INTRODUCTION

Human skin is a controllable tissue in which to explore epithelial-mesenchymal interactions during development and in postnatal life. Hair dermal papilla cells (DPCs) are specialized mesenchymal cells that exist in the dermal papilla (DP) located at the bottom of hair follicles. These cells play significant roles in hair formation, growth, and cycling. Hair follicle formation is usually direct by an aggregation of dermal mesenchymal cells, the origin of DPCs, in the embryonic skin. Cultured DPCs also have hair-forming activity and do not lose the activity even after long-term cultivation, provided cells that cultured with conditioned medium from keratinocytes without fibroblast growth factor or a medium-containing fibroblast growth factor.

ABSTRACT

Introduction: Nowadays, hair loss is becoming a major problem for a large number of people. The main factors affecting hair growth include racial, gender, genetics, and hormonal. Furthermore, environment, physiology, and nutrition are the tools that are affecting hair growth. Materials and Methods: This article analyses the effects of silymarin and Vitamin C on the proliferation of dermal papilla cells (DPCs), COX2, inducible nitric oxide synthase (iNOS), total anti-oxidant capacity (TAC), and reactive oxygen species (ROS). The DPCs of the hair follicle treated by 50 and 100 µM of silymarin and 100 µM of Vitamin C on days 2, 7, and 14. Results: A comparatively effect of silymarin and Vitamin C demonstrated that Vitamin C is more effective in the stimulation of dermal papillae in cell culture. Furthermore, the stimulation effects of silymarin are attributed to its antioxidant activity of the cells. Conclusion: The overall result shows that cell proliferation and TAC significantly increased. However, iNOS, ROS, and COX2 levels were decreased.

Key words: Antioxidant, dermal papilla, silymarin, Vitamin C

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women tends to diminish or even disappear from all areas, notably body sites, with the single exception of the face where, for some unknown reason, it increases.\[3\]

The turnover of hair follicles occurs in three steady phases, namely proliferation (anagen), involution (catagen), and resting (telogen). Anagen is an active growth phase of the hair follicle, thereby the length and strength of hair depend on the duration of this phase.\[4\]

Human hair proliferation controls by androgens, which response to hormones differently depending on body location. DPCs of the beard, and armpit, and scalp hair of people who are genetically predisposed to baldness were shown to be androgen target cells. DP is one population of mesenchymal cells in the skin and focus of interest because the DP not only stimulates hair follicle development and growth, but it also seems to be a reservoir of multipotent stem cells.\[4\]

Silybum marianum L. (Milk thistle) is an ancient medicinal plant that has been used for centuries as the treatment of different diseases such as liver and gallbladder disorders, protecting the liver against toxic agents.\[5\] For many years, silymarin used as a “hepatoprotection.” However, the mechanism of silymarin is not clear and fully understood; studies reported that silymarin has antioxidant, immunomodulatory, antifibrotic, anti-inflammatory, and antiviral properties. Due to the short half-life of silymarin and quick conjugation in the liver and principal excretion in bile, it should be used with high or repeated doses for controlling hepatic inflammation in vivo.\[6\]

Effects of silymarin or silibinin on breast cancer, ovarian cancer, lung cancer, skin cancer, prostate cancer, cervical cancer, bladder cancer, liver carcinoma, and colon cancer, have been reported. The mechanism of the cytoprotective activity of silybin is probably assigning to its antioxidative and radical scavenging effects.\[7\]

Vitamin C, known as ascorbic acid and L-ascorbic acid, is a vitamin found in various foods and sold as a different dietary supplement. Vitamin C is essential as a supplement that involved in the repair of tissue and the enzymatic production of certain neurotransmitters. Vitamin C, through the mechanism of angiogenesis, has the potential to enhance the density of DP. Topical Vitamin C may have the effects for partial corrections of the regressive structural changes associated with the aging process.\[8\]

In a study on epidermal cells, it has shown that the apoptosis and DNA damage caused by ultraviolet radiation was significantly decreased by silymarin by the nucleotide excision repair mechanism, which makes silymarin an excellent option for prophylaxis of skin cancers.\[9\] Cheon et al. in 2019 demonstrated that silibinin treatment elevated the spheroid formation of DPCs through the activation of protein kinase B and Wnt/\(\beta\)-catenin signaling pathways. Their data suggest that silibinin may have a potential effect on the treatment of hair loss by promoting hair growth induction in the DP.\[10\]

In this study, DP, as a stem cell source of the hair follicle, treated with silymarin to understand the protective role of this phytochemical extract against oxidative stress factors.

**MATERIALS AND METHODS**

**Study population**

In this study, dermal papillae were initially isolated from skin biopsies (\(n=5\)) from 5 individuals (male and female), who referred for treatments to the skin and hair clinic.

Skin biopsies are often full thickness and therefore have an epidermis, dermis, and dermal white adipose tissue, with the base of the hair follicles located in the adipose tissue. If the biopsy is from the scalp, then approximately 90% of these follicles are in anagen.\[10,11\]

**Study design**

**Cleaning and end-bulb isolation**

Isolation of the dermal papilla cells from human hair follicles carried out according to the procedure described by Topouzi et al (2017). Briefly, the skin biopsy was washed for 10 min in a petri dish-containing Dulbecco’s minimal essential medium with 2% antibiotics-antimycotics (ABAM). Following this, the skin biopsy transferred to a new petri dish containing Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1% ABAM. The cells were washed and trypsinized as to be regularly passaged. Using a sterile Pasteur pipette, put eight small drop, and one larger drop of DMEM supplemented with 1% ABAM onto the inverted lid of a petri dish. The drops were covered by placing the base of the petri dish inside the lid. If required, section tissue into manageable pieces using a sterile scalpel. Using sterile Noyes spring scissors and Dumont forceps, trim away any adherent adipose or connective tissue surrounding the lower section of the follicle to expose the end bulb located at the base of the follicle. The forceps can be used to gently pull up connective tissue and adipose tissue into a peak, away from each follicle, while the scissors can be laid parallel to each follicle to clean efficiently.\[10,11\]
Inversion

In this experiment, right-handed method was used for dissection. Using a fine needle held in a 1-ml syringe on one hand, the end bulb was fixed in place by pressing against the cylindrical cut top on its left side. With a fine needle held in a 1-mL syringe in one’s left hand, the end bulb should be fixed in place by pressing against the cylindrical cut top, on its left side. The second needle used in a 1-mL syringe in one’s right hand, pushed through the round bottom of the end bulb to invert the structure and expose the hair matrix and the DP residing within. When inverting the bulb, refrain from holding the needles at a steep angle. Instead, the needles held as close to the horizontal plane as possible.

The inverted end bulb was at the end of the right-hand needle. Used the left-hand needle to pull off the attached matrix. The matrix may have become dislodged during the inversion.

The left-hand needle used, gently prize the inverted end bulb off the right-hand needle by rumpling up the dermal sheath. The sheath held with the left-hand needle, and the DP brushed with the right-hand needle to remove any remaining matrix cells.

The inverted end bulb was transferred with the exposed and cleaned DP to the more considerable collection drop, which is at the top of the inverted petri dish lid. The procedure was repeated until the required number of inverted end-bulbs is contained within the significant drop of DMEM. Transection of DP was performed after the collection of all end bulbs, as individual bulbs due to their small size and shape they can be easily lost.

Adhesion and transection of dermal papilla

Eight-inverted end bulbs were lined up within the more considerable drop of DMEM/1% ABAM, replaced the used needles with new clean needles. The inverted sheath secured in place with the left-hand needle, the sharp edge of the right-hand needle used as a knife, and separated the DP from the connective tissue sheath. The DP can then be transferred to a 35-mm petri dish containing 2.5 mL of DMEM supplemented with 20% fetal bovine serum (FBS) and 1% ABAM. The DP were transferred between petri dishes, rotated the needle 180° while submerged in the new DMEM/20% FBS/1% ABAM containing a 35-mm dish. Eight DP was transferred to each 35-mm dish and disperse evenly on the bottom of the petri dish. If papillae are floating, use the needle edge to push them to the bottom of the dish. The 35-mm plates containing the isolated DP transferred to an incubator and set at standard culture conditions of 37°C 5% CO₂ in a humidified environment.

Silymarin

Silymarin extract was added in dosage at a level of 50 and 100 µmol to the prepared cell culture of the DPCs were recovered at time interval 2, 7, and 14 days.

Vitamin C: Vitamin C was added at the level of 50 µmol to a prepared culture of DP on the interval 2, 7, and 14 days.

For studying, four groups were chosen:

- Group 1: Control group: DMEM + FBS were added to cell culture without silymarin and Vitamin C
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- Group 2: DMEM + FBS were added to cell culture with 50 µmol aquatic extracts of silymarin
- Group 3: DMEM + FBS were added to cell culture with 100 µmol aquatic extracts of silymarin
- Group 4: DMEM + FBS were added to cell culture with 50 µmol of Vitamin C.

The cell proliferation was tested by 5-Bromo-2'-deoxyuridine (BrdU) assay, reactive oxygen species (ROS), inducible nitric oxide synthase (INOS), and COX2 were tested by different kits and methods.

**5-Bromo-2'-deoxyuridine assay**

The BrdU Cell Proliferation Assay Kit was used and detected BrdU that attached to cellular DNA during cell proliferation by using an anti-BrdU antibody. The cells were cultured by labeling medium that contains BrdU; this pyrimidine analog attached to the place of thymidine into the newly synthesized DNA of proliferating cells.

The cells (5000 cells/well) were seeded in 96-well plates. The cells were incubated with different concentrations of herbal extracts for 48 h. Then, 20 µl of the BrdU labeling solution was added, and the cells were incubated for an additional 4 h. After removing the BrdU labeling solution, the cells were fixed and denatured with the kit's FixDenat solution for 30 min at 25°C. Denaturation of the DNA is necessary to enhance the accessibility of the incorporated BrdU and facilitate its ability to locate the antibody. Samples were incubated for 90 min with a peroxidase-labeled anti-BrdU antibody (anti-BrdU antibody provided in the assay kit), which binds to the BrdU incorporated into newly synthesized cellular DNA. After washing off the unbound anti-BrdU-POD, the color reaction was developed for 3–5 min with the substrate solution and blocked by adding 25 µl 1 M sulfuric acid, and the optical densities of the samples were measured using a microplate reader at 450 nm.

![Figure 1: The effects of silymarin and Vitamin C on cell proliferation (BrdU assay).](image1)

*Figure 1: The effects of silymarin and Vitamin C on cell proliferation (BrdU assay). P = 0.016 compared to control group. Data are mean ± standard error of the mean of three samples arranged in duplicate. Details about treatment are as described in the method section.*

![Figure 2: The effects of Silymarin and Vitamin C on COX2 of cells.](image2)

*Figure 2: The effects of Silymarin and Vitamin C on COX2 of cells. P = 0.015 compared to control group. Data are mean ± standard error of the mean of three samples arranged in duplicate. Details about treatment are as described in the method section. Silymarin was dissolved in H$_2$O control group was treated with H$_2$O also.*

![Figure 3: Comparison of silymarin and Vitamin C effects on inducible nitric oxide synthase levels.](image3)

*Figure 3: Comparison of silymarin and Vitamin C effects on inducible nitric oxide synthase levels. P = 0.016 compared to control group. Inducible nitric oxide synthase is not time-dependent factor and normally decreased without any treatment. Data are mean ± standard error of the mean of 3 samples arranged in duplicate. Details about treatment are as described in the method section.*

![Figure 4: Comparison of silymarin and Vitamin C effects on total anti-oxidant capacity in dermal papilla cells.](image4)

*Figure 4: Comparison of silymarin and Vitamin C effects on total anti-oxidant capacity in dermal papilla cells. P = 0.016 compared to control group. Data are mean ± standard error of the mean of 3 samples arranged in duplicate. Details about treatment are as described in the method section. Silymarin was dissolved in H$_2$O control group was treated with H$_2$O also.*
The cells proliferation was calculated by the following formula:

\[
\text{Cell proliferation (\%)} = \frac{A\ (\text{treated cells})}{A\ (\text{control cells})}
\]

**Nitric oxide synthase assay**

Cells were collected and centrifuged, and then supernatant washed the isolated cells three times by PBS. The cells were washed in ice-cold PBS samples were divided into two parts. A small part of the sample was used for fixation in 4% paraffin. Aldehyde, paraffin embedding, and routine pathological examination, whereas the significant part of the sample were frozen in liquid nitrogen. The frozen samples were grounded in a specific mortar containing liquid nitrogen and suspended in the ice-cold buffer. The suspension was homogenized in three passes of 10 s at 22,000 rpm with Ultra-Turraxt and then sonicated with 10 brief pulses at 40% intensity. The resulting homogenate was centrifuged at 6000 3 g for 10 min at 48°C. The supernatant was kept on ice for the determination of protein concentrations. All samples were aliquoted and stored at 28°C. One hundred microliter of detection reagent B was added to each vial and incubated for 30 min at 40°C. PBS washed the sample for seven times. One hundred microliter of TMB was added to each vial. The blue color developed through the solution. One hundred microliter of stop solution was added to each vial, and the blue color was changed to yellow. The concentration of yellow color has shown the amount of COX2.

**Total anti-oxidant capacity assay**

TAC in the lysed cells was determined by anti-oxidant Caymon Chemical Company. One hundred microliter of metmyoglobin and 150 µL of chromogenic were added to each vial. Forty microliter of hydrogen peroxide was added to each vial. The samples were incubated for 5 min at room temperature. All the plates were measured by a plate reader at 450 nm.

**Determination of reactive oxygen species**

ROS was determined using a Fluorescent ROS Detection kit. One hundred microliter of 7-dichloro fluoro di acetate was added to cell culture after 30 min, cells were incubated at 37°C. PBS washed the cells. Ten Mm of EDTA was added to cells and were measured in 488 nm excitation and 525 nm emission by fluorescent microscope.

**RESULTS**

**The effects of Vitamin C and silymarin on the proliferation of dermal papilla cells by 5-Bromo-2'-deoxyuridine test**

The BrdU Cell Proliferation Assay Kit (Roche Diagnostics, East Sussex, UK) detects BrdU. The anti-BrdU antibody used was added to each vial and incubated for 10–20 min. The blue color was spread through the vials. Stop solution was added to each vial. After this section, the bluestem color changed to yellow. In the end, optical density was assessed by the micro-plate reader in 450 nanometers.
to incorporate into cellular DNA during cell proliferation. When cells cultured with labeling medium, this pyrimidine analog was incorporated in place of thymidine into the newly synthesized DNA of proliferating cells. After removing the labeling medium, cells fixed, and the DNA denatured with our denaturing solution. Then a BrdU mouse mAb is used to find the incorporated BrdU. Anti-mouse immunoglobulin, HRP-linked antibody is then added to recognize the bound detection antibody. HRP substrate TMB is added to develop color through the solution. In this article, the BrdU-based cell proliferation ELISA kit used. The proliferation of cells had meaningful raised with silymarin and vitamin C. There was no difference between the proliferation of the control group and 50 µM of silymarin on the 2nd day of the test. However, on days 7 and 14, significant rises of proliferation were shown. Also, by 100 µM silymarin significant rise of proliferation was detected. Vitamin C analysis showed a meaningful increase of DP proliferation in compare with the silymarin and control group [Figure 1].

**The effects of silymarin and Vitamin C on COX2 levels in dermal papilla cells**

The analysis by SPSS and COX2 kit (Assay designs and stress gene Inc. Michigan, USA) showed a significant decrease of COX2 in days 2, 7, and 14.

Treatment of DPCs with 50 and 100 µM of silymarin and 50 µM of Vitamin C on days 2, 7, and 14 in comparison with the control group caused a meaningful decrease of COX2. COX2 level also in the treatment group with a dose of 100 µM of silymarin in comparison with 50 µM had significantly decreased. Furthermore, in DPCs that treated with vitamin C in comparison with 50 and 100 µM of silymarin on days 2, 7 and 14 COX2 enzyme concentrations meaningfully decreased [Figure 2].

**The effects of silymarin and Vitamin C on the quantity level of inducible nitric oxide synthase in dermal papilla cells**

Data analysis with SPSS and iNOS kit (R&D Systems Inc., Minneapolis, USA) on days 2, 7, and 14 was showed a significant decrease of Nitric-oxide (NO) synthesize enzyme in cell culture.

DPC treatment with 50 and 100 µM of silymarin and 50 µM of Vitamin C had meaningfully decreased in comparison with the control group. 100 µM of silymarin had more effect on the decrease of NO synthetized compared with 50 µM of silymarin. Furthermore, the concentration of DPCs, which treated with vitamin C in comparison with 50 and 100 µM of silymarin, was meaningfully decreased [Figure 3].

**The effects of silymarin and Vitamin C on total anti-oxidant capacity of dermal papilla cells**

The following process was done on lysed cells to evaluate anti-oxidant capacity by Cayman Chemical Company kit (Cat. #709001, Ann Arbor, USA). Data analysis by SPSS showed elongation of time had not meaningfully effect on the level of anti-oxidant activity on the control group. However, treated groups by Vitamin C and silymarin had significantly increased in days 2, 7, and 14. Also, the effects of Vitamin C in comparison with 50 and 100 µM of silymarin on the increase of anti-oxidant activity were more visible [Figure 4].

**The effects of silymarin and Vitamin C on reactive oxygen species of dermal papilla cells**

For ROS determination, fluorescent reactive species detection kit used. Data analysis by SPSS showed that the level of ROS increased in the control group in cell culture. However, treatment of DPCs with 50 and 100 µM of silymarin and 50 µM of vitamin C have been decreased, bypassing the time on days 2, 7, and 14. The group that treated by Vitamin C has meaningfully decreased ROS on days 2, 7, and 14 in comparison with the silymarin group. Therefore, the effects of Vitamin C on ROS are more sensible than silymarin [Figure 5].

**DISCUSSION**

Currently, there are different approaches, such as using synthetic medicine, to prevent hair loss in human. It appears that phytochemicals are more efficient and safer for administration as an anti-hair loss. Even though there are several crude phytochemical extracts used for this purpose, but little is known about their mechanism of action of hair products available in the market.

Phytochemicals with relatively high anti-oxidant properties are assumed to stimulate hair follicles and DP. This issue can experimentally be approved either by treating DP in the cell culture or apply the active components directly to the skin. In the present study, the DP cell culture directly exposed to extract rich in silymarin revealed that cell proliferation is significantly promoted (BrdU assay) in the presence of the extract. The stimulatory action of silymarin on the cell culture was relatively less than that...
of vitamin C, which used as a positive control. However, the effect of silymarin on cell proliferation was concentration-dependent, and higher levels of silymarin (100 µM) caused >30% compared to the untreated cells. This preliminary data are in agreement with our previous study showing that the growth of the mesenchymal stem cells is stimulated when treated with selected herbal extracts. Similarly, Rodamun and co-workers showed that silymarin at a concentration of 100 µM had a significant effect on the proliferation of stem cells derived from bone marrow. When the silymarin data compared with those of Vitamin C treated cells, it was shown that vitamin C was relatively more effective in increasing cell proliferation (~50%). The stimulatory action of Vitamin C on cell proliferation is well established.

Interestingly, the stimulatory action of the phytochemicals on cell growth was associated with the amelioration of the anti-oxidant activity of the cells.[13] Furthermore, the increased anti-oxidant capacity of the cells in response to such herbal preparations resulted in the suppression of oxidative stress factors. For instance, when the stem cell growth stimulated due to silymarin, there was a significant suppression in lipid peroxidation products (Thiobarbituric acid reacting substances [TBARS]). Inline, Kim et al.[2009] reported that silymarin added to mesenchymal stem cells in culture could decrease the ROS levels.[15] Previously we have reported that silymarin (S. marianum) exerts hepatoprotective actions by improving anti-oxidant and anti-inflammatory factors.[13]

The silymarin extract stimulates DPC proliferation due to radical scavenging agents. In this article, we developed the effects of two different levels of silymarin on DP proliferation and compared them with Vitamin C effects. It is essential not only to understand the etiologic factors and molecular mechanisms behind hair loss but also to develop natural candidates for the treatment of this condition. It should be noted that silymarin can contribute to the antioxidant defences in different ways. First, by direct free radical scavenging. Secondary, by preventing free radical formation by inhibiting specific enzymes responsible for free radical production, or by maintaining the integrity of the electron-transport chain of mitochondria in stress conditions. Third, by participating in the maintenance of optimal redox status of the cell by activating a range of antioxidant enzymes and nonenzymatic antioxidants, mainly via transcription factors, including Nrf2 and Nuclear factor-κB. Finally, by enabling an array of vintages, responsible for the synthesis of protective molecules, including HSP, thioredoxin, sirtuins, and providing additional protection in stress conditions.[13] Specifically, these findings demonstrated that Vitamin C increased the gene expression ratio of BAX/BCL2, an index of apoptosis, under conditions of hypoxia. Furthermore, under increasing oxygen levels, Vitamin C increased the gene expression of BCL2, an antiapoptotic regulator, and decreased the gene expression of TP53, a proapoptotic factor, and the ratio of BAX/BCL2 mRNA, an apoptotic index. The knowledge that silibinin acts as a scavenger of ROS has led to its widespread dermatologic applications for the prevention of aging in skin. The DPs of alopecia patient has been shown the elevation of ROS.

The Wnt/β-catenin signaling pathway is linked to the maintenance of the anagen phase and the hair-inductive property of DP cells. Silymarin enhances Wnt/β-catenin signaling.[13]

As a consequence cell that treated with silymarin TAC was significantly elevated (P = 0.016), their increase was time and concentration-dependent. Silymarin is probably an ideal anti-oxidant for DPCs in culture. However, data shows significant improvement by vitamin C. Vitamin C behaves as an ROS scavenger and may be useful in combating oxidative damage under conditions of increasing oxygen concentrations and apoptosis.[16]

As shown, the cells were treated with 50 and 100 µM of silymarin and 50 µM of vitamin c; they compared with the control group. Our study demonstrated increasing of TAC but more effective with vitamin C.

Studies carried out on different proliferating cells show that both iNOS and COX-2 metabolites are potent modulators of cell growth. COXs are enzymes that are involved in the synthesis of prostaglandins (PG) from arachidonic acid. The two isoforms of COX (COX-1 and COX-2) differ in many aspects. COX-1 is expressed in most tissues and seems to be responsible for the baseline production of PG. COX-2 is not found in most normal tissues but is highly inducible by inflammatory and mitogenic stimuli. NO is an essential signaling and cytotoxic molecule that is synthesized from L-arginine by iso-forms of nitric oxide synthase (NOS). This enzyme exists in three isoforms, and therefore the inducible or immuno-logical (iNOS or NOS-2) isoform is notably distinguished from the constitutive isoforms by its prolonged production of a relatively large amount of NO.[17] The inhibitory effect of silymarin on iNOS and COX-2 expression suggests the possible application of
silymarin as a useful agent controlling inflammation, proliferation, and differentiation. Lee SK et al. studied the effects of Vitamin C on the proliferation of melanoma cells by the stimulation of COX-2 expression and the insulin-like growth factor (IGF)-I axis. As a result, we found that 1.0 mM Vitamin C inhibits the proliferation of SK-MEL-2 without the induction of apoptosis. At that moment, by the inhibition of COX-2 activity, IGF-II production was also down-regulated by Vitamin C treatment. It coincided with the result of the inhibition of COX-2 by NS-398 and COX-2 siRNA.[17]

Cells were treated by 50, and 100 µM of silymarin and 50 µM of Vitamin C. Significant decrease demonstrated in COX-2 and iNOS assay. Studies showed that 100 µM of silymarin had a better effect than 50 µM concentration and also Vitamin C had a meaningful impact on decreasing of COX-2 and iNOS.

CONCLUSION

In conclusion, a comparative study on the effects of silymarin and Vitamin C on the proliferation of DPCs show that Vitamin C is more effective in stimulation of the DPCs in culture. Evidences presented here show that the growth stimulation effect of silymarin is attributed to its positive role in antioxidant activity of the cells. Further experiments, particularly in vivo is required to confirm the usefulness of silymarin as a natural compound for hair regeneration.

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Conflicts of interest

There are no conflicts of interest.

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