Facilitating regeneration of tissues using plant-derived stem cells of *Cocos nucifera*

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Abstract: Stem cells are undifferentiated cells capable of self renewal and differentiation. The study aimed to use plant stem cells to treat human or animal diseases, in lieu of using stem cells from animal models. The method employed the isolation of plant cells from the bulb of at least a healthy one-year old *Cocos nucifera (coconut)* by introducing stress and simulating human condition *in vivo*, to initiate dedifferentiation to a stem cell state. The investigation used wound healing rate to assess the function of the undifferentiated cell in tissue regeneration in three different concentrations of stem cells. At day 7, stem cell, at thrice the original concentration (SCx3), showed an average of 0.00 mm of wound; thus, 100% healed rate at day 7. As for stem cells at twice the original concentration, at day 9; and SCx1 at day 10. Control groups showed an average of 0.50 mm (91.67%) for control positive with phosphate buffered saline and 0.89 mm (85.17%) for control positive without treatment. Histopathologic examination gave the effects (minimizing the wound faster than the control group) of stem cells at varying concentration against the baselines. Statistical analysis showed significant differences in wound healing measurement, a measurement of 1.47 cm difference at day 3 using the lowest concentration (3.47 cm; using 60 uL PBS with 0.6 uL x10 to the sixth and four 40 uL with 0.4 uL x 10 to the sixth concentration) against the control group (4.95 cm) and their healing rates at day 3 (10.78% and 42.22% repectively) at p=<0.05. Stem cells showed an enhanced regeneration as previously reported even at higher concentration as assessed by neovascularization.

Keywords: *Cell Culture, Dedifferentiation, Dedifferentiated Cells, Regeneration, Plant Stem Cell*

ISSN: 2094-5906

INTRODUCTION

There has been an interest in culture techniques which act as primary tools for the method of mass multiplication and conservation of the organism (Hassan et al., 2011). With the establishment of the first human embryonic stem cell (hESC), culture method and conditions for the derivation of cells has been described (Rajala et al., 2011). The capacity of the cells to sustain self renewal – the ability of the cell to produce daughter cells with the same properties as the parent cell – has been the unique characteristic of a stem cell (Sauvageau, Humphries and Iscove, 2004). To develop an environment that will maintain the self renewal properties of the stem cell, it is important to determine the signal and mechanisms that are responsible for the differentiation and potential cell fates (Grafi et al., 2011; Rajala et al., 2011; Barton, 2012).

By developing a novel method to generate MSCs from plant tissues, removing auxins, cytokinin, GCN5 histone acetyltransferase complex and WUSCHEL (WUS), which induces the re-entry in the cell cycle, amplifying cell proliferation, maintenance and formation of calli, is a critical step to rule out the threat of differentiation within the culture (Byrne et al., 2003; Hirakawa et al., 2008; Jiang et al., 2009; Kornet and Scheres, 2009; Nardmann et al., 2009; Vernoux et al., 2010; Grafi et al., 2011; Yadav et al., 2012).

Regeneration occurs via somatic embryogenesis (SE) rather than organogenesis (Wang et al., 2011). Because plants also have a unique property to produce new organs, an important concern in SE is the property of the callus cells to become totipotent making the stem cell populations active throughout life (Fletcher et al., 2003; Fulcher and Sablowski, 2009; Wang et al., 2011; Delporte et al., 2012). Cellular dedifferentiation is the withdrawal of cells from a differentiated state into a stem cell-like cell and through reentry into cell cycle and transdifferentiation and even cell death (Wang et al., 2011).

Acquisition of stem cell line lineages has drawn attention as it is a potential approach in regenerative medicine, in vitro toxicology, cell therapy, cell biology and tissue engineering (Lee et al., 2007; Grafi et al., 2011; Trounson et al., 2011; Mandal et al., 2012). Stem cell therapy has been used in lieu of pharmacotherapeutic drugs by overcoming its side effects (Kim et al., 2012). Application of stem cell therapy include cardiac repair, neurological applications, immunological application – therapy for Chronic Graft Versus Host Disease (GVHD) and in tissue transplant, and in genetic blood diseases - therapies in diseases such as Wiskot-Aldrich syndrome, sickle cell and β-thalassemia (Barnett, 2011; Trounson et al., 2011). Regeneration of urethral sphincter in an animal model by using Human amniotic fluid stem cells (HAFSCs) and with the absence of immunogenicity as well

ISSN: 2094-5906

as teratogenicity has also been one of the medical applications of stem cells (Kim et al., 2012).

Cocos nucifera, coconut tree, is a medium-sized, solitary herbaceous plant. Its trunks are composed of fibrous, stout, overlapping stems, and may grow to 25 m tall (80 feet), topped by a crown of pinnately compound leaves up to 4 meters (15 feet) long. It is a tree that is cultivated for its multiple utilities, mainly for its nutritional, industrial and medicinal values. All its parts are used in the daily life of the people in the traditional coconut growing areas. The various products of coconut include tender coconut water, copra, coconut oil, raw kernel, coconut cake, coconut toddy, coconut shell and wood based products, coconut leaves, coir pith *etc*. Coconut water and coconut kernel contain microminerals and nutrients, which are essential to human health, and hence coconut is used as food by the peoples in the globe, mainly in the tropical countries (DebMandal and Mandal, 2011). It is also called as the "Tree of life" here in the Philippines because of the said significance.

Cocos nucifera is also used in traditional medicine due to its following properties: antimicrobial, antioxidant, vasorelaxant, antihypertensive activity and inhibitory effect against oral microflora. As an antimicrobiotic agent, extracts from *C. nucifera* were used against methicillin-sensitive and resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa, Citrobacter freundii* and *Candida albicans*. Hot percolated ethanolic, cold macerated ethanolic, aqueous and dry distilled extracts showed that all extracts have an antioxidant property. The extracts also exhibit vasorelaxant effects when examined in vitro. In vivo anti-hypertensive activity, extracts revealed significant reduction in mean systolic blood pressure. Extracts of *C. nucifera* shell ash revealed inhibitory effects against oral microflora (Singla, 2012). Previous researches revealed that coconut kermel protein and water contain antidiabetic and cardioprotective properties (Chikku and Rajamohan, 2012). Evaluation of cardioprotective properties of coconut sprout were studied in induced myocardiac infarction in Sprague Dawley rats (Chikku and Rajamohan, 2012). The

Figure 1. *Cocos nucifera.* Left: Whole plant of a one year old *Cocos nucifera* plant. Right: Bulb section (cut) used for culture.

ISSN: 2094-5906

formation of callus in coconuts trees are determined by SERK protein expression as described by Nuñez et al, 2009.

The potential benefits of stem cells in facilitating tissue repair in cutaneous and subcutaneous wounds were studied. Cells that contribute in wound healing and tissue repair are the adipose-derived stromal cells due to multilineage mesodermal potential (Lim et al., 2009). The mesenchymal stem cells contribute to wound healing due to their multipotential properties (Badiavas et al., 2011). Connective tissue growth factor stimulate non-hematopoietic cell to undergo self renewal and enter the cell cycle and terminal differentiation, which are more likely contributes to the healing of skin structures like muscle and dermal stromal cells (Lee et al., 2010).

The primary objective of this research is to investigate if the plant derived stem cells (meristematic stem cell - MSCs) can be used as an alternative for animal stem cells (e.g. hematopoietic stem cells – hSCs; Human embryonic stem cell – HECs; mesenchymal stem cells – mSCs) by facilitating the regeneration of tissues in an area inflicted with a wound. One of our objective is to use *Cocos nucifera* (coconut tree) as a source of stem cells (Hugget and Tomlinson, 2012).

MATERIALS AND METHODS

Plant Material

The bulb of a one-year old coconut tree which was obtained from Lobo, Batangas City was used as true plant material. The criteria for choosing the specimen are as follows: length should be at least 30-50 cm (12-20 in); with first compound ear (resembles rabbit's ear), not twisted, not more than one shoot, not deceased; for its viability, green sprout and whitish or yellow- greenish bulb should be observed (Chan and Elevitch, 2006).

Animal

Eighteen four-week old male and female Sprague-Dawley rats from University of the Philippines-Manila were used. Four pre- and post-treatment animals were constantly maintained under room temperature, with free flowing water and access to food. Rats were caged individually. Cages were cleaned everyday for the duration of the observation. Anesthesia will be used should there be any problem in handling. Treatments were given once at Day 0 of the experiment.

Experimental Grouping

The rats were randomized in six groups, each with three rats. The groupings are as follows:

ISSN: 2094-5906

I. Control Negative
II. Control Positive
III. Control Positive with Phosphate Buffered Saline (PBS)
IV. Stem Cell (X1 cell number)
V. Stem Cell (X2 cell number)
VI. Stem Cell (X3 cell number)

Initial dosage of stem cell concentrations were 60 uL PBS with 0.60 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 0.40 uL x 10 to the sixth cells. For twice the concentration, 60 uL PBS with 1.20 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 0.80 uL x 10 to the sixth cells were used. For thrice the concentration 60 uL PBS with 1.80 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 1.20 uL x 10 to the sixth cells were used. The experiment were done in triplicates (Lim and Yoo, 2010).

Dissection of tissue

A section of the bulb was removed from the plant, from the lateral to the median of the bulb. The section removed was sliced through its medio-peripheral axis (Dissociation of Cells from Primary Tissue, 2013). A 3 x 3 mm from any part of the plant was prepared (Dissociation of Cells from Primary Tissue, 2013). The sections obtained were transferred in a flask with phosphate buffered saline (PBS: pH 7.4). Phosphate buffered saline was used as a transport medium.

Dissociation of tissue

To remove the cell wall that surrounds the plant cell membrane, cellulase was used to degrade the cellulose part of the cell wall (Ojumu et al, 2003; The University of Georgia, 2013). Cellulase (Biotechnology, University of the Philippines – Los Baños: 750 units/mL) was added in the flask. A concentration of one milliliter for every 100 milligrams of tissue was used. The solution was incubated at 4°C for four hours to maximize enzyme penetration with little enzyme activity, and for 12 hours at 37ºC to initiate enzyme activity. For the removal of oils and extracts, the solution was centrifuged at 200 rpm for five minutes. Supernatant was removed and the precipitate was reconstituted and washed with PBS. It was centrifuged again at 200 rpm for 5 minutes (Dissociation of Cells from Primary Tissue, 2013).

Cell Cloning

For primary culture, the diluted cells were transferred to a microwell using a pipette. Growth of cells was monitored for one week.

ISSN: 2094-5906

For secondary culture, at the end of the one week observation period, wells with none or more than one cell lines were discarded, while wells with only one line were transferred in to Murashige and Skoog culture media (Crismon Enterprises, Philippines). The culture media were changed every two to three days. Every change of culture media was considered a passage (Lee et al., 2007; Steiner et al., 2009; Taha et al., 2012).

Cell Count

Cells for treatment were counted. The solution used was 100x solution with cells. One drop was added to the slide and viewed under the microscope for at high power objective for ten fields (Dissociation of Cells from Primary Tissue, 2013).

In vivo transplantation

Wound was inducted 6 mm long wound and 1 mm deep at the right side of the nape of a four week old Sprague Dawley rat.

Cells were injected intradermal at four injection sites around the wound and one onto the wound bed.

60 *u*L of PBS was injected at four sites around the wound and 40 *u*L of PBS at the wound bed which represents the control group. For cell treated group, 60 *u*L of PBS containing 0.6 *u*L of dissociated cells from the sixth passage was injected at four sites around the wound and 40 *u*L containing 0.4*u*L of dissociated cells from the sixth passage(Lim and Yoo, 2010).

During induction of wound, laboratory animals were properly anaesthetized. General anesthesia was used if there is doubt that local anesthesia will work to reduce the pain that will be received by the laboratory animal while undergoing in vivo transplantation procedure (Lim and Yoo, 2010).

Cell Identification Algorithm

Macroscopic examination was used to rule out parasites and debris that may contaminate the medium in the process of the experiment and serves as a reagent control for the course of the research.

Parasites are detectable with the naked eye and are easily distinguishable from other microbes since it is larger than microbes. If detected, the culture is rejected, if undetectable microscopic examination is performed.

Microscopic examination is used to rule out bacteria, fungus, parasitic ova and cyst and viruses from plant cells that may contaminate the culture media. Bacteria and viruses are undetectable in an unstained environment at low, high and oil-immersed objective.

ISSN: 2094-5906

If detectable, presumably the organisms may be parasitic cyst or ova, fungus or plant. The specimen is cultured. If there is no growth, the organism is a parasitic ova or cyst. If there is growth, the organism may be a fungus and plant cell.

Growth in an anaerobic condition, incubated at 37ºC for 24 hours and 25ºC for 72 hours was checked. Shake the tube. If there is dissociation of culture, the cells are a plant cell The organism is presumed as a fungal contaminant The culture is solid and remains intact while when shaken (Dissociation of Cells from Primary Tissue, 2013).

Ten days Post Transplantation Assessment

For post-transplantation assessment, animal models were monitored for at least 10 days (Schneider et al., 2003; Lee et al., 2007; Steiner et al., 2009) for further observation.

The assessment of regeneration was calculated by tracing the wound margin of its area (Lim and Yoo, 2010). The rate of regeneration was calculated as follows: (Area of the original wound – Area of the remaining wound)/ Area of the original wound x 100 (Lim and Yoo, 2010).

Necropsy was done to all rats at the area of the wound after the assessment of tumor formation for at least 10 days (Lim and Yoo, 2010). Using a microscope, the specimens were visualized (Steiner et al., 2009). The assessments of regeneration and tumor formation marked the end of the experiment.

RESULTS AND DISCUSSIONS

I. Wound Healing measurement and rates

Table 1 shows measurements of wounds at day 0, 3, 5, 7 and 10. Measurements were done in triplicates. The measurements of the wound at day 0, 3, 5, 7 and 10 in control positive were 6.00mm, 4.95mm, 3.50mm, 2.28mm, and 0.89mm, respectively. For control positive with PBS, the measurements were 6.00mm, 4.78mm, 3.55mm, 2.33mm, and 0.50mm, respectively. In undifferentiated stem cell concentration of 60 uL PBS with 0.60 uL x 10 to the sixth cells and four dosage of 40 uL of PBS with 0.40 uL x 10 to the sixth cells were 6.00mm, 3.47mm, 2.17mm, 1.13mm, and 0.00mm, respectively. With a concentration of 60 uL PBS with 1.20 uL

x 10 to the sixth cells with four dosage of 40 uL of PBS with 0.80 uL x 10 to the sixth cells 6.00mm, 3.89mm, 2.17mm, 1.11mm, and 0.00mm with twice the number of cells in PBS. With a

ISSN: 2094-5906

concentration of 60 uL PBS with 1.80 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 1.20 uL x 10 to the sixth cells, the measurements were 6.00mm, 2.67mm, 0.83mm, 0.00mm, and 0.00mm respectively.

Table 1
Measurements of Wound for Ten days Post Incision (mm)
n=18

Groups	Day 0	Day 3	Day 5	Day 7	Day 10
Ctrl (-)	0.00	0.00	0.00	0.00	0.00
Ctrl (+) w/o Tx	6.00	4.95	3.50	2.28	0.89
Ctrl (+) w/ PBS	6.00	4.78	3.55	2.33	0.50
SCx1	6.00	3.47	2.17	1.13	0.00
SCx2	6.00	3.89	2.17	1.11	0.00
SCx3	6.00	2.67	0.83	0.00	0.00

Ctrl (-) : Control negative group
Ctrl (+) w/o Tx : Control positive without any treatment done
Ctrl (+) w/ PBS: Control positive with PBS
SCx1: 60 uL PBS with 0.60 uL x 10 to the sixth cells and four dosage of 40 uL of PBS with 0.40 uL x 10 to the sixth cells
SCx2: 1.20 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 0.80 uL x 10 to the sixth cells
SCx3: 1.80 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 1.20 uL x 10 to the sixth cells

Table 2
Differences in Measurements of Wound (mm)

Baseline	Day 0	Day 3	Day 5	Day 7	Day 10
Control Group	6.00	4.95	3.50	2.28	0.89
Group 1 Concentration x1)	0.00	1.48	1.33	1.15	0.00
Group 2 (Concentration x2)	0.00	1.06	1.33	1.17	0.00
Group 3 (Concentration x3)	0.00	2.28	2.67	2.28	0.00

Control Group: Control positive without any treatment done
Group 1: 60 uL PBS with 0.60 uL x 10 to the sixth cells and four dosage of 40 uL of PBS with 0.40 uL x 10 to the sixth cells
Group 2: 1.20 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 0.80 uL x 10 to the sixth cells
Group 3: 1.80 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 1.20 uL x 10 to the sixth cells

ISSN: 2094-5906

As shown Table 2, the control group had a difference of 1.48 mm against group 1 at day 3. At day 7, the control group had a difference of 1.15 mm against group 1. At day 10, the wound of the control group and the test group have been closed, thus having a zero difference in measurement. The differences of measurement of the control group against group 2 are 1.06 mm, 1.33 mm and 1.17 mm at day 3, day 5 and day 7 respectively. The results revealed a faster recovery than the control group.

Table 3
Comparison of the measurement of wounds

	Group	p-value	Interpretation
Group 1 (Positive Control)	Group 2 (Vehicle Control)	0.999	NS
	Group 3 (Concentration x1)	0.075	NS
	Group 4 (Concentration x2)	0.101	NS
	Group 5 (Concentration x3)	0.006	S
Group 2 (Vehicle Control)	Group 1 (Positive Control)	0.999	NS
	Group 3 (Concentration x1)	0.105	NS
	Group 4 (Concentration x2)	0.140	NS
	Group 5 (Concentration x3)	0.008	S
Group 3 (Concentration x1)	Group 1 (Positive Control)	0.075	NS
	Group 2 (Vehicle Control)	0.105	NS
	Group 4 (Concentration x2)	1.000	NS
	Group 5 (Concentration x3)	0.507	NS
Group 4 (Concentration x2)	Group 1 (Positive Control)	0.101	NS
	Group 2 (Vehicle Control)	0.140	NS
	Group 3 (Concentration x1)	1.000	NS
	Group 5 (Concentration x3)	0.409	NS
Group 5 (Concentration x3)	Group 1 (Positive Control)	0.006	S
	Group 2 (Vehicle Control)	0.008	S
	Group 3 (Concentration x1)	0.507	NS
	Group 4 (Concentration x2)	0.409	NS

Table 3 shows that there is no significant difference between groups 1, 2, 3 and 4, where group 1 is the positive control and group 2 is the vehicle control. When group 1 is compared to group 5, it showed a significant difference in measurements of wounds. Group 2 has no significant difference in measurement of wounds between groups 1, 3

ISSN: 2094-5906

and 4, but showed significant difference in measurement between group 5. This indicates that group 5 is more has a reduced size in measurement of wound. There was no significant difference when group 3 is compared between all groups. This implies that group 3 has the same or no difference as the control group and vehicle group. Group 4 when compared to all groups showed no significant difference in measurement of wounds. This may imply that the group has no difference in reduction of wound size. There has been a significant difference between group 5 and group 1 and group 2, but no difference between groups 3 and 4. There is a difference in reduction of wound size.

As seen from Table 3, it was found out that it was statistically significant since the computed p-value of 0.001 < 0.05 level of significance, thus there was considered difference on the wound measurement when tested according to group. It was also supported using post hoc analysis (Scheffe method), where the difference on the each group was also observed.

Majority of the wound healing studies showed the ability of stem cells to hasten the healing process. Previous researchers used hematopietic stem cells, (Sauvagaue, et al, 2004), adipose stem cells, (Lim & Yoo, 2010), human embryonic stem cells, (Steiner et al., 2010), mesenchymal stem cells (Badiavas and Badiavas, 2012), human amniotic fluid stem cells, (Kim et a.l, 2012) for the regeneration studies.

Several studies proved the regeneration of plant stem cells. Geier et al., 2008 and Delporte et al., 2012 had investigated the mechanisms of plants to stabilize stem cell number even in variations in cell proliferation. Wang et al., 2011, had studied about the ontogeny of embryonic callus which relates the proliferation and differentiation of the pluripontential procambial cells of *M. trucatula*. Lastly, Hossain et al., 2011, experimented the in vitro regeneration of *Phlocanthus thyrsiflorus* in Murashige and Skoogg culture media which showed a positive result.

In this study, we used undifferentiated stem cells from *Cocos nucifera*, to replicate the result of previous experiments and to test if the said undifferentiated cells facilitate regeneration in a non-plant model. One of the purpose of the experiment is to establish an alternative source of stem cells in the Philippines.

Comparisons of the measurements of wound with undifferentiated stem cells in varying concentration are shown in Table 1. Using three times the concentration showed a smaller or a closed wound at day 4 than the other concentration which closed the wound at days 6 and 7 for the original and twice the concentration, respectively.

ISSN: 2094-5906

As illustrated in Table 4, wound healing rates were computed for days 0, 3, 5, 7, and 10. Further, the rates were 0%, 10.78%, 41.67%, 62.06%, and 85.17% for control positive, respectively. Rates for control positive with PBS were 0%, 20.34%, 40.78%, 61.11%, and 91.67%, respectively. For days 0, 3, 5, 7 and 10 had a rates of 0%, 42.22%, 63.89%, 81.11%, and 100% respectively using the initial concentration of dosage (60 uL PBS with 0.60 uL x 10 to the sixth cells and four dosage of 40 uL of PBS with 0.40 uL x 10 to the sixth cells). The rates were 0%, 35.22%, 63.89%, 81.50% and 100% respectively for days 0, 3, 5, 7 and 10 with a wound closure at day 9 using 60 uL PBS with 1.20 uL x 10 to the sixth cells and four dosage of 40 uL of PBS with 0.80 uL x 10 to the sixth cells. Using 60 uL PBS with 1.80 uL x 10 to the sixth cells and four dosage of 40 uL of PBS with 1.20 uL x 10 to the sixth cells, the rates were 0%, 55.56%, 86.11%, 100%, and 100% respectively.

Table 4
Percent Wound Healing Rate (%)
n =18

Groups	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10
Ctrl (-)	0.00	0.00	0.00	0.00	0.00	0.00
Ctrl (+) w/o Tx	0.00	0.00	10.78	41.67	62.06	85.17
Ctrl (+) w/ [PBS]	0.00	0.00	20.34	40.78	61.11	91.67
SC x1	0.00	16.67	42.22	63.89	81.11	100
SC x2	0.00	16.67	35.22	63.89	81.50	100
SC x3	0.00	11.11	55.56	86.11	100	100

Ctrl (-) : Control negative group
Ctrl (+) w/o Tx : Control positive without any treatment done
Ctrl (+) w/ PBS: Control positive with PBS
SC x1: 60 uL PBS with 0.60 uL x 10 to the sixth cells and four dosage of 40 uL of PBS with 0.40 uL x 10 to the sixth cells
SC x2: 1.20 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 0.80 uL x 10 to the sixth cells
SC x3: 1.80 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 1.20 uL x 10 to the sixth cells

All of the groups had a reduction in wound as time passes. At day 5, the cell treated group reached a healing rate of more than 50.00% of the original wound, while the control group reached less than 50.00% of the wound size. The cell treated group had a 20 – 40% faster healing rate than the control group. The increase in healing rate may be associated with presence of administered cells. The highest

ISSN: 2094-5906

concentration gave the fastest regeneration rate than the other concentration of cell treatment. The sixth group gave a faster rate than the control group, like the fourth and fifth group as mentioned. The regeneration may be associated with the concentration of 1.80 uL x 10 to the sixth cells. The higher the number of cells transplanted, the faster the rate of healing as supported. The number of cells implanted for 60 uL PBS with 0.60 uL x 10 to the sixth cells at 100x concentration was 3-7 cell clusters per hpf with an average of 4.4 cell clusters. For the dosage of 40 uL of PBS with 0.40 uL x 10 to the sixth cells the concentration was 2-7 cell clusters per hpf with an average of 4.4 cell clusters.

Table 5
Comparison of wound healing rate (in %)

Group		p-value	Interpretation
Group 1 (Positive Control)	Group 2 (Vehicle Control)	1.000	NS
	Group 3 (Concentration x1)	0.081	NS
	Group 4 (Concentration x2)	0.108	NS
	Group 5 (Concentration x3)	0.006	S
Group 2 (Vehicle Control)	Group 1 (Positive Control)	1.000	NS
	Group 3 (Concentration x1)	0.106	NS
	Group 4 (Concentration x2)	0.140	NS
	Group 5 (Concentration x3)	0.008	S
Group 3 (Concentration x1)	Group 1 (Positive Control)	0.081	NS
	Group 2 (Vehicle Control)	0.106	NS
	Group 4 (Concentration x2)	1.000	NS
	Group 5 (Concentration x3)	0.515	NS
Group 4 (Concentration x2)	Group 1 (Positive Control)	0.108	NS
	Group 2 (Vehicle Control)	0.140	NS
	Group 3 (Concentration x1)	1.000	NS
	Group 5 (Concentration x3)	0.418	NS
Group 5 (Concentration x3)	Group 1 (Positive Control)	0.006	S
	Group 2 (Vehicle Control)	0.008	S
	Group 3 (Concentration x1)	0.515	NS
	Group 4 (Concentration x2)	0.418	NS

*The mean difference is significant at the 0.05 level
NS = Not significant
S = Significant
Group 3: 60 uL PBS with 0.60 uL x 10 to the sixth cells and four dosage of 40 uL of PBS with 0.40 uL x 10 to the sixth cells
Group 4: 1.20 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 0.80 uL x 10 to the sixth cells
Group 5: 1.80 uL x 10 to the sixth cells with four dosage of 40 uL of PBS with 1.20 uL x 10 to the sixth cells

ISSN: 2094-5906

Table 5 shows, when group 1 was compared to groups 2, 3 and 4, there were no significant difference in percent in wound healing rate. When group 1 is compared to group 5, it showed a significant difference in wound healing rate. Group 2 showed no significant difference in wound healing rate when compared against groups 1, 3 and 4; and showed significant difference in percent in healing rate when compared against group 5. Groups 3 showed no significant difference in wound healing rate when compared against groups 1, 2, 4 and 5. There is no significant difference in wound healing rate when group 4 is compared against groups 1, 2, 3 and 5. Lastly, when group 5 is compared to groups 1 and 2, it showed a significant difference while when compared to groups 3 and 4, there were no significant difference. This presented a significant difference in healing rate. Group 5 is the most effective in increasing the wound healing rate.

Based from the output of Table 5, it was found out that there is a significant difference exists on the wound healing rate when grouped according to treatment. This was supported by the obtained p-value (0.002) < 0.05 level of significance, thus the mean percent differs across treatment.

Various step and plans were used to establish treatment regimen to physiologic and pathologic condition using various cell-based therapies (Lim and Yoo, 2010). In the experiment, groups that were injected with undifferentiated stem cells from *Cocos nucifera* heals faster than those of the control group. The undifferentiated stem cell using varying concentrations, has reduced wound measurement at day 1 and at day 3, 5, and 7 after wound induction, the treatment group has significantly smaller wounds compared to control positive group. As for wound healing rates in all groups increase with time, but wound healing rates in treatment group were higher compared with the baseline group at 0, 3, 5, 7 and 10 after wounding. The result confirms the result of Lim and Yoo, 2010, the adipose stem cells is used to test its regenerative property, were the healing rates of the test group is significantly higher than the control group with time. This may be due to the substitution of deficient cells after the *C. nucifera* undifferentiatied cells differentiated as stated by previous reports.

II. Histopathologic Examination

Microsection Description

In a blind manner, assessment and identification of morphology of the tissue were done by a single observer. The tissue will be assessed by its capillary density and inflammation, for neovascularization. Capillary density will be given a grade of 1+ for 0-3 capillaries per high power field; 2+ for 4-6 capillaries per high power

ISSN: 2094-5906

field; 3+ for 7-9 per high power field; and 4+ for 10 or more capillaries per high power field. Assessment of inflammation will also be graded as follows: 1 – 3, for no to minimal granulation tissue or epithelial travel with no to minimal cell accumulation, with 1 as its lower limit and 3 as the upper limit; 4 – 6, for disclosing immature granulation, few fibroblasts, few collagen deposition and capillaries, minimal epithelial travel dominated by inflammatory cells, with 4 as its lower limit and 6 as its upper limit; 7 – 9, for moderate epithelial travel, wide-ranging neovascularization and more fibroblasts, moderately thick granulation tissue and dominance of inflammatory cells, with 7 as its lower limit and 9 as its upper limit; and 10 – 12, for thick granulation tissue and extensive collagen deposition with the epithelium partially to completely casing the wound dominated by fibroblasts, with 10 as its lower limit and 12 as the upper limit (Lim and Yoo, 2010).

For the control negative, Hematoxylin and Eosin stained microsection disclosed at high power magnifications, no cell accumulation, granulation or epithelial travel as shown at Figure 2 – Top right with a score 1. As for capillary density, the pathologist graded the density with 1+. The score of the control negative group is the baseline for the experiment. Control positive group with no treatment had a score of 3+ for capillary density and 8+ for the inflammation. The microsection disclosed tissue fragment with moderately thick granulation tissue and moderate epithelial travel with wide ranging neovascularization and more fibroblasts and dominant inflammatory cells. Control positive with a vehicle control of phosphate buffered saline gave a score for capillary density of 1+ and 1 for inflammation. At high power magnification, Hematoxylin and Eosin stained microsection disclosed no cell accumulation, granulation or epithelial travel. The scoring and pathologist interpretation is the same as the control negative, this suggests that there is no significant difference between the control negative without treatment (sig. 0.999). The first test group with 60 uL PBS with 0.6 uL x10 to the sixth and four 40 uL with 0.4 uL x 10 to the sixth concentration gave a score of 3+ for capillary density and 6 for inflammation. Microsections disclosed tissue fragments with few fibroblasts, capillaries and collagen deposition with minimal epithelial migration. Thin granulation and dominance of imflammatory cells is also noted. Compared to the control negative group, presence of fibroblasts and higher capillary density is noted. This may be due to the fact that higher capillary density may mean increased circulation of nutrients to the injured site which is needed by the cells. The second test group using 1.2 uL x 10 to the sixth cells and four 40 uL PBS with 0.8 uL x 10 to the sixth cells concentrations has a result of 2+ for capillary density and 1 for inflammation. The microsection disclosed tissue fragments with none

ISSN: 2094-5906

to minimal granulation tissue and epithelial travel with minimal to no cell accumulation. Compared to the first test group 60 uL PBS with 0.6 uL x10 to the sixth and four 40 uL with 0.4 uL x 10 to the sixth concentration, there is less capillary density than the first test group. This may have been the time period where the tissue is reestablishing normal morphology at day 10 of the experiment. Test group with thrice the concentration of cells, 1.8 uL x 10 to the sixth and four 40 uL PBS with 1.2 uL x 10 to the sixth cells, showed grossly a closed wound and had a score of 2+ for capillary density and 7 for inflammation. Hematoxylin and Eosin stained microsection disclosed tissue fragments at high power magnification with moderately thick granulation tissue dominated by inflammatory cells and more collagen deposition, with extensive neovascularization and moderate epithelial travel. Compared to the first test group it has less capillary density. It is also noted that it has higher scoring in the inflammation compared to the second test group. Neovascularization is evident in the third test group, which may hasten regeneration as there are more fibroblast and collagen deposition as they are components of a tissue and higher capillary density as to provide nutrients to the damaged cells. Migrating epithelium suggests that the wound is closed and is continuing the regeneration of the wounded area.

Histopathologic report

Figure 2 shows the differences of skin tissue samples between the baseline and different concentrations of undifferentiated stem cells which are apparent. Control negative shows the normal morphology of the tissue, unaltered and unwounded, with intact skin. Control positive shows the presence of inflammatory cells. Control positive with PBS shows the appearance of the tissue regenerated after 10 days (wound 0.50 mm to a completely healed surface). Using the treatment injected with 60 uL PBS with 0.6 uL x10 to the sixth and four 40 uL with 0.4 uL x 10 to the sixth cells shows the completely healed surface with blood vessels. Treatment with 60 uL PBS with 1.2 uL x 10 to the sixth cells and four 40 uL PBS with 0.8 uL x 10 to the sixth cells showed a healed surface and more blood vessels. Lastly, using 60 uL PBS with 1.8 uL x 10 to the sixth and four 40 uL PBS with 1.2 uL x 10 to the sixth cells showed a completely healed wound. Increasing concentration of undifferentiated cells showed it had a contrary effect on the healing process. A high concentration applied may cause a delay in healing – substitution to active granulation tissue instead of healing the internal tissue.

ISSN: 2094-5906

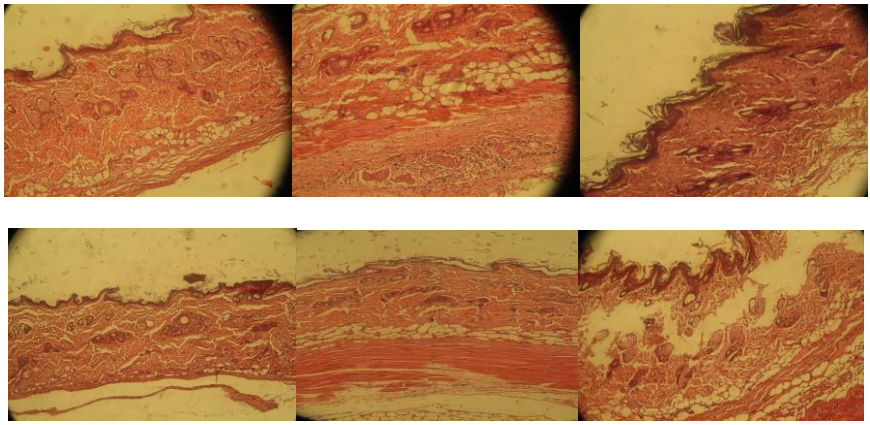

Figure 2. The pictures shows differences of skin tissue samples between the baseline and different concentrations of the stem cells. A is the Control negative; B is control negative, with an active inflammatory cell; C is a control with PBS; D is stem cell x1 concentration, with blood vessels; E is stem cell x2 concentration, with more blood vessel ;and F with thrice the original concentration.

Neovascularization is a crucial step and a part of wound healing process as it is necessary to sustain newly formed tissue and is survival (Lim and Yoo, 2010). Badiavas and Badiavas, 2012 stated that mesenchymal stem cells (MSCs), due to their multipotent property, tmay differentiate into various cell types. Secretions of cytokines are beneficial in wound healing such as epidermal growth factor, vascular endothelial growth factor-ɑ and angiopoietin. These are secreted in increased amounts by the MSCs under hypoxic conditions to be involved in tissue growth and angiogenesis (Badiavas and Badiavas, 2012). As reported by Lim and Yoo , 2010, adipose-derived stem cells (ADSCs) when used for treatment had significantly higher capillary densities, which suggests that ADSC promote neovascularization which is also a factor in promoting regeneration. In the experiment, *C. nucifera*-derived undifferentiated stem cell (cnDUSC) showed a faster regeneration rate as previously stated and, like ADSC and MSC, promote neovascularization as shown in the histopathologic examination on Figure 2. Based from the statement of Badiavas and Badiavas ,2012, cytokines and growth factors that may be secreted by the cnDSC may contribute to the healing process but the exact detail on what kind of growth factor it may be is still unknown and is subjected to further study.

ISSN: 2094-5906

CONCLUSIONS

In conclusion, the study showed that the plant-derived stem cell can hasten wound regeneration at concentration using 60 uL PBS with 1.8 uL x 10 to the sixth and four 40 uL PBS with 1.2 uL x 10 to the sixth cells, such concentration was also considered as best in hastening regeneration.

RECOMMENDATIONS

The researchers recommend the following, based on our findings and conclusions:

1. Increase the number of test subjects, include treatment using drugs and other stem cells to form a comparative study.
2. Prolong observation post transplantation and post healing to check for any possible tumor growth.
3. Research on other application that uses this principle.
4. To test the minimal therapeutic dosage and tumor forming dosage of cnDSCs.

Acknowledgement

We thank the following for making the research possible, staff of Biotechnology, University of the Philippines Los Baños for providing the enzymes needed as well as the staff of Biological Sciences of UPLB; Dr. Carina R. Magbojos for the unwavering support and critical reading of the manuscript; Mr. Oliver Shane Dumaoal for the equipment and reagents we used; Ms. Annalie Pateña for the statistical analysis needed for the research paper. Mr. Dean Lee for providing materials needed for the experiment; Dr. Katherine Reyes, Dr. Lloyd Asor, and Dr. Gay Bautista in assisiting us in making a microsection description; Ms. Karen Sumaray for the consultation of microbes and microscopy and all the staff and faculty of College of Allied Medical Profession who assisted us during the duration of the research.

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