Effectiveness of Alpha linolenic acid (ALA) and Gamma linolenic Acid (GLA) on ER positive mammary gland carcinoma and redefining its mechanism of action through mitochondria mediated death apoptosis pathway

SUMMARY

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Summary

Controversy exists regarding the role of dietary fat in breast cancer etiology. We have investigated the association of dietary PUFAs (ω-3 and ω-6 PUFAs) with *in vitro* and *in vivo* models of mammary gland carcinoma.

The *in vitro* studies on ER+ MCF-7 cells affirmed the significant cytotoxic and apoptotic potential of ALA and GLA when scrutinized through MTT assay, AO/EtBr and JC-1 staining. The ALA and GLA treated AO/EtBr stained cells were evident for the presence of apoptosis as visualized with nuclear shrinkage, chromatin condensation, fragmented nuclei and membrane blabbing. Considering the fact that mitochondria participates in apoptosis, the effect of ALA and GLA on mitochondrial membrane potential was validated through cationic dye JC-1.Therefore, JC-1 is considered to be an indicator of mitochondrial potential and decrease in mitochondrial membrane potential is an indicative of apoptosis as perceived after the ALA and GLA treatment. Treatment with GLA and ALA demonstrated cell cycle arrest in G0/G1 phase by presenting a lower % of G0/G1 cell population in comparison to control with a higher population of cell in G2/M phase. Subsequent studies affirmed the cell cycle arrest in G2/M phase by the ALA and GLA treatment. Translocation of PS to the outer leaflet of the cellular membrane is a key marker of apoptosis and was validated through Annexin-V labeled with FITC and PI in ALA and GLA treated group which indicate the initiation of programmed cell death in ER+ MCF-7 cells.

Subsequently, the effect of PUFAs was evaluated against chemical carcinogen induced mammary gland carcinoma models. In the present study, two carcinogens viz. DMBA and MNU were used. DMBA is a polycyclic aromatic hydrocarbon and the tumors produced from DMBA are morphologically and histopathologically very similar to

human tumors. DMBA is an indirect carcinogen, and requires metabolic activation by cytochrome P450 enzymes to reactive metabolites, i.e. dihydrodiolepoxides and forms mutagenic DNA adduct. Mainly two enzymes viz. cytochrome P4501B1 (CYP1B1) and microsomal epoxide hydrolase (EPHX1) are responsible for DMBA bio activation. Whereas, MNU is a direct carcinogen and it doesn't require any intermediate. Electrophilic diazonium cation formation of an intermediate of alkylnitrosourea is a well establish mechanism in mammalians, which further needs cytochrome P450 activation to exhibit its mutagenic potential. The possible site of alkylation in guanine base pair is either in nitrogen or oxygen because of high abundance of electron. This alkylation leads to generation of a fast (N (7)-methyl guanine) as well as a slow (O (6)-methyl guanine) intermediate. The hydrogen bonding properties of guanine was changed due to methylation at the O (6)-position and thereby inducing guanine to adenosine transition followed by DNA damage. The MNU and DMBA induced rat models are very much similar with human ER+ breast cancers in terms of histopathology and other hormonal manipulations.

Afterwards, the effect of ALA and GLA was evaluated upon autonomic dysfunction. It was a well known fact that autonomic dysfunction, cardiovascular complications, poor quality of life and premature mortality is very common to several types of cancer chemotherapeutic regimen and it may be shepherded to increased sympathetic activity and decreased vagal tone. In fact, autonomic dysfunction is now a day is considered as a non-invasive prognostic marker for chemotherapeutic regime The HR, R wave amplitude and RR interval are the crucial markers of autonomic dysfunction, which were positively modulated after ALA and GLA treatment. Treatment group embarked a marked positive

regulation of the HRV factors, which is indicative of the positive regulation of autonomic dysfunction by both PUFA and also ensure its long term safety in chemotherapeutic regimen.

The biochemical markers could be majorly categorized as the ones associated with antioxidant defense or to the physiological mechanisms, and authors validated both. ROS are constantly produced in all aerobic cells and are counter balanced by the antioxidant enzymatic defense. However, during anaerobic/hypoxic conditions like cancer (due to increased cellular proliferation), the counter balance effects of antioxidant enzymes are subsided. The damage to the cellular lipids and proteins can be validated through increased production of TBARs and PC respectively; which was very well evident after the DMBA and MNU treatment. The increased ROS production also inhibits the enzymatic antioxidant defense of GSH, SOD and catalase, as they all work in tandem to curtail ROS through series of peroxidation, dismutation and oxidation reactions. The decrease in the enzymatic defense of SOD, catalase and GSH suggest their increased utilization which was profoundly evident after the DMBA and MNU treatment. It would be appropriate to remark that ALA and GLA administration curtailed the levels of TBARs and PC with restoration of enzymatic antioxidant defense of SOD, catalase and GSH.

The mammary gland tissues were further evaluated in terms of morphological analysis using carmine staining, H&E staining and SEM. Cancer progression is defined by two hallmarks named as cellular proliferation and angiogenesis. The results of carmine staining were event with increased in number of ABs and lobules and the same was subsided after ALA and GLA treatment. In case of H&E staining, there was a scattered

pattern of CEC whereas LCT and DCT were hard to identify along with loss of duct and MEC. ALA and GLA treatment restored all the cellular architecture to the normal. Marked proliferation after the toxicant administration was observed with increase in micro vessel formation, loss of intra-arterial cushion and vascular conglomeration, when perceived through SEM analysis. ALA and GLA treatment demarcated a marked impression on cellular architecture and morphology of the mammary gland tissue and decreased the growth of enlarged capillaries (the sign of rapidly growing tumors). Henceforth, ALA and GLA imparted dose-dependent curtailment of cellular proliferation and therefore warrants further validation through more stringent markers.

Afterwards, the metabolic analysis of the serum was evaluated using ^{1}H NMR studies. The results shows that the increased concentrations of serum acetyl-glycoproteins (both NAG and OAG) (acute phase anti-inflammatory proteins expressed during inflammation and immune response) was recorded after the toxic treatment and is in line with previous investigations in liver disease, inflammatory disease and cancer. As expected, ALA and GLA (an anti-inflammatory PUFA) curtailed the expression of acute phase proteins expressed during inflammation. The increased levels of amino acids like arginine, glycine, histidine, tyrosine, creatine and phenylalanine indicates abnormal/aberrant biosynthesis of amino acids in toxicant treated rats. The increased levels of arginine and citrulline suggest protein catabolism, deriving overall picture of high metabolic activity, a hallmark for tumor progression. The high metabolic activity as evident through increased levels of amino acids was also endorsed through increased levels of formate (a product of glycine metabolism through glycine succinate pathway). The decreased level of glucogenic amino acids (glutamate, glutamine, proline, isoleucine and alanine) in

toxicant treated group expressed their increased utilization in energy production. Proline metabolism is especially important in nutrient stress, as it is interchangeably converted into glutamate and glutamine. Concomitant drug treatment further diminished the levels of glucogenic amines which could be accounted to the fact that exogenous PUFA would have provided a faulty lipid to the fastly growing tumour cells. Whereas the requirement for the amino acids as a building block for cellular membranes was prevalent till such time. The deregulated metabolites represent altered cancer cell energy metabolism including amino acid metabolism (glutamate, glutamine, alanine, etc.), glycolysis or gluconeogenesis (glucose, and lactate,) and lipid metabolism (LDL, VLDL, choline, and acetate) and are associated with high rate of glycolysis. Most of the metabolic changes in toxicant treated animals were reset back to normal after PUFA administration, suggesting that the ALA and GLA have potential to balance the metabolic abnormalities in fastly growing cells.

To evaluate the proteomic signal from different identified protein associated with mitochondrial mediated death apoptosis pathway, hypoxic microenvironment, calcium influx and involvement of anti-cholinergic pathway, immunoblotting and qRT-PCR assay were performed. In terms of apoptosis, both drugs have produced marked protection against toxicant induced carcinogenesis. Treatment groups indicate decreased expression of anti-apoptotic proteins (Bcl-2 and Bcl-xl) along with positive modulation of BAD and BAX, a pro-apoptotic member to the Bcl-2 family. Similar findings were found with mRNA expression when scrutinized with qRT-PCR. Cytochrome c is released due to loss of channel integrity and the decreased expression of VDAC validates the same. After ALA and GLA treatment, the protein and mRNA expression of VDAC and cytochrome c

confirm the same. Cytochrome crelease then triggers the congregation of cytoplasmic apoptosome. The apoptosome is a complex formed of Apaf-1, cytochrome c and procaspase 9. Apoptosome formation decreased the cytosolic levels of Apaf-1 and procaspase 9 and the same findings were observed after ALA and GLA treatment. Procaspase 9 was cleaved with the formation of apoptosome and leads to the formation of active caspase 9which results in the activation of caspase 3 and 8. The caspase activation leads to activation of downstream caspase cascade and leading to apoptosis. ALA and GLA treatment increased cytosolic caspase 3 and 8 and thereby accredits apoptosis.

It is well known that tumor cells require energy from glycolysis due to hypoxic condition of the cells (Warburg effect). HIF-1α regulates the hypoxia and is further regulated by 2-oxoglutarate (2-OG) and iron dependent hydroxylases enzyme PHD-2. It was previously reported that the increased glycolytic activity in tumor cells is combined with increased FASN to meet the fatty acid requirements through de novo fatty acid synthesis. ALA and GLA treatment increased the expression of PHD-2 and thereby curtailed the expression of HIF-1α when scrutinized through immunoblotting and qRT-PCR studies. The decreased expression of HIF-1α was cross validated through the decreased expression of NFκBp65and UCHL-1. During deubiquination of HIF-1α, NFκBp65 imparts positive modulatory effect upon HIF-1α and UCHL-1 stabilizes HIF-1α. ALA and GLA treatment downregulated the protein and mRNA expression forNFκBp65 and UCHL-1; suggesting curtailment of cellular hypoxia. ALA and GLA treatment also decreased the expression of FASN and SREBP-1c, the markers for de novo fatty acid synthesis.

The Ca^{2+} influx regulating proteins play a pivot role in angiogenesis and trigger so many intercellular checkpoints to increase metastasis and invasiveness. There is a positive

correlation between Ca^{2+} channel influx with cellular migration and vascular invasiveness. The decreased expression of VDAC after drug treatment suggests stabilization of membrane potential transition pore (MPTP) and in Ca^{2+} influx. Subsequently, the role of Ca^{2+} influx was validated trough α7nAchR proteins by which cholinergic anti-inflammatory pathway have been regulated. In normal cell, Ca^{2+} trigger is requisite for maintaining cell physiology. The production of inflammatory cytokines is controlled by Ach and nicotine via α7nAchR. α7nAchR have four transmembrane domains (TM1-4). A regulatory intracellular domain is located between TM3 and TM4 and forms a hetero or homo pentamers of α7nAchR which maintain integrity of central ion channel in transmembrane junction. The entry of different cations (Na^+, K^+ and Ca^{2+}) is regulated through nAchRs group of proteins and it is much more selective for Ca^{2+} influx. The cations influx reduces the negative charge on intracellular side causing membrane depolarization. After initiation of membrane depolarization, the gates on the intracellular side of plasma membrane is open for the entry of voltage gated Ca^{2+} and leads to downstream activation of various intracellular angiogenic cascade [vascular endothelial growth factor (VEGF), endothelial growth factor (EGF) and PI3AkT]. Present study elucidates biphasic regulation of cancer cells by GLA and ALA through inhibition of Ca^{2+} influx and activation of cholinergic anti-inflammatory pathway. The α7nAchR mediated cholinergic signaling after drug treatment was also pathway was also confirmed by increased expression of TNF-α.

From the above findings, it was concluded that ALA and GLA ameliorate the morphological, biochemical and associated biological effects of MNU and DMBA. In summary, the present study has assessed the anticancer effects of ω-3 and ω-

6PUFAswhen consumed or treated individually. ALA and GLA can differentially inhibit mammary tumor development by changing the cell membrane fatty acid composition, suppressing eicosanoid biosynthesis and influencing signaling transcriptional pathways to inhibit cell proliferation and induce apoptosis. This study also provided evidence for using ω-3 and ω-6PUFAsas a nutritional intervention in the treatment of ER+ breast cancer to enhance conventional therapeutics, or potentially lowering effective doses. Our results suggest the possible therapeutic potential of PUFAs against mammary gland carcinoma without any untoward effect. It last, it was also conclude that the mechanism of action of ALA and GLA is mitochondrial mediated death apoptosis pathway, inhibition of hypoxic pathway along with inhibition of anti-inflammatory cholinergic pathway.