-time dependent changes in PCNA was observed by silymarin and radiation.

Cancer cells are known to have high level of intrinsic antioxidants. Ionizing radiation and O_2- produced during cellular processes can give rise to weakly bound electrons, resulting in DNA damage, genomic mutations and ultimately leads to cancer in cell [28]. Silymarin itself is an antioxidant but in transformed cells, time dependent upregulation and downregulation of antioxidant enzymes play important role in radiation sensitization. Antioxidants are known to induce cell death when they are in high concentration [28]. Present investigations suggest that transformed cells are susceptible to the combination induced radiation sensitization [29].

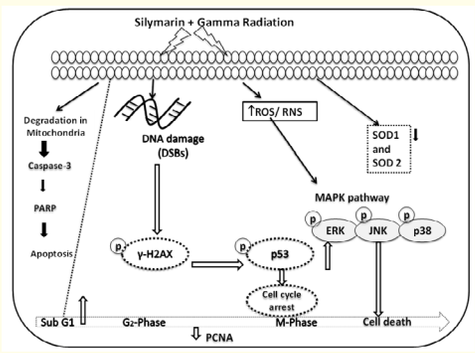

Figure 5: Proposed signalling pathway indicates molecular mechanism of radiation sensitization efficacy by Silymarin in Human colon carcinoma cells. Activation of cell death pathways of apoptosis by combination (silymarin +Radiation) therapy results in DNA damage by phosphorylation and activation of p53, stabilized p53 can upregulate apoptotic signals through mitochondria and activate death effector kinase caspase-3, JNK (MAPK). Activated p53 cellular response leads to growth arrest and apoptosis. Intrinsic reductive sources such as antioxidants (SOD2), superoxide produced in cellular processes, can give rise to weakly bound electrons, leading to reductive DNA damage. Nitric oxide mediated DNA damage triggers p53 accumulation and induces apoptosis. Influence of oxidative stress and ROS overproduction contributes apoptosis. Increase in phosphorylation of γ-H2AX and concomitant decrease in cell proliferation protein (PCNA) results in apoptosis. All these factors might have collectively contributed towards radiation sensitization by silymarin in colon carcinoma cells.

Summary and Conclusion

In summary our study demonstrates that silymarin in combination with IRs, induced cell death (apoptosis). The apoptosis induction could be additive effect of both silymarin and radiation. Moreover, the induction of apoptosis was also accompanied by strong activation of ERK MAPK kinase. These results support the hypothesis that silymarin may have potential in colon cancer treatment. Radiotherapy is one of the most effective ways in treating human cancers. This study describes the design and application of silymarin as radiotherapy sensitizer to enhance gamma rays induced inhibitory effects on HCT-15 and RKO cells. The results show that, pretreatment of the cells with silymarin enhances gamma rays induced growth inhibition. Results of western blotting demonstrated that combined treatment induced cell death and involvement of RNS and their association with DNA damage. Taken together, current investigation suggests that silymarin can act as radiosensitizer with potential application in human colon cancers. Radiation is known to induce ERK mapkinase and phosphorylation of JNK [30]. Which was further increased in cells pretreated with silymarin. Hence it is proved that ionizing radiation utilizes JNK as well as ERK pathway to induce cell death in both HCT-15 and RKO cells. The changes in levels of SOD2 enzyme expression in combined treatment group were found to be crucial in radiation sensitization.

Increase in phosphorylation of gamma H2AX (biomarker of DSBs) by combined treatment group in both the cells were observed which is indicative of DNA damage and repair responses. The levels of nitric oxide in both cells were found to be higher after treatment with silymarin + radiation. This indicates that high levels of nitric oxide are cytotoxic for cells. All together it is evident that silymarin triggers different signalling pathways in thereby cell death which could be due to additive effects of both silymarin and radiation, and the hypothesis may of use to consider silymarin as potential radio-sensitizer.

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Data Availability

The original data shown figures 1–5 used to support the findings of this study are available from the corresponding author upon request.

Declaration of Competing Interest

The authors declare no conflicts of interest

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Author Contributions

Mitu performed all Experiments followed by complete analysis.

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