

Effects of Silibinin on Hair Follicle Stem Cells Differentiation to Neural-like Cells

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ABSTRACT

The hair follicle stem cells are an abundant and easily accessible source of pluripotent adult stem cells. Hairfollicle stem cells differentiate into neurons and glial cells *in vitro* and express stem cell markers such as tubulin, RIP, P75, and S100. Silibinin, a flavonolignan and the active component of Milk thistle (*Silybum Marianum*) has been used as a dietary supplement and herbal medication. The aim of the present study was to investigate the effects of Silibinin on hair follicle stem cells differentiation to neural-like cells. The bulge region of the rat whisker was isolated and cultured in Dulbecco's modified Eagle's medium supplemented with different concentrations of silibinin and Neurotrophin-3. One week later, bulge cells immunostained with Nestin and p75 and then with tubulin. The morphological and biological features of cultured bulge cells were observed by light microscopy. Present data demonstrated that silibinin ($0.5 \mu\text{g mL}^{-1}$) can promote differentiation of hair follicle stem cells to neural-like cells.

Key words: Silibinin, β III tubulin, hair follicle stem cell, nestin, neurotrophin 3

INTRODUCTION

The neural crest is a transient embryonic tissue that generates a variety of cell types and tissues in the vertebrate, including the autonomic and enteric nervous systems, neurons, dorsal root ganglia, sympathetic ganglia (Le Douarin and Kalcheim, 1999) meninges, endocrine cells, cardiac muscle, vessels, pigment cells, and tooth papilla (Rochat *et al.*, 1994; Hu *et al.*, 2006).

Hair follicles are self-renewing structures. The bulge region of the hair follicle serves as a repository of multipotent stem cells that support hair follicle cycling (Mignone *et al.*, 2007; Ohyama *et al.*, 2006). The bulge approximates the attachment site for the arrector pili muscle and marks the bottom of the permanent portion of the follicle during cycling (Sulewski and Kirsner, 2010).

There are at least two distinct populations of cells in the bulge that possess stem properties (Blanpain *et al.*, 2004; Blanpain and Fuchs, 2006; Morris *et al.*, 2004; Tumber *et al.*, 2004). The

first population is in contact with the basal lamina at birth and maintains this contact at later stages; the second is suprabasal and appears during the postnatal hair cycles. Furthermore, the bulge region of the whisker follicle also contains cells of neural crest origin which can self-renew and differentiate to produce neurons, Schwann cells, smooth muscle cells and melanocytes (Sieber-Blum and Grim, 2004). The population of cells in the bulge region, contain nestin (intermediate filament) as a marker of neural stem cells and early progenitor cells (Hoffman, 2006). The hair follicle stem cells were studied by Blanpain (2010). Li *et al.* (2003) made a fundamental observation that the hair follicle stem cells may be highly pluripotent (Li *et al.*, 2003; Limat *et al.*, 2003). Li *et al.* (2003) observed that nestin, a neural stem cell marker, was expressed in both neural stem cells and hair follicle stem cells. This observation suggested that hair follicle stem cells could be converted into neurons. Therefore, the hair follicle stem cells may be used for therapeutic purposes in regenerative medicine. Also Limat *et al.* (2003) suggested that multipotent and highly proliferative bulge cells may also be an important source of stem cells for the treatment of recurrent leg ulcers (Limat *et al.*, 2003; Ohyama *et al.*, 2006).

The hair follicle stem cells are a suitable source of the stem cells for regenerative medicine since, (1) they are easily available (2) they are highly pluripotent, (3) they be easily cultured, (4) they have been demonstrated to be differentiated to neurons, (5) they do not carry the ethical issues with embryonic stem cells and fetal stem cells. Further potential applications could be spinal cord injury, neurodegenerative diseases such as Parkinson's and Alzheimer's (Hoffman, 2006).

Herbal remedies are being increasingly used in the world as dietary supplements and therapeutic agents. Milk thistle (*Silybum marianum*) is a herb with known anti-cancer activity (Kittur *et al.*, 2002; Katiyar *et al.*, 1997; Zi and Agarwal, 1999). It has been used experimentally and clinically in the treatment of alcoholic liver disease (El-Kamary *et al.*, 2009) immune disorders (Tager *et al.*, 2001) and as an anti hepatotoxic agent (Saller *et al.*, 2001). Milk thistle contains many diverse chemicals, with antioxidant activities. Among the active components, silymarin has been the most intensively studied compound (Luper, 1999). Silymarin has been known to decrease oxidative stress and protect cells against apoptosis (Manna *et al.*, 1999; Kittur *et al.*, 2002) Neurotrophic and neuroprotective effects of milk thistle extract, suggesting possible beneficial effects of this herbal dietary supplement in the nervous system (Yaghmaei *et al.*, 2009). The active complex in milk thistle, Silymarin, is a lipophilic fruit extract, composed of several isomer flavonolignans: silybin (also spelled silybinin or silibinin), isosilybin, silychristin, silydianin, and dehydrosilibinin (Ding *et al.*, 2001; La Grange *et al.*, 1999). Silibinin is the main component and responsible substance for silymarin biological activity.

There are a small number of reports on the effects of silymarin on central nervous system, such as protective effect against ethanol-induced fetal rat brain injury (Neese *et al.*, 2004; Wang *et al.*, 2002) or protective effect on dopaminergic neurons (Sieber-Blum *et al.*, 2006). Nevertheless, the effects of Silibinin on stem cells differentiation have not yet been evaluated. The aim of the present study was to investigate the effects of Silibinin on hair follicle stem cells differentiation to neural-like cells.

MATERIALS AND METHODS

Animals: All animals experiment were carried out according to the guidelines of the Iranian Council for Use and Care of Animals and were approved by the Animal Research Ethical Committee of Iran University of Medical Sciences. Female Wistar rats (n = 20) weighting 180-200 g were purchased from the Razi Institute of animal facility, Tehran, Iran. The rats were permitted free access to water at all times and were maintained under 24 h light/dark cycles.

Media: Media used were Dulbecco's modified Eagle's medium and Ham's F12 medium (3:1) (Sigma), supplemented with 10% fetal bovine serum, antibiotics (100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin, 0.5 µg mL⁻¹ amphotericin B), 10 ng mL⁻¹ epidermal growth factor (EGF, Sigma-Aldrich), 10⁻⁹ M cholera toxin (Sigma-Aldrich), 0.5mg mL⁻¹ hydrocortisone, 3.4 mM L-glutamine, 5 µg mL⁻¹ insulin and 0.135 mM adenine. Silibinin was purchased from Aldrich Chemical (Milwaukee, WI, USA). Its purity was claimed to be 97% detected by a UV-spectrometer at the wavelength 252-288 nm by the company. The drug was recrystallized in methanol and being dried under vacuum at 353 K over 24 h and then analyzed by HPLC. All other reagents were from Sigma.

Hair follicle Isolation: The rats were sacrificed with ether and the whisker follicles were dissected as described by Sieber- Blum (Yang *et al.*, 1993; Sieber-Blum and Grim, 2004). Briefly, the animal head was rinsed in a mixture of 1:1 betadine and hydrogen peroxidase for 3 min. After trimmed into small pieces (4×8 mm²) and the samples were incubated in 2 mg mL⁻¹ collagenase I/dispaseII solution (Sigma-Aldrich) at room temperature. Most of the connective tissue and dermis around the follicles was removed and the whisker follicles lifted out. Then, the follicles were transferred into a 35 mm dish. The bulge region was then amputated from the upper follicle by making two transversal cuts respectively at the site of the enlargement spots of ORS with a fine needle, and the collagen capsule was incised longitudinally. The culture procedure used was that previously described with a slight modification Yang *et al.* (2003) and Bedada *et al.* (2006). After an additional two rinses, the bulges were transferred into a new dish at a density of 20 per dish. Briefly, 20 isolated bulges were cut into small pieces, plated into 6-well culture plates precoated with collagen type¹ (Sigma) prior to cultivation (the plates were pre incubated for 2 h with medium then the medium was removed) and immersed in a 3:1 supplemented mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) containing 10% fetal bovine serum as described. The bulges were allowed to attach to the collagen for 1 h. All dissection and cultivation procedures were performed under sterile conditions and incubation was at 37°C and 5% CO₂.

Subculture and proliferation: Within approximately 4 days, initiation of the outgrowth of bulge cells from the bulges was observed. One week after onset of this outgrowth the bulges were removed from the culture plates and the cells collected by incubation with a 1:1 mixture of 0.125% trypsin (Sigma) and 0.02% EDTA (Sigma) for 20 min at 37°C. The dispersed cells were centrifuged for 10 min at 2000 rpm and placed in other collagen coated plates and incubated for another week with a medium change every 3-4 days. Cells were routinely passaged every 7 days.

Preparation of incubations with silibinin: Hair follicle stem cells obtained from whisker follicles was divided into four groups. In the first step, different concentrations of silibinin (1, 10, 30 and 100 µg mL⁻¹) were used which resulted in population cell death. In the second step, lower concentrations of silibinin (0.05, 0.1, 0.4, 0.5, 0.7 and 1 µg mL⁻¹) were used. Every 4 days, the medium was changed, and EGF and silibinin were added. After 14 days we have determined the effect of different doses of Silibinin on hair follicle stem cells differentiation.

Quantification of Cell Differentiation: Following addition of differentiation medium containing EGF to HFSc cultures, for rapidly differentiation, we used silibinin only and silibinin plus 10 ng mL⁻¹ neurotrophin 3. Silibinin was added to the cultures with final concentrations of 0.05, 0.1, 0.4,

0.5, 0.7 and 1 $\mu\text{g mL}^{-1}$. Every 2 days, the medium was changed, and EGF and silibinin were added. Cell differentiation was evaluated by counting cells with immunocytochemistry technique.

Immunocytochemical Staining: Cells plated on collagen coated cover slips were washed 3 times for 5 min with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min. The fixed cells were then washed with PBS and incubated in blocking buffer (10% Goat serum, Invitrogen/0.3% Triton X-100, Fluka) for 30 min at room temperature. They were then incubated overnight at 4°C with the following primary antibodies: mouse monoclonal β III-Tubulin (1:200; Chemicon), mouse monoclonal RIP antibody (1:50,000; MAB1580, Chemicon), and mouse anti-nestin monoclonal antibody (1:200; MAB353, Chemicon). Subsequently they were incubated for 2 h at room temperature with the following secondary antibodies: sheep anti-mouse fluorescein isothiocyanate (FITC) conjugate IgG (1:200 F2266; Sigma), Alexa Fluor 546-conjugated goat anti-mouse (1:400; Invitrogen). After a final cycle of rinsing, they were incubated for 15 min with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen).

After washing, cover slips were removed from the 6 wells and mounted on a slide with mounting media for visualization using a fluorescence microscope. To examine the specificity of the nestin antibody, 3T3 fibroblast-like cells were used as negative control cells (Pasteur Institute, Tehran); PC12 cells were used as positive controls for β III-Tubulin (Pasteur Institute, Tehran). Labeled cells were identified using fluorescent microscopy (Olympus Ax70) and cell colonies were observed using inverted microscopy.

Statistical analyses: All the experiments were performed in triplicates and the data analysis values were expressed as Mean \pm SD. The results were analyzed using Student's t-test with one-way ANOVA followed by either a t-test with Bonferroni test, a modified least significant difference test by SPSS-Software. Comparisons were made between different of groups. The difference between groups was considered to be significant at $p < 0.05$.

RESULTS

In this study, we demonstrated the effects of different concentrations of silibinin on differentiation of hair follicle stem cells to neural-like cells in culture. Hair follicle stem cells obtained from whisker follicles. In the first step, we used silibinin (1, 10, 30, and 100 $\mu\text{g mL}^{-1}$) which resulted in population cell death (Fig. 1). In the second step we added silibinin to the cultures with much lower concentrations of 0.05, 0.1, 0.4, 0.5, 0.7 and 1 $\mu\text{g mL}^{-1}$. We determined that 1 $\mu\text{g mL}^{-1}$ silibinin was toxic dose and stopped the cell growth. But in the concentration of 0.5 $\mu\text{g mL}^{-1}$, silibinin significantly increased the rate of hair follicle stem cells differentiation. When neurotrophin 3 was added to silibinin, a synergistic effect on cell differentiated was observed on day 14 of the culture (Fig. 2). To examine the specificity of the β III-Tubulin antibody (Fig. 3), 3T3 fibroblast-like cells and PC12 cells were used as negative and positive control cells respectively. Following addition of differentiation medium containing EGF to HFSc cultures, for rapidly differentiation, we used silibinin only and silibinin plus 10 ng mL^{-1} neurotrophin 3. Silibinin was added to the cultures to final concentrations of 0.05, 0.1, 0.4, 0.5, 0.7 and 1 $\mu\text{g mL}^{-1}$. Then, we added silibinin and neurotrophin 3. Every 2 days, the medium was changed, and EGF and silibinin were added. Cell differentiation was evaluated by counting cells with immunocytochemistry technique. Silibinin at concentrations of 0.5 $\mu\text{g mL}^{-1}$ significantly increased the rate of hair follicle

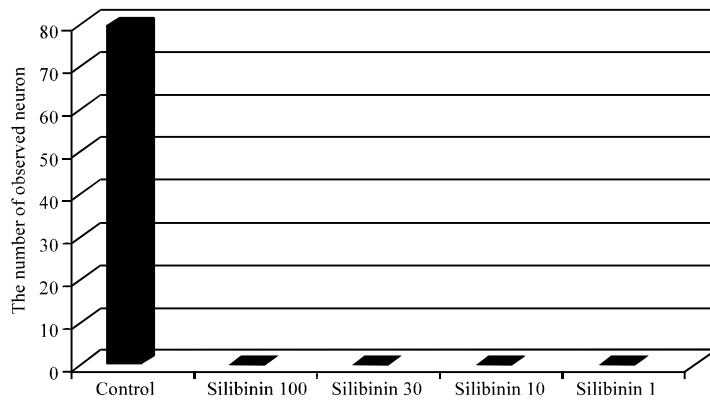


Fig. 1: Determination of the number of differentiated neurons from hair follicle stem cells (HFSCs) at increasing concentrations of silibinin compared with control group

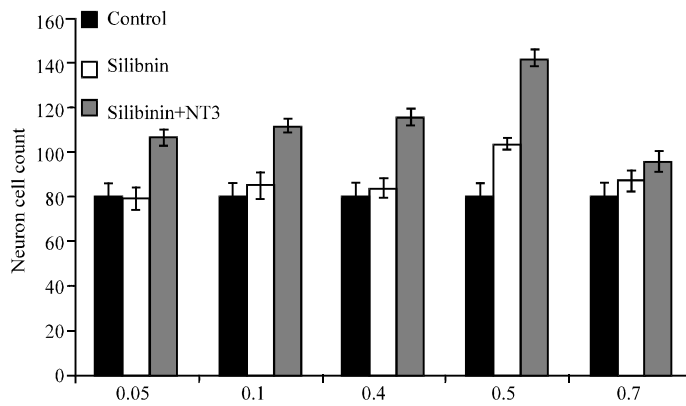


Fig. 2: The neurotrophic effect of Silibinin and Silibinin-neurotrophin 3 on the proliferation and differentiation of the rat HFSCs to neural-like cells. The number of neurons was counted after 14 days *in vitro*. Values represent the mean \pm SEM. * $p < 0.05$, ** $p < 0.005$ vs with untreated control

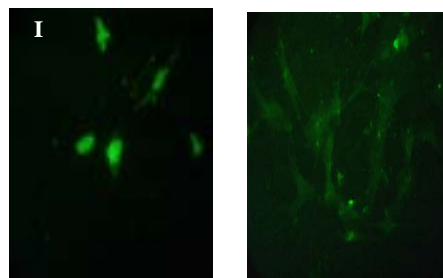


Fig. 3: Photomicrograph of the differentiated rat HFSCs to neurons. A) PC12 in cell culture for positive control againsts β -III tubulin, and B) NIH/3T3 fibroblast in cell culture for negative control againsts β -III tubulin. Scale bar = 200X

stem cells differentiation at day 14 after culture. Silibinin at lower concentration (0.7, 0.4, and 0.1 $\mu\text{g mL}^{-1}$) also induced an increase in the number of differentiated cells on day 14 of culture,

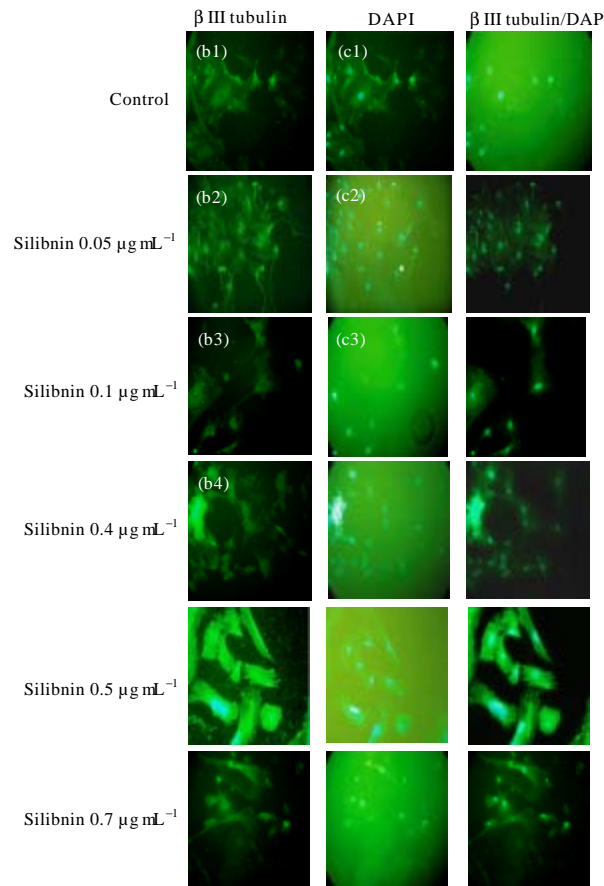


Fig. 4: β -III tubulin and DAPI immunoreactivity on differentiated rat HFSCs in control and silibinin treated. (a1-b1-c1): As a control, differentiated rat hair follicle stem cells have no β -III tubulin immunofluorescent. (a1): β -III tubulin fluorescein fluorescence. (b1): Corresponding DAPI nuclear stain. (c1): Merged images. Immunofluorescence is predominantly nuclear. (a2-b2-c2): Differentiated rat HFSCs treated by Silibinin $0.05 \mu\text{g mL}^{-1}$. Most cells, including neuronal cells in aggregates, have lost β -III tubulin immunofluorescence. Rare cells show asymmetric β -III tubulin immunofluorescence. (a2): β -III tubulin fluorescein fluorescence. (b2): Corresponding DAPI nuclear stain. (c2): Merged images. (a3-b3-c3): Differentiated rat HFSCs treated by Silibinin $0.1 \mu\text{g mL}^{-1}$. All HFSCs show low levels of β -III tubulin-immunoreactivity. (a3): β -III tubulin fluorescein fluorescence. (b3): Corresponding DAPI nuclear stain. (c3): Merged images. (a4-b4-c4): Differentiated rat HFSCs treated by Silibinin $0.4 \mu\text{g mL}^{-1}$. Some cells in clusters(neuronal cells) still expressed β -III tubulin. (a4): β -III tubulin fluorescein fluorescence. (b4): Corresponding DAPI nuclear stain. (c4): Merged images. (a5-b5-c5): Differentiated rat HFSCs treated by Silibinin $0.5 \mu\text{g mL}^{-1}$. The most of HFSCs expressed β -III tubulin. They are intensely β -III tubulin-immunofluorescent. (a5): β -III tubulin fluorescein fluorescence. (b5): Corresponding DAPI nuclear stain. (c5): Merged images. (a6-b6-c6): Differentiated rat HFSCs treated by Silibinin $0.7 \mu\text{g mL}^{-1}$. All rat HFSCs show low levels of β -III tubulin-immunoreactivity. (a6): β -III tubulin fluorescein fluorescence. (b6): Corresponding DAPI nuclear stain. (c6): Merged images. Scale bar = 200X

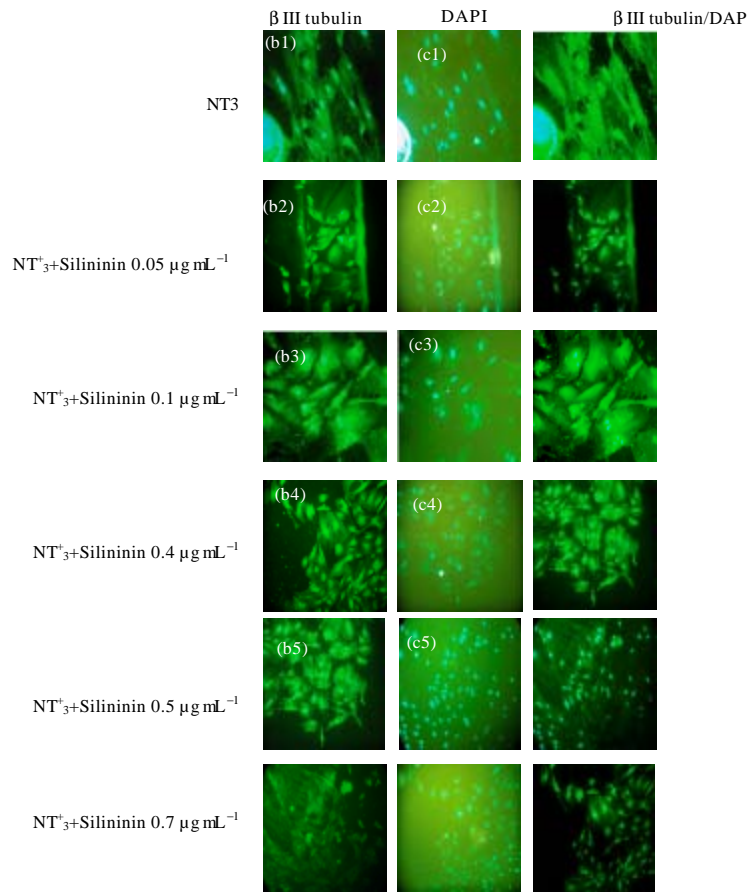


Fig. 5: β -III tubulin and DAPI immunoreactivity on differentiated rat HFSCs in NT3 and Silibinin-NT3 treated. (a1-b1-c1): As a NT3 control differentiated rat hair follicle stem cells have no β -III tubulin immunofluorescent. (A1): β -III tubulin fluorescein fluorescence. (b1): Corresponding DAPI nuclear stain. (c1): Merged images. Immunofluorescence is predominantly nuclear. (a2-b2-c2): Differentiated rat HFSCs treated by NT3-Silibinin $0.05 \mu\text{g mL}^{-1}$. Most cells, including neuronal cells in aggregates, have lost β -III tubulin immunofluorescence. Rare cells show asymmetric β -III tubulin immunofluorescence. (A2): β -III tubulin fluorescein fluorescence. (b2): Corresponding DAPI nuclear stain. (c2): Merged images. (a3-b3-c3): Differentiated rat HFSCs treated by NT3-Silibinin $0.1 \mu\text{g mL}^{-1}$. All HFSCs show low levels of β -III tubulin-immunoreactivity. (a3): β -III tubulin fluorescein fluorescence. (b3): Corresponding DAPI nuclear stain. (c3): Merged images. (a4-b4-c4): Differentiated rat HFSCs treated by NT3-Silibinin $0.4 \mu\text{g mL}^{-1}$. Some cells in clusters (neuronal cells) still expressed β -III tubulin. (A4): β -III tubulin fluorescein fluorescence. (b4): Corresponding DAPI nuclear stain. (c4): Merged images. (a5-b5-c5): Differentiated rat HFSCs treated by NT3-Silibinin $0.5 \mu\text{g mL}^{-1}$. The most of HFSCs expressed β -III tubulin. They are intensely β -III tubulin immunofluorescent. (a5): β -III tubulin fluorescein fluorescence. (b5): Corresponding DAPI nuclear stain. (c5): Merged images. (a6-b6-c6): Differentiated rat HFSCs treated by NT3-Silibinin $0.7 \mu\text{g mL}^{-1}$. All rat HFSCs show low levels of β -III tubulin immunoreactivity. (a6): β -III tubulin fluorescein fluorescence. (b6): Corresponding DAPI nuclear stain. (c6): Merged images. Scale bar = 200X

which was not statistically significant. We next determined the effects of silibinin plus neurotrophin 3 on cell differentiation. At day 7 day 14 after treatment, cell viability was increased in a concentration-dependent manner by silibinin. When hair follicle stem cells were differentiated by treatment with EGF, silibinin and neurotrophin 3, they became dependent on this neurotrophic factor. As seen in Fig. 4, silibinin protected hair follicle stem cells for rapid differentiation to neural like cells. Control hair follicle stem cell cultures not treated with silibinin showed a decrease in number of differentiated neurons (Fig. 4). In contrast, during the same period of time, hair follicle stem cells treated with $0.5 \mu\text{g mL}^{-1}$ silibinin showed an increased number of differentiated neurons. Hair follicle stem cells treated with $0.05 \mu\text{g mL}^{-1}$ of silibinin plus neurotrophin 3 showed an increase in number differentiated neural-like cells (Fig. 5). Thus, silibinin protected neural cells for early differentiation induced by neurotrophic factor. These findings demonstrate that silibinin and also nerotrophin 3 can differentiate hair follicle stem cells to neural like cells.

DISCUSSION

Stem cells derived from many numbers of adult tissues have demonstrated unexpectedly vast differentiation potential in cultivation *in vitro* or transplantation (Bedada *et al.*, 2006; Pomerantz and Blau, 2004; Szeder *et al.*, 2003). The embryonic neural crest, from which epiderma neural crest stem cells EPI-NCSC are derived, originates from the neural folds during neurulation, and changes from an epithelial to mesenchymal transformation, and subsequently construct the embryo that it generates numerous structures of the adult organism, for example the autonomic and enteric nervous systems, most primary sensory neurons, peripheral glia, melanocytes and Schwann cells, Merkel cells, and the cranial mesenchyme (Richardson and Sieber-Blum, 1993; Rochat *et al.*, 1994). Neural crest cells invade the epidermis early in embryonic development, as early as day 3 of incubation in avian trunk skin (Kobayashi *et al.*, 1993) and day 9.5 of gestation in murine facial skin (Lyle *et al.*, 1998). Similar to embryonic stem cells, the bulge of hair follicle is a multilayered region, (Spyridonidis *et al.*, 2005) which is continuous with the basal layer of the surface epidermis. The bulge is a known niche for keratinocyte/epidermal stem cells, and can form new epidermis, sebaceous gland, and hair (Ito *et al.*, 2004) Hair follicle stem cells as epidermal neural crest stem cells (EPI-NCSC) exhibit a high degree of physiological plasticity and they can be proliferated *in vitro*. In contrast to other types of adult stem cell, EPI-NCSC do not raise ethical concerns, and since the patients can use own EPI-NCSC for autologous transplants, therefore there would be no graft rejection. Furthermore, EPI-NCSC are very accessible. They can be isolated from the bulge of hair follicles (Sieber-Blum and Grim, 2004; Sieber-Blum *et al.*, 2004). In this study we evaluated the effects of different concentrations of silimarin on differentiation of hair follicle stem cells to neural-like cells in culture. Hair follicle stem cells obtained from whisker follicles. In the first step, we used different concentration of silibinin. In high concentration of silibinin, all of the cell populations s were died. We hypothesized that EPI-NCSC are likely to be compatible with any tissue as a candidate for cell replacement therapies. Our data support this notation. Proliferation and differentiation of stem cells need too many factor. Organic factor, non-organic factor, synthetic factor and herbal medicine are very important in treatment neurodegenerative disease. Silybum marianum is a member of the aster family Asteraceae have been used for more than 2000 years to treat liver and gallbladder disorders and to protect the liver against poisoning from chemical and environmental toxins. Active component of Silybum marianum is obtained from its seeds containing approximately 70 to 80% of the silymarin flavonolignans and approximately 20 to 30% chemically

undefined fraction, comprising mostly polymeric and oxidized polyphenolic compounds. The main component of the silymarin complex is silybin, synonymous with silibinin, sometimes incorrectly called silybinin. Recently, silymarin/ silibinin received attention due to its alternative beneficial activities. These include mostly cardioprotective, hepatoprotective and neuroprotective activities (La Grange *et al.*, 1999). silibinin or silymarin may be useful in treatment and prevention of some neurodegenerative diseases. Wang *et al.* (2002) demonstrated that silymarin could effectively protect dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting microglia activity that acting in the CNS (Sieber-Blum *et al.*, 2006). There is growing evidence that activated microglia contribute to neuropathological changes in several CNS diseases including multiple sclerosis, Parkinson's disease, Alzheimer's disease and AIDS dementia. Silymarin also inhibits the production of inflammatory mediators, such as tumor necrosis factor- α and nitric oxide and thus reduces damage to dopaminergic neurons. Moreover, the compound protected primary hippocampal neurons against oxidative stress-induced apoptosis (Krena and Walterova, 2005).

In the present study, we have isolated EPI-NCSC from the bulge of rat whisker follicles. Then we evaluated the effects of silibinin and neurotrophin 3 on differentiation of hair follicle stem cells to neural-like cells. To our knowledge, this is the first work showing biological activity of silibinin on hair follicle stem cells.

The neurotrophic effects of silibinin demonstrated in the present study suggest a potential for use of the drug as a neuroprotective agent in the treatment of neurological disorders.

Based on many studies, antioxidant properties of the silibinin are responsible for its neuroprotective effects. Neurotrophic factors and antioxidants are being studied for their potential use in the treatment of neurodegenerative disease such as Alzheimer's disease, Parkinson's disease, spinal cord injuries, traumatic brain injury and stroke. Studies on the potential efficacy of silibinin in animal models of CNS disorders is therefore warranted. An interesting study aiming at neuro-immunomodulation mediated by silybin was accomplished by Sakai (2001). Major histocompatibility complex (MHC) I is usually suppressed in neuronal cells and neuroblastoma cells and this may lead to persistent viral infections. Induction of MHC I molecules in neuronal cells can stimulate the immune system to be able quickly to identify intracellular pathogens by cytotoxic T cells and remove the viruses from the central nervous system. Silymarin treatment resulted in the expression of MHC I in cells. Therefore, it was proposed that silymarin may be useful in the treatment of encephalitis. More studies, both *in vitro* and *in vivo* are, however, required (Krena and Walterova, 2005).

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