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SYNERGISTIC OF SILIBIN AND RUTIN ENHANCES RUNX2 AND ALP EXPRESSION OR NOT

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Abstract
A flavonoid substance called silibin is obtained from milk thistle (Silybum marianum). Studies have been done on it to see whether it has any anti-inflammatory, antioxidant, or hepatoprotective qualities. A flavonoid substance called rutin can be found in many fruits and vegetables.In some preclinical experiments, rutin has been shown to enhance osteoblastic activity, encourage bone formation, and prevent bone resorption. Normally, silibinin and rutin were bought separately, and 25 M of silibinin and 0.1 M of rutin was used to determine their synergistic effects (the amount was derived from linked papers). MG-63 cells were collected, and they were cultured for 3 days straight in both normal medium and osteogenic medium. Comparing Rutin, silibinin, and a combinational complex revealed fold alterations, and the expression of Runx2 and the ALP gene was also examined.Synergistic effects of silibin and Rutin enhances expression Runx2 and ALP especially in the osteogenic medium.

Keyword: alkaline phosphatase, bone, osteogenic, osteoporosis , PCR, Rutin, silibin.

Introduction

The ability of a material to promote bone growth ectopically or de novo is known as osteoinductivity. Progenitor cells were treated with dexamethasone, glycerophosphate, and ascorbic acid 2 phosphate to stimulate osteogenic differentiation[1]. Within 4 days of being maintained in this media, progenitor cells showed phenotypic changes, losing their spindle-shaped phenotype and developing an osteoblastic appearance with finger-like cytoplasmic excavations. The cells began to group at 16 days and developed the usual lamellar bone-like structures. These developed cells exhibit the ability to precipitate calcium, which is a key characteristic of osteoblasts in terms of functionality. Alkaline phosphatase (ALP) production and calcium deposition by differentiated osteoblasts from the progenitor cells are consistent with the differentiation of bone. Progenitor cells without differentiation lacked this capability[2]

The medicinal plant Silybum marianum, sometimes known as milk thistle, yields silibinin, which has long been used to treat liver disorders. A number of in vitro and in vivo studies, including skin, breast, lung, colon, bladder, prostate, and kidney carcinomas, have recently shown these orally active flavonoid compounds to have considerable anti-neoplastic effects[3].As a bioactive antioxidant, silymarin and silibinin have been proven in several animal experiments to have hepatoprotective effect against toxins and oxidative attack. Human hepatic stellate cells taken from the human liver exhibit anti-inflammatory and anti-fibrogenic properties when exposed to silibinin [4]. According to a different study on silibinin, silibinin can induce BMPs and RUNX2 in hBMSCs to promote osteogenic differentiation, and when silibinin was added at concentrations of 1, 10, or 20 μmol/l silibinin, human bone marrow stormal cells proliferation was not different from the control group, indicating that silibinin had no cytotoxic effects on cells at these concentrations.[5]

Rutin is a dietary flavonoid that is abundantly found in vegetables and fruits. It has a wide range of medicinal benefits that are mainly attributable to its strong anti-inflammatory and

antioxidant characteristics. These fantastic health benefits, which include the prevention of skin cancer, cardiovascular ailments, and neurological problems, have been proven in numerous research. However, the amount and bioavailability of rutin for absorption can have an impact on its health advantages. Rutin's low water solubility has been improved in a number of methods, making it possible to include it in nutritious foods like bread goods. Rutin can be found in a variety of places. Consequently, we can include rutin in our diet. It is a dietary flavonoid phytochemical that is widely present in food plants, has advantageous qualities, and offers significant health benefits.([6]).Osteoporosis is a bone condition characterized by a decline in bone density and is linked to an increased risk of fracture [7]. This illness appears to be a developing health problem that affects senior citizens everywhere. When bone resorption by osteoclasts outpaces bone synthesis by osteoblasts, osteoporosis is shown [8]. Osteoclast-mediated bone resorption inhibition is emphasized in all therapeutic approaches for the treatment of osteoporosis. According to Hodsman et al. (2005), the only types of substances that "stimulate bone formation" are parathyroid hormones[9]. Rutin induced human osteoblast-like MG-63 cell growth and differentiation in assays pertaining to osteogenesis. Alkaline phosphatase activity, collagen type I expression and mineralization level all increased . [10]

RUNX2 (Runt-related transcription factor 2) is a member of the group of transcription factors known as RUNXs, which are thought to be associated with runts [11]. The heterodimeric RUNXs' DNA-binding subunit is encoded for by mammalian RUNXs. RUNX1, RUNX2, and RUNX3 are proteins that have the 128-amino-acid-long "Runt domain" as their distinguishing characteristic. Both the heterodimerization with the non-DNA binding component and the DNA binding are controlled by this domain.[12] . Runx2 is a crucial transcriptional regulator of osteoblast differentiation and it is essential for the development and homeostasis of osteoblasts. Despite having a normal skeletal pattern, runx2-null mice lack osteoblasts and, as a result, bone tissue. Humans that have runx2 gene mutations get cleidocranial dysplasia. Runx2 functions as a transcription factor and is a member of the Runx family. It binds to the osteoblast-specific cis-acting element 2 (OSE2), which is located in the regulatory region of all major osteoblast-related genes and regulates the expression of these genes. Runx2 integrates and merges various signaling pathways that regulate osteoblast function, whereas its expression and/or activity are controlled by a variety of diverse external inputs. [13]

ALP, also known as alkaline phosphatase, is a widely distributed glycoprotein that is membrane-bound and catalyzes the hydrolysis of phosphate monoesters at basic pH levels. Alkaline phosphatase is divided into four isozymes based on the tissues in which it is expressed: intestine, placental, germ cell, and tissue nonspecific alkaline phosphatase (L/B/K) ALP. The intestine and placental ALP loci are close to the end of the long arm of chromosome 2, while the L/B/K ALP locus is at the end of the short arm of chromosome 1. Even though ALPs have been studied for many years and are present in many mammalian tissues, little is still known about them.The transport of phosphate into gut epithelial cells is thought to be facilitated by the intestinal isoenzyme, while the calcification of mammalian bones may be facilitated by the bone isoenzyme.[14]There is not necessarily a correlation between free phosphate localization and phosphatase activity. The enzyme appears to have a close relationship with the metabolism of pre-osseous cells and the formation of a chemically calcified bone matrix. It is still possible, nevertheless, that phosphatase contributes to the calcified matrix's ability to access inorganic salts. If this function does exist, it is secondary because the formation of bone matrix, which is always associated with phosphatase activity, can and does occur in the absence of calcification. If the enzyme is missing, calcification might take place later.According to data, cartilage matrix is used to create bone matrix.Only when there are living cells present does phosphatase become physiologically active. Where it may be seen in the absence of living cells, like in the metaphyseal cartilage fragments, it seems to be biologically inactive. Because phosphatase is momentarily inactivated in weakly acidic media and easily reactivated by alkaline solutions, it is conceivable that the enzyme may persist in weakly acidic tissues in an inactive physiological state and still be used for histochemical demonstration in vitro.[15]

This study aims at checking the efficiency and effectiveness of synergistic effects of Silibin and Rutin and to check if the synergistic effect can enhance Runx2 and ALP genes which increase osteogenic activity.

Materials and methods

Materials Needed

1. Human MG-63 cells
2. Silibinin (25 µM) and Rutin (0.1 mM) stock solutions
3. Normal medium (appropriate for MG-63 cells)
4. Osteogenic medium
5. TRIzol reagent or a similar RNA isolation reagent
6. cDNA synthesis kit
7. Real-time PCR master mix (containing fluorescent DNA-binding dye)
8. Gene-specific primers for Runx2, ALP, and a reference gene (e.g., GAPDH)
9. Real-time PCR machine
10. Consumables: Pipettes, microcentrifuge tubes, PCR tubes, PCR plates, etc.

Cell Culture and Treatment

MG-63 cells are seeded in the proper culture plates with regular growth medium, and they are given the night to adhere. Fresh regular growth media containing rutin (0.1 mM) and silibinin (25 M) should be used in its stead. the cells for three days. Maintain control cells devoid of therapy. If desired, change the medium to osteogenic media containing silibinin and rutin after three days to provide more stimulation for osteoblast differentiation. Keep control cells in an osteogenic media devoid of rutin and silibinin.

RNA Isolation

Aspirate the medium and wash the cells with phosphate-buffered saline (PBS) after the treatment time is over. As directed by the manufacturer, lyse the cells in TRIzol reagent. Utilizing conventional techniques, extract total RNA from the lysate. Using a spectrophotometer, determine the isolated RNA's concentration and purity.[16]

Real-time PCR Analysis

Utilizing readily available tools or databases, create gene-specific primers for Runx2, ALP, and the reference gene (such as GAPDH). Make certain that the primer pairs are highly specific and effective. Create a master mix with real-time PCR master mix, forward and reverse primers unique to each target gene, and cDNA templates for each reference gene.Depending on the quantity of samples and controls, distribute the master mix into PCR tubes or plates. Follow the real-time PCR instructions provided by the manufacturer of your real-time PCR

apparatus. Set up the device for fluorescence detection and use proper cycling conditions.Utilize the machine's software to gather and examine the real-time PCR data. Use the reference gene as a normalizer and the comparative Ct method (2-Ct) to determine the relative gene expression.

Examine the relative levels of Runx2 and ALP gene expression in treated cells (those receiving Silibinin and Rutin) compared to control cells (those receiving no treatment), both in osteogenic and normal settings. To ascertain the significance of the variations in gene expression, statistical analysis can be carried out using the relevant techniques (e.g., t-test or ANOVA).

Data Analysis

Result:

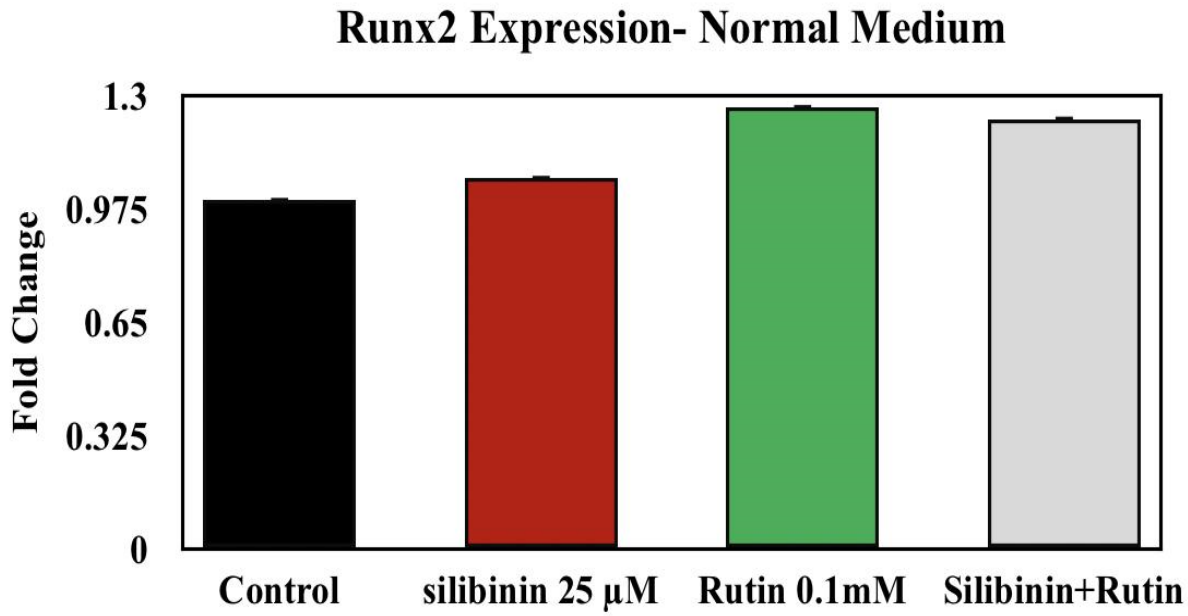

Figure 1A: Rutin shows upregulation of Runx2 in normal medium

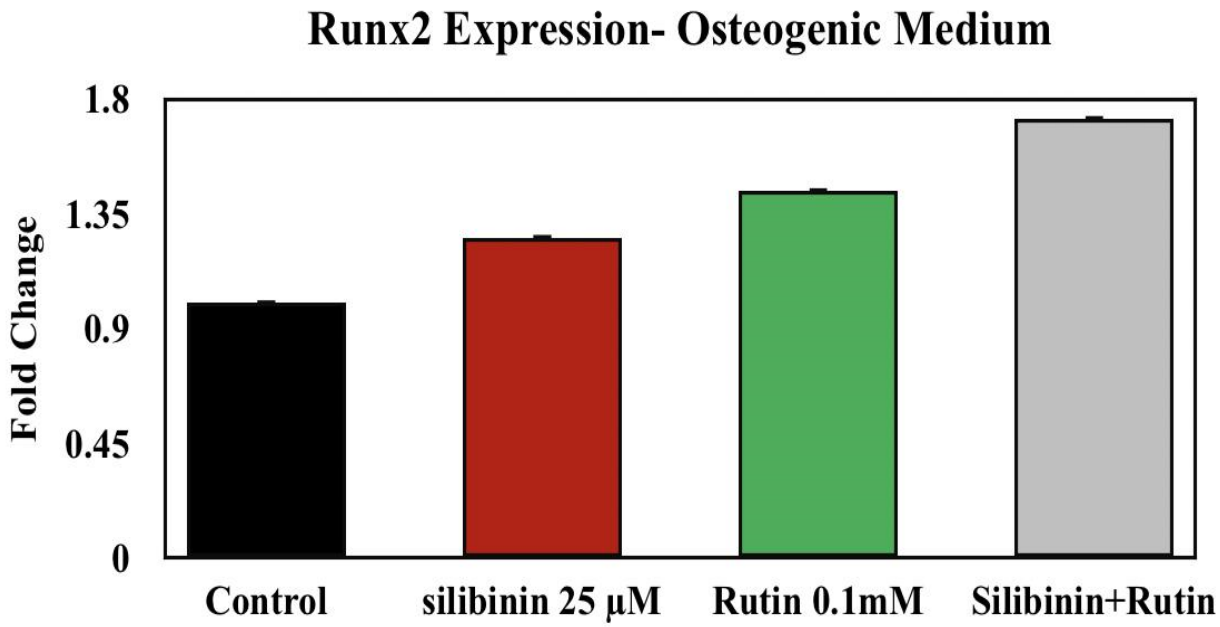

Figure 1B: Synergistic effect of silibin and rutin has shown up regulation of Runx2 in osteogenic medium

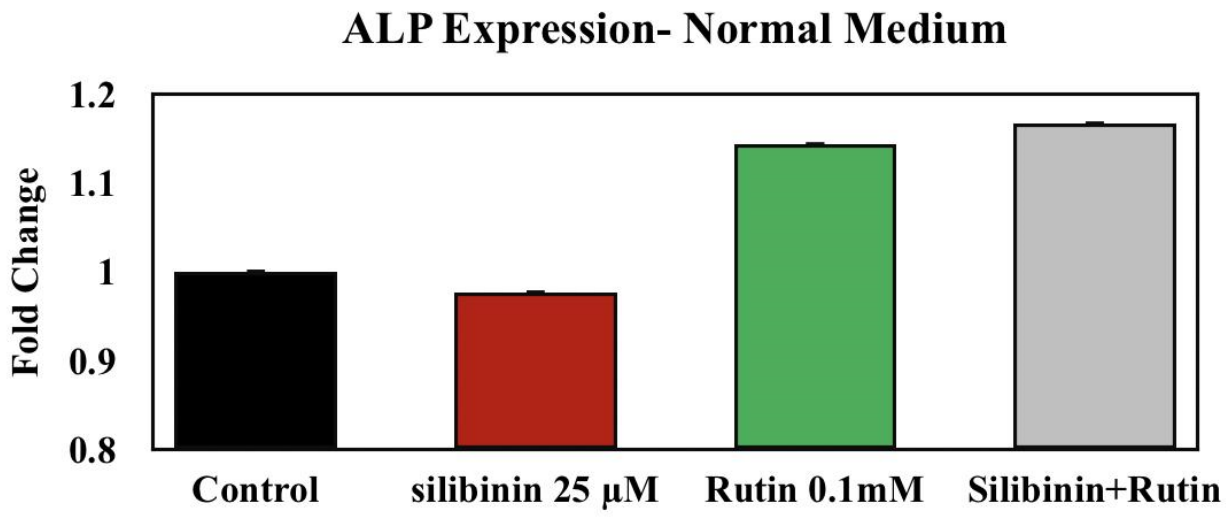

Figure 2A: There is marginal increase in both Rutin and synergistic products and shows upregulation of ALP in normal medium

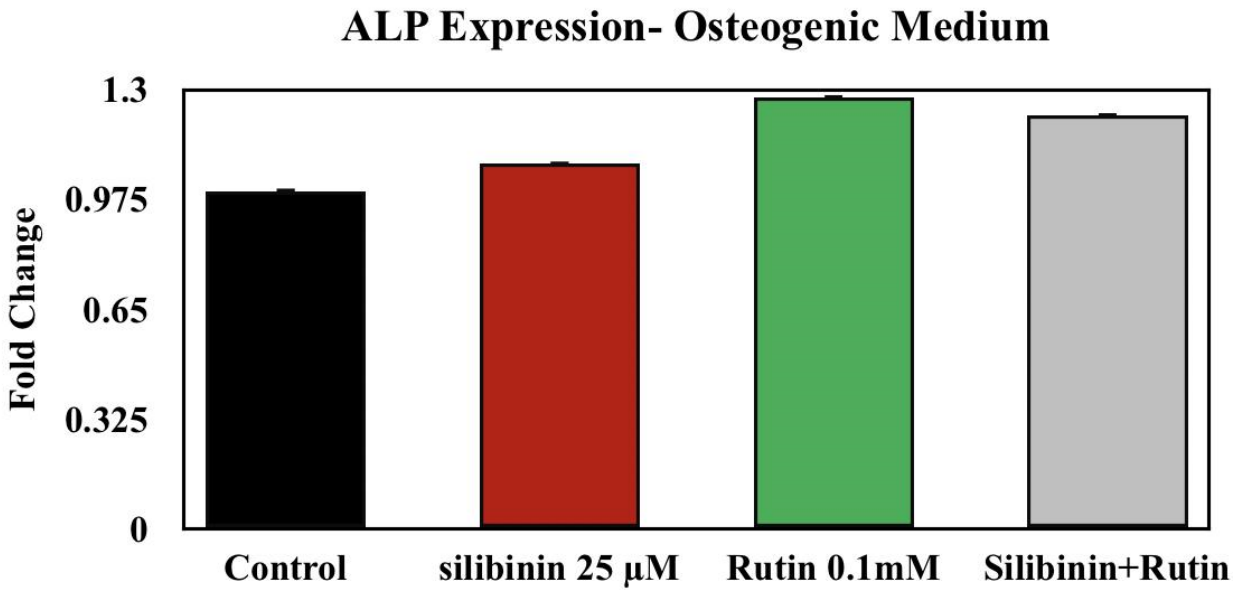

Figure 2B: Synergistic effect of silibin and rutin has shown up regulation of ALP in osteogenic medium.

Discussion:

Based on the results, it can be stated that the synergistic effect of Silibin and Rutin was showing up regulation in case of Runx2 expression in both normal and osteogenic conditions. And in ALP expression, synergistic effect was up regulated in normal conditions but in osteogenic conditions it can be observed that there was up regulation of Rutin than that of the synergistic effect of silibin and rutin. Not much researches were done based on synergistic effects of these flavonoids before but studies were done separately to check for expression of ALP and Runx2

A study was conducted to see if rutin, which was isolated from the plant Chrozophora tinctoria, improved the ossification markers and bone cell proliferation, the study concludes that although numerous flavonoids with anti osteoporosis properties were isolated by the study of C. tinctoria extract. Rutin was particularly encouraging because it demonstrated ossification in bone cells and had punctate proliferative activity with little impact on cell cycle distribution. To put it another way, rutin may be further investigated as a potential antiosteoporotic agent with negligible anticipated mutagenesis consequences.All ossification markers' activity and concentrations, as well as those of the ALP enzyme, OCN hormone, and active vitamin D3 concentration, were all significantly increased by rutin.[17]

Another research done on overview of the function of the rutin-zinc (II) complex in zebrafish and human dental pulp stem cells during the development of bone correlates with the results obtained from this research. The research concludes by stating that at the molecular level, the mRNA expression profile of osteoblast indicators such Runx2, type 1 col, OC, and ON was examined after rutin and rutin-Zn(II) exposure. Additionally, real-time RT-PCR analysis was used to examine the expression of inhibitors of osteoblast growth like Smad7, Smurf1, and HDAC7.[18]

Another research was done on the topic which majorly focuses on osteoblasts, osteoclasts and flavonolignan whether silibinin promotes osteoblastogenesis and osteoprotection or not. This

research shows correlating results with the results obtained from our research. The research concludes by stating that Silibinin enhanced bone nodule formation via calcium deposits, which sped up cell proliferation and aided matrix mineralization. In addition, alkaline phosphatase, collagen type 1, connective tissue growth factor, and bone morphogenetic protein-2 osteoclastogenic indicators were further induced by silibinin.[19]
The synthesis and characterization of zinc-silibinin complexes, which have the potential to be bioactive compounds with angiogenic and antibacterial capabilities for bone tissue engineering, have been the subject of research. This study found that silibinin and Zn-silibinin complexes promoted osteoblast development both at the molecular and cellular levels by upregulating Runx2, type 1 col, ALP, and OC mRNA expression and calcium deposition and ALP activity. Zn-silibinin complexes have also demonstrated potential impacts on the growth of osteoblasts via modulating the miR-590/Smad7 signaling pathway.[20]
Not much research was conducted against the results obtained from this research and most of the research supported the results presented in this research hence further strengthening the research with proofs from other researches.

Conclusion:
According to research, silibin and rutin may work synergistically to increase the expression of Runx2 and ALP, two essential proteins involved in the formation and mineralization of bones. This suggests a possible advantage for osteogenesis and bone health. It's crucial to remember that the amount of existing knowledge is limited and that additional research is required to confirm these results. It is important to do a thorough investigation of variables such as dosage, timing, specificity to cell types or animal models, and overall effect on bone health.

Future scope:
Future research opportunities include opportunities for in vivo and clinical studies, combination therapy, and translational applications related to the synergistic effects of silibin and rutin on Runx2 and ALP expression. By addressing these issues, we can improve our knowledge of bone health and perhaps create brand-new therapeutic strategies for conditions related to bones.

CONFLICT OF INTEREST: There is no conflict of interest.

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