INDUCED PLURIPOTENT STEM CELLS TRANSPLANTATION AS WOUND THERAPY ON A MOUSE MODEL

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ABSTRACT

Stem cell transplantation represents a relatively new approach of treating wounds, with several studies reporting positive results. Yet these studies used stem cells that are limited by the following: differential potency, ethical permissibility, or histocompatibility. To overcome these obstructions, induced pluripotent stem (iPS) cells, a type of stem cell that can be generated from cells of a patient, was used in this study instead. This study aims to culture and graft iPS cells into mice with the splinted wound mouse model and determine the effectiveness of the treatment. 18 clinically healthy female mice were immunosuppressed with a daily intramuscular injection of dexamethasone at 1 μg/g for three consecutive days. Under anaesthesia, two sterile wounds were incised on shaved backs of each mouse via biopsy punch. Subsequently, six intradermal injections were made around the two wounds before the wounds were adhered with splints and wound dressing. The mice were divided into two groups; a treatment group that was given 7×10^5 iPS cells in each injection, and a control group that was given 0.9 % sodium chloride. Wound closure rates were determined through timed scaled photography and subsequent analyses with GNU Image Manipulation Program. Three mice were euthanised from each group at every seven days post-wounding, at which point wound beds and blood were harvested. Total leucocyte counts and differential leucocyte counts were conducted on the blood. Wound beds were fixed, processed, blocked, and sectioned. Sections were used in fluorescence *in situ* hybridization (FISH) to detect iPS cells of male origin in female hosts. Sections were also stained with H&E and Masson's Trichrome, as well as immunolabeled for CD31 and KI67. The stained sections were then subjected to an evaluation under compound microscopy and subsequently scored for several variables. FISH revealed that the transplanted iPS cells were successfully grafted and may survive permanently. KI67-immunostained sections revealed no difference between groups; together with the fact that no tumours were found throughout the study, the risk of teratoma formation seems to be low. Total and differential leucocyte counts showed no difference between groups. The wound closure curve of the treatment group was stiffer, yet the difference was not significant, however individual results were significantly different for day 7 ($p = 0.038$). From the evaluation and scoring, the treatment group scored better in chronic inflammation, fibroblast proliferation, granulation tissue, and collagen deposition, albeit not statistically significant. However, the treatment group did score significantly better in angiogenesis ($p = 0.006$ for day 7) and hypodermis regeneration ($p = 0.006$ for day 21).

ABSTRAK

Pemindahan sel induk merupakan pendekatan yang agak baharu bagi merawat luka, dengan beberapa kajian yang melaporkan keputusan positif. Namun, pengunaan sel induk di dalam kajian-kajian tersebut terhad oleh sebab-sebab berikut: potensi pembezaan terhad, masalah etika, dan, keserasian tisu. Untuk mengatasi masalah-masalah tersebut, sel induk pluripotent teraruh (sel iPS), sejenis sel induk yang boleh dijana daripada sel-sel pesakit, telah digunakan dalam kajian ini. Kajian ini bertujuan untuk mengkultur sel iPS dan mencantumkannya ke atas tikus-tikus yang dilukakan berasaskan model luka bertuap serta menentukan keberkesanan rawatan tersebut. 18 ekor tikus betina yang sihat secara klinikal telah disekat imuniti dengan diberikan suntikan harian 1 μg/g dexamethasone selama tiga hari berturutan. Dalam keadaan bius, dua luka steril dibuat pada bahagian belakang tikus yang telah dicukur menggunakan alat pembuat lubang. Selepas itu, enam suntikan dibuat di sekeliling dua luka tersebut sebelum luka tersebut dilekat dengan tuap dan pembalut luka. Tikus-tikus ini dibahagikan kepada dua kumpulan; kumpulan rawatan yang diberi 7×10^5 sel iPS dalam setiap suntikan, dan kumpulan kawalan yang diberi 0.9 % natrium klorida. Kadar penutupan luka ditentukan dengan fotografi berskala waktu dan dianalisa dengan program GNU Image Manipulation. Tiga ekor tikus daripada setiap kumpulan dimatikan pada setiap tujuh hari selepas dilukai, dan tisu luka dan darah diambil pada masa yang sama. Pengiraan leukosit keseluruhan dan pengiraan leukosit pembezaan dibuat ke atas darah tersebut. Tisu luka difiksasi, diproses, dibenam, dan dihiris. Hirisan-hirisan tersebut digunakan dalam proses hibridisasi pendarfluor *in situ* (FISH) untuk mengesan sel iPS yang berasal jantan di dalam perumah betina. Selain itu, hirisan-hirisan juga diwarnai H&E dan Masson's Trichrome, dan diimun-label untuk CD31 dan KI67. Selepas itu, hirisan-hirisan tersebut dinilai di bawah mikroskop majmuk dan beberapa pemboleh ubah telah diberi markah. FISH mendedahkan bahawa sel iPS yang dipindahkan telah berjaya dicantumkan dan berkemungkinan boleh hidup secara kekal. Hirisan immun-label KI67 menunjukkan tiada sebarang perbezaan antara kumpulan; bersama dengan tiadanya pembentukan tumor sepanjang penyelidikan ini, oleh itu risiko pembentukan teratoma nampaknya rendah. Lengkungan penutupan luka kumpulan rawatan lebih kaku, namun perbezaan tersebut tidak ketara; tetapi, secara individu, perbezaan antara kumpulan dianggap signifikan pada hari ke-7 ($p = 0.038$). Berdasarkan penilaian dan pemberian markah, kumpulan rawatan mendapat markah yang lebih baik dalam keradangan kronik, percambahan fibroblast, tisu granulasi, dan pemendapan kolagen, walaupun perbezaan markah tidak signifikan secara statistik. Walaubagaimanapun, kumpulan rawatan mendapat markah yang lebih baik dan signifikan dalam pembentukan salur darah ($p = 0.057$ bagi hari ke-7) dan pembentukan semula hipodermis ($p = 0.006$ bagi hari ke-21).

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of variance
APS0004	-	Animal iPS no. 4
CD31	-	Cluster of differentiation 31
CD68	-	Cluster of differentiation 68
DAB	-	3,3'-diaminobenzidine
DMEM	-	Dulbecco's modified eagle medium
DMSO	-	Dimethyl sulfoxide
DPX	-	Distyrene plasticizer xylene
ES	-	Embryonic stem
FISH	-	Fluorescence in situ hybridization
HRP	-	Horseradish peroxidase
HSCT	-	Hematopoietic stem cell transplantation
H&E	-	Hematoxylin and eosin
ID	-	Intradermal
IM	-	Intramuscular
IP	-	Intraperitoneal
iPS	-	Induced pluripotent stem
KI67	-	Kiel, clone no. 67
LIF	-	Leukemia inhibitory factors
MEF	-	Mouse embryonic fibroblast
NEAA	-	Non-essential amino acids
PBS	-	Phosphate buffered saline
SNL 76/7	-	STO-Neo-LIF clone 76/7

LIST OF SYMBOLS

α	-	Significance level
%	-	Percentage
β	-	Beta
mm	-	Millimetre
cm	-	Centimetre
s	-	Second
min	-	Minute
h	-	Hour
H_0	-	Null hypothesis
H_1	-	Alternate hypothesis
d	-	Day
° C	-	Degree Celsius
µg	-	Microgram
mg	-	Miligram
g	-	Gram
µL	-	Microliter
mL	-	Mililiter
$\times g$	-	Times gravity
mM	-	Milimolar
Q_1	-	Quartile 1
Q_3	-	Quartile 3
U	-	Unit
v/v	-	Volume per volume
w/v	-	Wight per volume

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CHAPTER 1

RESEARCH BACKGROUND

1.1 Introduction

The skin covers the entire surface of our bodies and among other functions, act as a layer of protection to everything underneath. Understandably, a wound would compromise the integrity of the skin and expose the body to microbial infections.

When it comes to wounds, perhaps the most important classification is whether a wound is acute or chronic. Put simply, **acute wounds** are wounds that heals in a timely and orderly fashion (as will be discussed in Section 2.1.2), whist **chronic wounds** do not (Lazarus et al., 1994). For practicality, chronic wounds can also be defined as wounds that take more than 3 weeks to heal (Hermans and Treadwell, 2010)

Broadly speaking, chronic wounds can be caused by medical conditions, or certain infections and tropical diseases; venous leg ulcers, ischemic ulcers, diabetic foot ulcers, and pressure ulcers are amongst the predominant types of chronic wounds (Hermans and Treadwell, 2010). Acute wounds on the other hand, are wound that are a result of incisions such as surgical incisions, or of trauma such as burns or falls (Ather et al., 2019). Of course, acute wounds can become chronic wounds as well.

There is no one single way of grading or classifying the severity of a wound, instead, an assessment of several parameters provides a better understanding to formulate the appropriate response. These parameters include wound size, wound edge characteristics, wound site properties, wound bed granulation tissue appearances, wound depth, surrounding skin characteristics, infection, haemostasis, presence of foreign matter, and the presence of necrotic tissue, slough, and eschar (DeBoard et al.,

2007; Grey et al., 2006). Burns in particular are often characterised by depth; from first degree burns which is of a simple sunburn; to fourth degree burns which have the dermis and epidermis destroyed, and subcutaneous and deeper tissue substantially damaged (Hermans and Treadwell, 2010).

Sadly, chronic wounds are staggeringly prevalent in the population. Diabetic foot ulcers, a type of chronic wound and a major complication of diabetes mellitus, is estimated to have a history prevalence of 5.1% and a lower-limb amputation prevalence of 1.2% in the United Kingdom (Abbott et al., 2005). In fact, their National Health Service is estimated to spend £ 5.3 billion on managing wounds annually (Guest et al., 2015). In the United States, it is estimated that 1.3 to 3.0 million of the population have pressure ulcers and that 10% to 15% of the 20 million diabetic patients are at risk of developing diabetic ulcers (Kuehn, 2007). Besides that, a study conducted in a rural village of Sweden established that the prevalence of healed or non-healed chronic leg ulcers is over 12.6% for the elderly over the age of 70 (Marklund et al., 2000). In Europe, approximately 1% of the population is affected by chronic wounds (Schreml et al., 2010). If the complication of delayed healing was not enough, it was found that 68.9% of patients do not survive the 6 months after first detecting nosocomial pressure ulcer; by the end of the first year, the percentage of mortality increases to 78.4% and 83.8% at the end of year 2 (Brown, 2003).

Wounds may considerably disrupt the daily routine of a patient. Such is caused by the frequent dressing change, sleep deprivation-caused continued fatigue, mobility restriction, discomfort, pain, unpleasant wound odour, infection, polypharmacy's effects on the body and mind, and reduced independency. Suffice to say, in many cases, the quality of life of wound patients is harshly compromised (Grey et al., 2006). That, however, assumes that the patient survives the trauma in one piece. It was found that foot ulcer patients have a 6-fold increased risk of low extremity amputation (Davis et al., 2006). On top of that, it is estimated that only approximately 30.7% diabetic amputation patients survive 5 years post amputation (Faglia et al., 2006). In a quality of life assessment consisting of 36 questions called the short form (36) health survey, the score of healed and unhealed leg ulcer patients were on par with patients with

chronic obstructive pulmonary disease, osteoarthritis, or angina (Kahn et al., 2004). Indeed, wound complications are a nightmare to the patients, but the nightmare may not end with the complete healing of the wound, that is if they heal at all. In the aftermath of a wound episode, wounds may leave behind scars.

Scars, especially those situated at eye-catching positions can be quite disfiguring and harm the self-esteem of a patient. As a matter of fact, in our society that emphasizes on appearance, a scared face may frighten away possibilities of social connections with others.

While humans have complications with the recovery of a simple wound, urodele amphibian can regenerate amputated limbs with ease while invertebrates like planarians and starfish can even regenerate bidirectionally (Brockes, 1997). However, to say that humans have completely lost, over the course of evolution, the ability to regenerate a body part as complicated as a limb would not be most accurate. For example, children are known to regrow fingertips should they be severed (Illingworth, 1974). As for the matter of scarring, the human foetus is capable of healing cutaneous wounds so flawlessly to the point of leaving no scars at all (Longaker et al., 1994).

But how far could we theoretically go? Take a chicken for example, similarly with a long history of evolution, similarly not known to be capable of limb regeneration; in one study, partial regeneration of an amputated limb in a developing chick embryo was made possible by manipulation of the spatiotemporal of β-catenin expression (Kawakami et al., 2006). In African clawed frogs, an additional eye could be generated via manipulating the transmembrane voltage potential (Pai et al., 2012). Unrelated as it sounds, this suggests that given the right genetic manipulation and conditions, humans too could theoretically regenerate the lost limb or an extra eye, though the latter is probably not preferable in normal situations. Compared to that, wound regeneration should be relatively straight forward. Moreover, it was found in a recent study that a single clonogenic neoblast (a type of planarian pluripotent stem cell) is all it took to restore regeneration and completely replace all cells in a lethally

irradiated planarian host (Wagner et al., 2011). Although planarians are known to regenerate from tiny body fragments, the revelation that a single pluripotent stem cell alone is capable of such a task would make one wonder what pluripotent stem cells are capable in wound therapy.

Induced pluripotent stem cells (iPS cells), are pluripotent stem cells generated from somatic cells by viral transfection of 4 transcription factors (Takahashi and Yamanaka, 2006). With the ability to generate pluripotent stem cells from simple somatic cells, not only does iPS cells offers a source of stem cells free of ethical implications associated with the use of embryonic stem cells (ES cells), but they may also be generated from cells derived from specific patients (who requires transplant) and avoid the roadblock of immune rejection. Also, iPS cells can be generated from virtually any somatic cell, such as from blood cells and hair, making them amongst the least invasive to procure (Aasen et al., 2008; Kim et al., 2016). That being said, there has yet been any research done to address the use of iPS cells in wound therapy.

1.2 Statement of Problem

As discussed earlier, wounds, especially chronic wounds, greatly lower the quality of life of a patient, and may graduate towards extremity amputation and even death; paired with the prevalence of the condition, the need for wound care and treatment represents large sums of spending, on top of the misery caused on a personal level. Of potential treatments, cell-based wound therapies represent an interesting relatively new method of wound treatment. Of the many types of cells, or more specifically, stem cells available, iPS cells are ethical, pluripotent, possibly patient homologous, and non-invasive to procure; thereby circumventing roadblocks that may limit other types of stem cells. In fact, iPS cell have been differentiated into different types of skin cells (Ohta et al., 2011; Hewitt et al., 2011; Bilousova et al., 2011; Itoh et al., 2011; Bilousova and Roop, 2013); and in some, skin structures (Kim et al., 2018; Itoh et al., 2013); despite their effectiveness in these studies, as of this writing, iPS cells have yet been evaluated in wound therapy in an *in vivo* setting.

1.3 Significance of Study

Induced pluripotent stem cells are a type of potentially patient-homologous pluripotent stem cells that can be generated non-invasively and from an ethical source; due to their potency, their use in wound therapy should, in theory, result in wound recovery superior to other cell-based wound treatments, due to having a wider coverage of the types of cells they can differentiate into, without the risk of immune rejection.

iPS cells promise a great many uses wound healing. As a fellow pluripotent cell line, iPS cells may be beneficial to diabetic wounds just as ES cells have (Lee et al., 2011). If the use of iPS cells is advantageous to diabetic wound healing, then perhaps such benefits extend to chronic wounds as well. On top of that, while it is naïve to believe that iPS cells alone could replicate scarless wound healing demonstrated by foeti (Larson et al., 2010), the 'less differentiated' iPS cells may be able to mitigate scarring, and in doing so promises some use in hypertrophic scaring mitigation, which is often seen in burn victims.

However, a successful grafting of iPS cells must first be established before any of these potential applications can be explored. Additionally, an understanding how iPS cell transplantation affects the various aspect of wound healing will determine the types of wounds iPS cell transplantation therapy is suited for.

1.4 Objectives

The objectives of the study are:

a. To culture and transplant iPS cells into mouse wounds.
b. To assess the potential of iPS cell transplantation as a type of wound therapy with the following analyses:
    i. Quantitative analyses: Wound closure rate, total leucocyte count, and differential leucocyte count.

ii. Qualitative analyses: iPS cell grafting successfulness, transplanted iPS cell proliferation.

iii. A score system to interpret qualitative data in a quantitative way: Inflammation, angiogenesis, fibroblast proliferation, granulation tissue formation, collagen deposition, reepithelialization, and hypodermis regeneration.

1.5 Scope of Study

The iPS cell line used was APS0004, an iPS cell line that was generated via introduction of 4 transcription factors via plasmids (Riken BioResource Center, 2014). The iPS cells used were not generated from cells acquired from the test mice for this study, and therefore, the mice were immunosuppressed to avoid immune rejection of the grafted cells. The wound model used was full-thickness incisional wounds on the back of immunosuppressed mice, with attached splints and wound dressing (Wang et al., 2013).

Wound closure rate was approximated via scaled photography. A graft was considered successful if the transplanted cells or their descendants were detected in wound bed samples. Proliferation was deemed present as indicated by the expression of KI67 (Borue et al., 2004). Angiogenesis was deemed present from the presence of blood vessels in the wound beds (Sivan-Loukianova et al., 2003). Immune response was evaluated from blood leucocyte and from the presence of macrophages and monocytes in the wound beds (Stepanovic et al., 2003). The degree of which inflammation, angiogenesis, fibroblast proliferation, granulation tissue formation, collagen deposition, and hypodermis regeneration, are different between groups were assessed via a score system (Abramov et al., 2006; Loh et al., 2020).

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