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Evaluating the effect of autologous fat grafting on wound healing - An animal study

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1.) Title

Evaluating the effect of autologous fat grafting on wound healing - An animal study

Submitted in partial fulfilment for the degree of

Master of Medicine

Department of Plastic and Reconstructive Surgery

University of the Witwatersrand, Johannesburg, South Africa

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02 June 2021

2.) Declaration

I declare that this dissertation is my own authentic work. This dissertation is submitted for the degree of Master of Medicine in Plastic and Reconstructive Surgery to The University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any University before.

I declare that the idea of using impurified, unexpanded, uncultured fat obtained from routine liposuction used to accelerated wound healing was an idea formed from observation in clinical management of wounds over 4 years of general surgery and 4 years of plastic surgical training.

I declare the reason for the study is to prove a cost-effective method to manage wound healing.

I declare that the demonstration of viable adipose stem cells in the lipoaspirate was done by myself in the flow cytometry laboratory of the Department of Haematology, Charlotte Maxeke Academic Hospital, Johannesburg. The presence of adipose derived stem cells was analysed and confirmed by Professor M. Hosie, Department of Anatomical Sciences, University of the Witwatersrand.

I declare that the wound biopsy specimens were embedded in paraffin wax moulds by myself together with the help of Consultants in the Department of Anatomical Pathology, University of the Witwatersrand.

I declare that the cutting and staining of the wax specimens to haematoxylin and eosin slides were done by myself using a microtome under guidance of the Department of Anatomical Pathology, University of the Witwatersrand.

I declared that I sustained a laceration of my hand during the above slide cutting at which time Dr. G Hariparsad, executive board member and chairman of LANCET laboratories offered assistance in the staining of the last 50 slides.

I declare that Professor Simon Nayler from the Department of Anatomical Pathology, Specialist pathologist at Gritzman & thatcher inc. interpreted the histological measurements to exclude bias.

I furthermore declare that collagen staining was done in the department of Haematology but suspended due to equipment failure of the Electron Microscope of the Department of Haematology, University of the Witwatersrand.

I declare that the processing of the histological specimens although it was done by myself was not fully reported on in this MMED study as this is a known procedure and offers no new contribution to the practice of Plastic and Reconstructive Surgery.

I declare that financial assistance in obtaining the animals in the study was provided by a Research Fund obtained from the Department of General Surgery University of the Witwatersrand.

I declare that the Department of Anatomical Pathology (University of Witwatersrand), Department of Haematology (University of Witwatersrand), Dr G Hariparsad, Executive Board Member and Chairman of LANCET laboratories, Professor Simon Nayler from the

Department of Anatomical Pathology, Specialist Pathologist at Gritzman & Thatcher inc. offered their services free of charge provided I was personally involved and present during every step of the study.



Dr Marisse Venter

02 June 2021

3.) Dedication

I dedicate this dissertation to my parents, Ms. Stephane Venter and Mr. Chris Venter, who have supported me through four decades of academic toiling.

I also dedicate this this to my husband Dr Adrian Kelly who has shown me that no mountain is too high to climb.

4.) Acknowledgements

I would like to firstly thank Jesus and, through the Son, his Father for giving me the privilege and allowing me to grow under the light of God's amazing and forgiving grace.

I am extremely grateful to Professor Elias Ndobe whom has been like a father to me since my arrival in the Department of Plastic Surgery. Professor Ndobe's belief in me has, on a daily basis, inspired me to achieve academic excellence. Since the first day of my arrival and myself witnessing our first Plastic Surgery Departmental Academic discussion, I was so happy because what I had been yearning for, I had finally found- a place where I could attain truly deep academic and practical knowledge and gain valuable insight into the field of Plastic and Reconstructive surgery from Professor Ndobe and my peers.

I would also like to thank Professor Lukhele for his ongoing and unfaltering support since the first time I walked into his orthopaedic department with an idea for research. Professor Lukhele is a Research Champion and a perfectionist and this came forth time and time again as he tirelessly reviewed and re-reviewed the many versions of my protocol.

5.) Abstract

Animal Study: The Effects of Adipose Extract on Wound Healing

Investigator **Dr. M Venter**

Supervisors Professor Elias Ndobe, FC (Plastic and Reconstructive Surgery) SA

Dr A Rooi SA, FC (Plastic and Reconstructive Surgery) SA, Mmed (Plast Surg) Wits

Background:

Adipose derived stem cells have been shown to enhance skin wound healing. The stem cells are harvested , purified, cultured and the viability assessed to provide adequate cellular yield. The isolation process requires trained laboratory staff, intensive procedures utilizing multiple purification solutions and expensive equipment for culturing and interpretation of viability of the isolated stem cells. The aim of the study was to investigate the effect of simple lipo-aspirate on wound healing.

Methodology:

This is a prospective , interventional study to investigate the effect of adipocyte extract on wound healing. Young, healthy female large white pigs were used in the study. Fat was harvested using standard liposuction technique and injected around the defects created. Skin defects were evaluated for wound healing.

Results:

Histological evaluation shows accelerated wound healing with the treatment of adipose tissue compared to control groups. There was an increased rate of wound surface reduction

and epidermal thickness in the wounds treated with lipo-aspirate. Bacteriology results showed no significant differences.

Conclusion:

Results indicate a benefit in the treatment of wounds with the use of lipo-aspirated extract. Fat processed via routine liposuction without isolation, culture and expansion of adipose derived stem cells allowed for accelerated wound healing. The procedure allows for a cost-effective method to enhance wound healing in a developing country.

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7.) Ethical considerations

The protocol was approved by the Animal Ethics Screening Committee (AESC)- clearance certificate number 2011/39/04. All animals were treated in compliance with the National research council criteria as subject to AESC guidelines for the use and care of animals as provided by the Animal Ethics Research Committee of the University of the Witwatersrand, Johannesburg, South Africa (Appendix C).

Approval was given on the 5th of September 2011 and the pigs were purchased on the 6th of September 2011. The Clinical component of the study, i.e. obtaining specimens from the animals was completed by the 15 October 2011.

Before commencement of the study: The Effects of Adipose Extract on Wound and Bone Healing the Ethics committee had an additional requirement that we demonstrate the presence of stem cells in the lipo-aspirated, please see condition 1 in the Ethics certificate.

This could have been done either clinically or by providing literature. Unfortunately prior to this study there were no experiments done on harvesting lipo-aspirate from the **dorsal hump** of South African pigs or in fact the dorsal hump of any other porcine model as a source of adipose derived stem cells.

The demonstration of the viable adipose derived stem cells is described below as the first part of the study and conformation by Flow Cytometry. The lipo-aspirate used for demonstration of the viable adipose derived stem cells was obtained from the 1 pig used in the pilot study assessing the effects of lipoaspirate on bone healing.

The pilot study on 2 pigs assessing the effects of lipo-aspirated on bone healing was done by the Department of Orthopaedic Surgery and does not from part of this MMED report.

This MMED report describes the effect of Adipose Extract on wound healing in 6 pigs as approved by the Animal Ethics Committee.

8.)Introduction

Non-healing wounds remain a significant challenge in the field of Plastic and Reconstructive surgery. In the United States of America, for example, more than 600,000 people suffer from chronic wounds and venous ulcers. This issue of chronic wound healing constitutes a problem that currently carries with it not only a considerable socio-economic burden within the community and to the families of these patients, but also places a considerable burden on limited Hospital resources.

The use of tissue engineering techniques, such as stem-cell therapy and gene therapy, to promote wound healing is a promising strategy. It has been proven that the isolation, purification and population of adipose derived stem cells accelerates wound healing.

The isolation, purification and population of adipose stem cells is a complex process, involving multiple steps, qualified personnel, and specialized laboratory equipment. The financial implications are not within reach of a developing country. The harvesting of subcutaneous fat is a rather standard procedure easily practiced by all plastic and reconstructive surgeons. The liposuction technique does not require expensive equipment and laboratory facilities.

By evaluating specific variables pertaining to wound healing to which unprocessed fat containing stem cells is applied, and by comparing these to similar control wounds to which unprocessed fat is not applied, this study aims to assess the significance of unprocessed fat on several recognized variables involved in wound healing.

In this study we quantitatively evaluate the efficacy of adipose tissue obtained by liposuction, without further costly processing namely stem cell isolation, purification, and population, on wound healing as compared to control wounds in an animal model.

9.)Literature Review

The author conducted a review of the English literature on autologous adipose harvesting, grafting, and adipose derived stem cell therapy. PubMed and ClinicalTrials.gov were the search engines employed in this review to identify and evaluate current literature. The search terms utilized were "autologous fat grafting wound healing "; "lipo-injection wound healing"; "adipose extract wound healing "; "autologous fat transfer wound healing"; "adipose-derived stem cells wound healing"; "fat harvest wound healing " ; "stem cell therapy wound healing"; "animal model wound healing"; "lipoaspirate wound healing"; "lipo-transfer wound healing", and combinations thereof. The studies selected were limited to English language articles indexed as animal studies, human studies, clinical trials, systematic reviews, case series and case reports. Letters to the editor and expert opinion were omitted from inclusion.

Studies have shown that wound healing remains a significant burden to the health sector. The economic and psychological implications of delayed wound healing on both the health sector and the patient remains an investigative field of interest. Biological therapies such as topically applied growth factors have been shown to promote wound healing. The conclusion by Cherubino et al is that growth factors cannot be used in isolation and should be used in combination with dermal substitutes [1].

The limitations of topically applied growth factors led to the investigation of wound healing through cell therapies such as stem cell therapy [2]. Stem cells are cell populations possessing self-renewal, long term viability, and multilineage potential. There are two types of stem cells

namely embryonic stem cells and adult stem cells. Embryonic stem cells have limited applications due to immune rejection, tumorigenicity, as well as ethical and political considerations. Thus, the attention currently is on adult derived stem cells [3]. Adult stem cells are noted by Weissman et al to be able to multiply and differentiate into phenotypes of essentially any cell type, including cells needed in wound healing [4].

At the turn of the millennium stem cell lineages were limited to hematopoietic stem cells, umbilical cord blood stem cells, bone marrow mesenchymal stem cells, neural stem cells and muscle satellite cells [2]. Of the above mentioned, bone marrow derived mesenchymal stem cells have been shown to promote wound healing. Despite this positive effect, the limitations of bone marrow mesenchymal stem cells are cited to be the inability to culture large quantities. The invasive harvesting, low yield, deceased marrow population with patient age, and degradation outside the body over time, of bone marrow mesenchymal stem cells makes their use problematic [1].

In 2001 the adipose derived stem cell was added to the list of useful stem cell lineages in a land-mark paper [2]. Adipose derived stem cells are precursor cells which can be obtained by enzymatic digestion of the vascular stromal fraction of fatty tissue [6]. Since then several studies have reported that adipose tissue contains the highest percentage of adult stem cells, in fact surpassing the percentages of stem cells contained in the other stem cell lineages [7,8]. Studies have furthermore proven that adipose derived stem cells have multilineage mesodermal potential. These cells have been demonstrated to have a significant positive effect on tissue repair and regeneration. Useful applications of adipose derived stem cells include ischemic revascularization, cardiovascular tissue regeneration, urinary tract reconstruction, peripheral nerve repair, acute spinal cord injuries, liver injury repair, diabetes,

slowing the progression of these numerous chronic conditions [5]. In wound healing, adipose derived stem cells have been proven to significantly improve angiogenesis [1]. Several studies have further reported that adipose derived stem cells significantly promote the proliferation of dermal fibroblasts and stimulate paracrine secretory functions, thus enhancing specifically cutaneous wound healing [17-19]. In a rodent model using mice, the application of adipose derived stem cells to iatrogenically inflicted cutaneous wounds significantly accelerated the rate of wound healing [22]. With regards to cutaneous wound healing, Ebrahimian et al in a rodent animal study demonstrated that adipose derived stem cells have the ability to produce vascular endothelial growth factor thereby accelerating angiogenesis, as well as the ability to differentiate into keratinocytes. The angiogenic properties of the vascular endothelial growth factor was also demonstrated to be independent of the mode of administration. The conclusion of this study was that the secretory activity of adipose derived stem cells injected into wound sites creates an ideal environment where chronic inflammation could be overcome, and thereafter, furthermore initiated tissue regeneration [10]. Other animal studies using mice, one of which used diabetic mice and the other, mice with irradiated skin wounds, both demonstrated that adipose derived stem cells applied to cutaneous wounds accelerated wound healing [10,11]. Blanton et al used a healthy pig model reported that the injection of adipose derived stem cells alone only demonstrated a significantly improved rate of re-epithelialization in well vascularized tissue and did not demonstrate a significantly improved rate of re-epithelialization in poorly vascularized tissue. The study further reported the importance of the addition of platelet derived growth factor in poorly vascularized tissue for a significant advantage to be seen [12].

The terms autologous fat grafting and adipose derived stem cell therapy should not be used interchangeably. The first refers to simple harvesting, processing, and the injection of the

stromal vascular fraction into recipient tissue. The latter refers to a complex process of isolation, purification and population of adipose derived stem cells. The importance of distinguishing these two processes has major cost and time implications [13].

Autologous fat grafting is a procedure extensively used in plastic and reconstructive surgery to restore volume deficits secondary to tissue loss from disease, surgery, trauma or aging. Fat is harvested from the subcutaneous fat compartments in the body by mechanical loosening with a blunt-tipped atraumatic needle. Thereafter, it is drawn into a syringe utilizing a syringe aspiration technique [23]. The fat from the syringe is then divided into multiple syringes for centrifugation which separates it into 3 distinct layers. The top oil layer and the bottom blood layer are both discarded. The middle stromal vascular fraction is then transferred into additional syringes, used in small aliquots, and injected into the desired areas. The exact technique of fat harvesting, size of cannulas selected, size of syringes selected, aspiration pressures chosen, and centrifuge rates, differ from surgeon to surgeon. The most well-known and widely employed atraumatic technique is that described by Coleman [14].

Adipose derived stem cell therapy on the other hand, while employing the same process of fatty tissue harvesting, subsequently requires multiple steps and expensive laboratory equipment to firstly isolate viable cells, purify those isolated, culturing the purified cells, and ultimately multiplying the cells cultured, to produce a quantity of adipose derived stem cell that is useful [15]. The methodology underpinning adipose derived stem cell therapy rests on the histological examination of raw lipo-aspirate which has been shown to be composed of mature adipocytes, extracellular matrix, adipose derived stem cells, endothelial cells, and mural cells (pericytes and vascular smooth muscle cells). When enzymatically digested, the cellular fraction forms the stromal vascular fraction (SVF). This SVF contains enough adipose

derived stem cells to have a positive effect on wound healing, with adipose derived stem cell therapy assuming that this by necessity needs adipose derived stem cells to be isolated and expanded [15]. Several studies elaborate on the complex methodology of adipose derived stem cell processing. The harvested lipoaspirates are firstly washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. The washed aspirates are then treated with 0.075% collagenase in PBS for 30 min at 37°C employing gentle agitation. The collagenase is inactivated with an equal volume of DMEM/10% foetal bovine serum (FBS) and the infranatant centrifuged for 10 min at a low speed. The cellular pellet is resuspended in DMEM/10% FBS and filtered through a 100-mmesh filter to remove debris. The filtrate is then centrifuged and plated onto conventional tissue culture plates in control medium. The control media is subsequently incubated to expand the culture and obtain the desired cell lineage. The process can take from a few days to several weeks depending on the volume of the cell lineage required [15,16].

Studies show that a porcine wound model most accurately models the human wound model. The porcine skin and the human skin share greater anatomical and physiological similarities compared to the similarities between human skin and the skin of smaller mammals. Both porcine and human skin have a similarly thick epidermis, a similar dermal-epidermal thickness ratio, rete-ridges, dermal papillary bodies and likewise an important abundance of subcutaneous fat. The epidermal turnover time in human skin and porcine skin is furthermore very similar. Physiologically, human skin and porcine skin share another important characteristic namely wounds heal by re-epithelization, rather than the pattern of wound healing seen in smaller mammals which heal by wound contraction. The major organ systems and body size in humans and swine is also similar, making procedures such as general anaesthesia and wound dressings comparable [17].

Fu X et al a porcine animal model, fat was removed from the posterior cervical region of miniature pigs. The subcutaneous adipose tissue obtained was washed with Hank's balanced salt solution and thereafter visible blood vessels were removed. The harvested adipose tissue was cut into 3mm x 3mm x 3mm pieces, purified by washing with HBSS, and filtering through four-layer gauze to remove blood, lipid, and cell fragments. The fat pieces were then sharply threaded mechanically. The threaded adipose was then mixed with a 10cc volume of 0.01 mol/L cold PBS (pH 7.2), homogenized under 41 degrees Celsius, and centrifuged at 1000rpm. The isolated supernatant was then filtered through a 0.22mm micropore film to eliminate microorganisms. The resultant adipose paste was then applied to the same open wounds created on the posterior cervical region of each miniature pig. The findings of this study were that the epithelial border was at the edge of the healing wound and was easily discernible from the moist granulation tissue. During the first 3 days, while all wounds demonstrated a paucity of granulation tissue formation as well as a paucity of epithelial-tissue growth, the wounds did become smaller. At the end of day 3 wound examination confirmed all wounds in all groups to contain haemorrhage and a complete, albeit only 3-5mm thick, layer of granulation tissue. Another finding was that there was new epithelial growth at the edge of all wounds. By day 7, and more so on day 14, there was a significant difference in the formed epithelium area in the adipose treated wounds as compared to the wounds treated with growth factor alone as well as that of a control wound. The study reported a significant reduction in wound surface area, epidermal tissue thickness, increased presence of vascular nets, and an accelerated rate of wound healing in those wounds which receive the adipose tissue treatment as compared to those wounds treated with growth factor alone as well as that of a control wound [18].

Huss et al evaluated the effect of autologous fat grafting on inflammation. The study challenged the thinking that autologous fat grafted tissue will not replicate, eventually will die, and generate harmful inflammatory responses. The study finding was that a significant proportion of autologous fat graft cells had a pro-inflammatory modulating effect limiting the amount of inflammation and promoted wound healing [19].

In a prospective cohort study that considered 20 patients with radiation induced skin damage who were injected with purified autologous fat graft lipoaspirate from a healthy donor site followed up for 31 months, concluded progressive regeneration, neovascularization and improved hydration in patients [20].

Considering the human model, one study examined the effect of autologous fat grafted adipose tissue on small ulcer defects of less than 3x3 cm and chronic burn scar management. This study challenged the notion that with regards to burn wounds the application of autologous fat grafted adipose tissue would be harmful, due to the relatively poor circulation compared to conventional wounds, which would result in early adipose liquefaction and cause infection. The study findings were that the application of autologous fat grafted adipose tissue to burn wounds accelerated wound healing and did not result in a significantly increased infection rate compared to the infection rate in similar conventional wounds treated with adipose tissue. The study conclusion was that autologous fat grafting accelerated tissue remodelling, increased pro-angiogenic growth factors, and decreasing ongoing inflammation, resulting in improved burn wound healing [21].

Considering collagen deposition, a human study on a regenerative approach to scars and ulcers treated with autologous fat grafting, reported that significantly increased collagen deposition occurred. [21]. In a porcine model evaluating tissue expanded skin flaps, those

injected with adipose derived stem cells demonstrated significantly more expansion. Histological evaluation showed significantly increased collagen content with the use of adipose derived stem cells [24]. The finding by Wang et al that adipose derived stem cells augment collagen deposition is supported by the findings of Branski et al [23].

This study aimed to quantitatively evaluate the effect of autologous fat grafting in a porcine wound model compared to similar control wounds inflicted in the same animal. The study hypothesis was that neither additional processing techniques nor cell culture expansion was needed to facilitate accelerated wound healing.

10.)Research Question

1.) Does adipose tissue obtained from routine liposuction contain a significant amount of viable adipose derived stem cells?

2.) What is the influence of injection of unprocessed fat into wounds on recognized wound healing variables?

3.) Is there a difference in the bacteriology of wounds that receive injection of unprocessed fat, compared to control wounds that do not receive injection of adipose tissue, in an animal model?

11.)Aims of the Study.

The aim of the study is to determine the effect of adipose tissue harvested from routine liposuction techniques on wound healing. Without the isolation, purification, and population of adipose derived stem cells.

12.)Objectives of the Study

1. To determine if adipose tissue obtained from routine liposuction contain significant amounts of viable adipose derived stem cells.

2. To investigate if adipose tissue without isolation, purification, and population of adipose derived stem cells injected into wounds significantly influence wound size in an animal model.

3. To determine if the injection of adipose tissue change the bacteriology of wounds treated with adipose extract.

13.)Research Methodology

a.) Study design

Prospective clinical control cohort study in an animal model.

b.) Study population

Seven healthy adult pigs ranging from 32-64 Kg in weight constituted the study population.

One pig was used to confirm the presence of viable adipose derived stem cells in the lipo-aspirate obtained from the dorsal hump of a large South African Pig.

**This Pig was used as part of the pilot study assessing the effect of lipo-aspirate on bone healing. The effect of lipo-aspirate on bone healing was reported on in a separate MMED report done in the Department of Orthopaedics, University of the Witwatersrand.*

The remaining six pigs were used to assess the effect of lipoaspirate on wound healing compared to control wounds.

c.) Sample size

No formal sample size calculation was performed as this is the first study of its kind. This study will provide valuable information that does not exist at this point in time.

d.) Data collection

The seven pigs were purchased one-week prior to commencement of surgical procedures for observation to ensure all were in good health.

Upon commencement of the study the pigs were allowed to fast for 8 hours before surgery.

Pigs (35 - 50 kg) were anaesthetized using injectable Dormicum (0.3 mg/kg) and Anaket V (11 mg/kg). Maintenance of the animal in the anaesthetic state was achieved using Isofor as the inhalation agent. Anaesthetic technique was kept uniform and each pig was placed under general anaesthesia with sodium pentothal (40mg/kg) injected intravenously.

The standardised surgical preparation technique involved clipping of the hair on the back of each pig with an electric razor where-after the skin was sterilized with 75% alcohol. The surgical field area was then draped with sterile towels.

Liposuction was performed from the dorsal hump of each pig whereby fat harvesting was performed using a 10 ml Syringe connected to a 2 ml Coleman cannula via the dry technique. As such no tumescent fluid was used. The fat was processed using a centrifuge at 1200 rpm for 3 minutes. Once the fat was processed, the 3 layers of fat were easily discerned. The top

oil layer was wicked off, the lowest blood level discarded and only the middle vascular stromal fraction was used in the study (Figure 1).

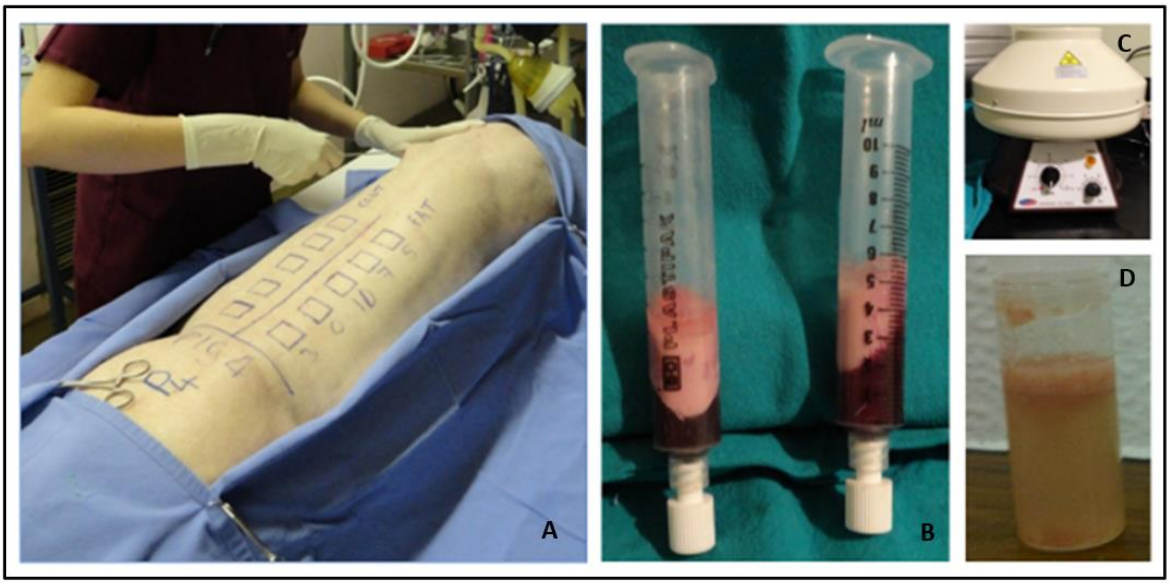


Figure 1. Liposuction of the dorsal hump using 2mm blunt tip cannula, no tumescent solution used **(A)**. Autologous fat graft consisting of 3 layers, top oil layer, middle vascular stromal fraction, lowest blood level. The middle vascular stromal fraction used in the study **(B)**. Centrifuge using 1200 rpm for 3 minutes **(C)**. Collagenase digested lipo-aspirate for evaluation of Adipose derive stem cells via immunohistochemistry and flow cytometry **(D)**.

The first part of the study: - Demonstration of viable adipose stem cells from the dorsal hum of a South African pig.

Isolation of the adipose derived stem cells: 10 ml of processed lipo-aspirate was digested with mixed collagenase. The stromal vascular fragment was labelled using immunohistochemistry and the fraction evaluated via flow cytometry.

Flow cytometry as a science provides the unique ability to measure the optical and fluorescence characteristic of single cells, micro-organisms or cellular components in a fluid stream when passed through a light source [25]. Flow cytometry is used in various applications based on the detection of the external characterizing features, internal morphological composite structures, cell cycles, membrane flux and action potentials. Flow Cytometry may also sort cells of interest.

The underlying principle is related to light scatter and fluorescence emission as light from an excitation source strikes labeled moving particles of interest.

Light scattering is related to structural and morphological properties of the cell while fluorescence emission is proportional to the amount of fluorescent probe bound to cellular component of interest.

The components of flow cytometers and cell sorters are fluidics, optics (excitation and collection), an electronic network (detectors) and a computer.

The fluidics direct liquid containing particles to the focused light source. The excitation optic focuses the light source on the cells while collection optics transmits the light scatter or fluorescent light of the particle to an electronic network. The electronic network detects the signal and converts the signals to a digital data that is proportional to light intensity. The data is analyzed via a computer network [26].

In this study the operator selects the fluorescent cell of interest using a computer as they are passing through the laser beam. The target cells will be be marked with a fluorochrome and charged, light is reflected as the fluidic passes the light source. Light is recorded either as forward or side scatter and converted to electric current. The electric current travels to an amplifier where it is converted into a voltage pulse which is first converted into an analog signal and eventually a digital signal. The signals become digital data which can be displayed as plots of histograms.

The number of intrinsically fluorescent compounds in the cells and organisms are limited. To delineate specific cells by fluorescence, cells are stained with fluorescent probes called fluorochromes. These fluorochromes act as amplified identifiers of the variable under investigation.

Fluorochromes are used in a wide range of applications such as identification of different cell populations, cell surface receptors, intracellular organelles, cell sorting, immunophenotyping, determining nucleic acid content and measuring enzymes. For this

reason, there are different groups of fluorochromes e.g., those that label proteins such as cell surface antigens, nucleic acids, or receptor molecules.

To label proteins covalently, the probe is commonly selected as an antibody. Proteins such as a lectin, hormone, avidin or streptavidin, or even c-DNA may be labelled using various fluorochromes. The most widely used fluorochromes for labelling antibodies include FITC, phycoerythrin (PE) and allophycocyanin (APC) [26]

The data analysis from Flow cytometry has been considered as the most critical parameter for biological experiments. The major principles of data analysis are to selectively show the cells of interest and to find out more about your cells. This method is called ‘‘gating’’ in flow cytometry. The results may also be differentiated into different gates.

A gate is a numerical or graphical boundary that can be used to define the characteristics of particles for analysis.

Cell surface antigens localized on the plasma membrane of the cells are the most common proteins to use for flow cytometry to identify and characterize the cell types. Plasma membrane proteins are easily accessible to the antibody for cell surface staining.

In our study we were only interested in living Adipose derived stem cells . Thus, to determine if the method of harvesting adipose extract from the dorsal hump of pigs, yields living cells.

The harvested fat was processed by enzymatic digestion and centrifugation to identify the Stromal Vascular Fragment. Extensive washing with phosphate-buffered saline (PBS) and digestion of lipoaspirate with 0.075% collagenase to release the stromal vascular fraction (SVF) of cells. Collagenase digestion remains the gold standard among the currently used methods for isolating Adipose derived stem cells (ADSC) [25].

Please note we did not culture or expand the adipose derived stem cells as we were not creating large amount of adipose derived stem cells, we were merely interested in their presence.

The characteristics of adipose derived stem cells are commonly evaluated by flow cytometric analysis of cell surface markers.

The International Society for Cellular Therapy (ISCT) and the International Federation for Adipose Therapeutics and Science (IFATS) specify minimal criteria for defining Adipose Derived Stem Cells:

(1) cells must be plastic-adherent.

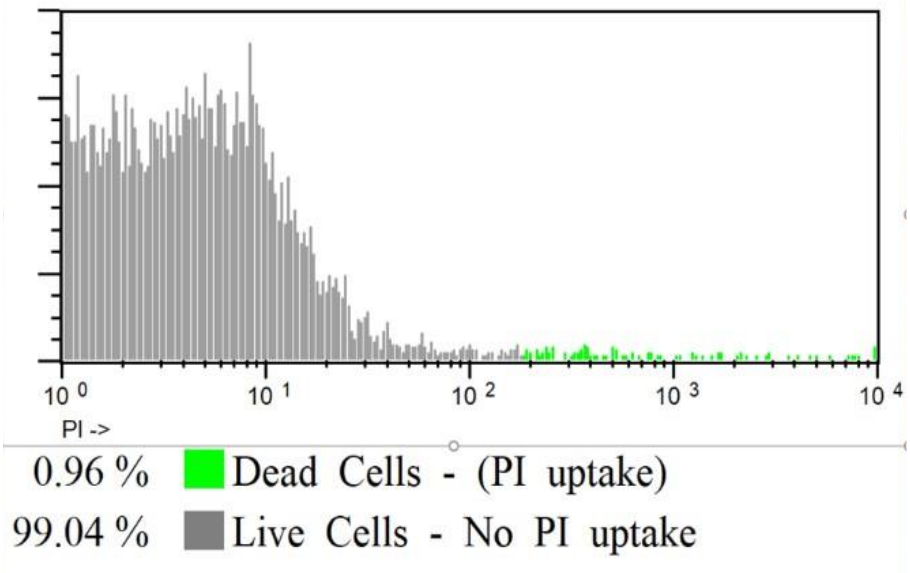
(2) they must express CD73, CD90, and CD105 and lack the expression of CD14, CD11b, CD45, CD19, CD79, and human leukocyte antigen-DR (HLA-DR)

The Stromal Vascular fragment was labeled with a combination of cell surface markers as directed by The International Society for Cellular Therapy (ISCT) and the International Federation for Adipose Therapeutics and Science (IFATS). Marking was performed by using fluorophore-conjugated rabbit IgG isotype.

- Anti-CD 34, Rabbit IgG1, Conjugated flurophore, Phycoerythrin (PE), Brand and Catalog number, BD Pharmingen 555822
- Anti-CD 45 : Rabbit IgG1. Conjugated flurophore fluorescein – 5 – isothiocyanate (FITC), Brand and Catalog number, BD Pharmingen 555482
- Anti-CD 73 : Rabbit IgG1. Conjugated flurophore PE , Brand and Catalog number, BD Pharmingen 550257
- Anti-CD 90 : Rabbit IgG1. Conjugated flurophore , FITC Brand and Catalog number, BD Pharmingen 555595
- Anti-CD 105 : rabbit IgG1. Conjugated flurophore , , peridinin-chlorophyll-protein (PErCP). Brand and Catalog number, BD Pharmingen 560819

Flow cytometry was performed, FACScan argon laser cytometer, via passage of adipose stem cells for detection of surface antigenic markers CD34, CD45, CD73, CD90, and CD105 The data was converted to digital format as explained above and recorded on a histogram figure 9 below.

Figure 9. A single Parameter Histogram. The x-axis represent the parameter's signal value in channel numbers (Count) and the y-axis represents the number of events per channel number.
Live re-print from Flow Cytometer Department of Haematology, Charlotte Maxeke Academic Hospital, Johannesburg



For this purpose, the researcher utilized the flow cytometry laboratory of the Department of Haematology, Charlotte Maxeke Academic Hospital, Johannesburg. The presence of adipose derived stem cells was analysed and confirmed by Professor M. Hosie, Department of Anatomical Sciences, University of the Witwatersrand

For the second part of the study; 6 Pigs were used.

The researcher created ten full thickness defects, 2.5 x 2.5 cm on the dorsal surface of the pigs whilst under general anaesthesia. A sterile template of 2.5cm x 2.5cm was placed on the back, 3 cm away from the midback, and a full thickness wound to the deep fascia corresponding to the template was made by excising the skin. Five interventional and 5 control wounds on each side of the midline were created (Figure 2).

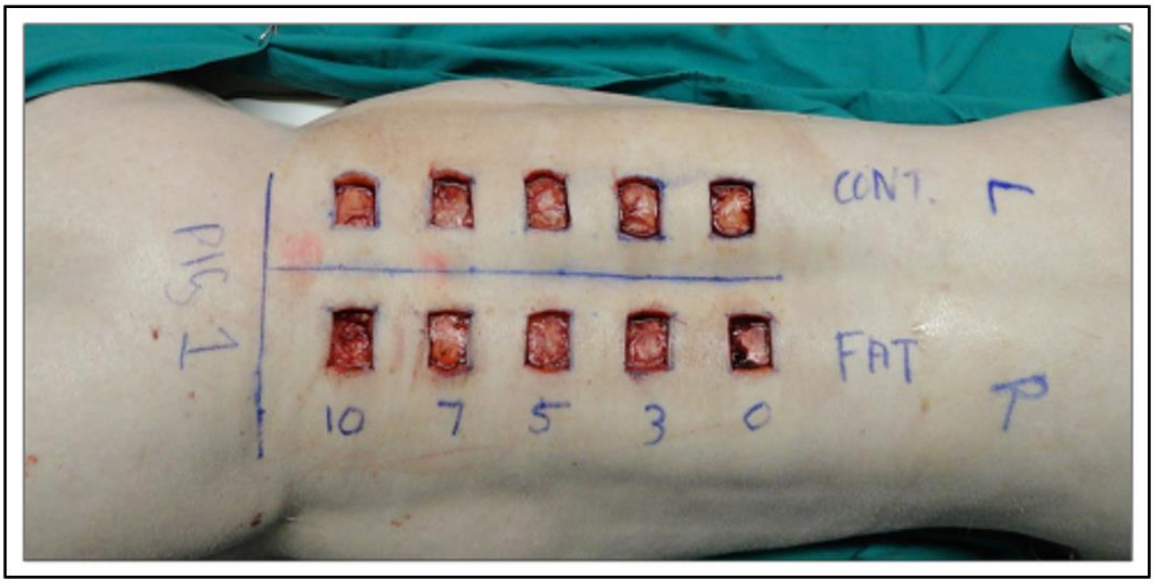


Figure 2. Ten full thickness defects, 2.5 x 2.5 cm were created on the dorsal surface of each pig. A sterile template of 2.5cm x 2.5cm was placed, 3 cm from the midback, and a full thickness wound to the deep fascia corresponding to the template was made by excising the skin. Five interventional and 5 control wounds on each side of the midline were created.

On one side of the midline the wounds were injected with 2 ml of processed fat 0.5 ml per side (Figure 3). The defect was rectangular, 0.5 ml was injected into each side.

4 x 0.5ml = 2ml per wound of lipoaspirate. The lipo-aspirate was injected on day 0. The fat injected wounds received lipo-aspirate from the same pig so as not introduce a possible of autoimmune rejection.

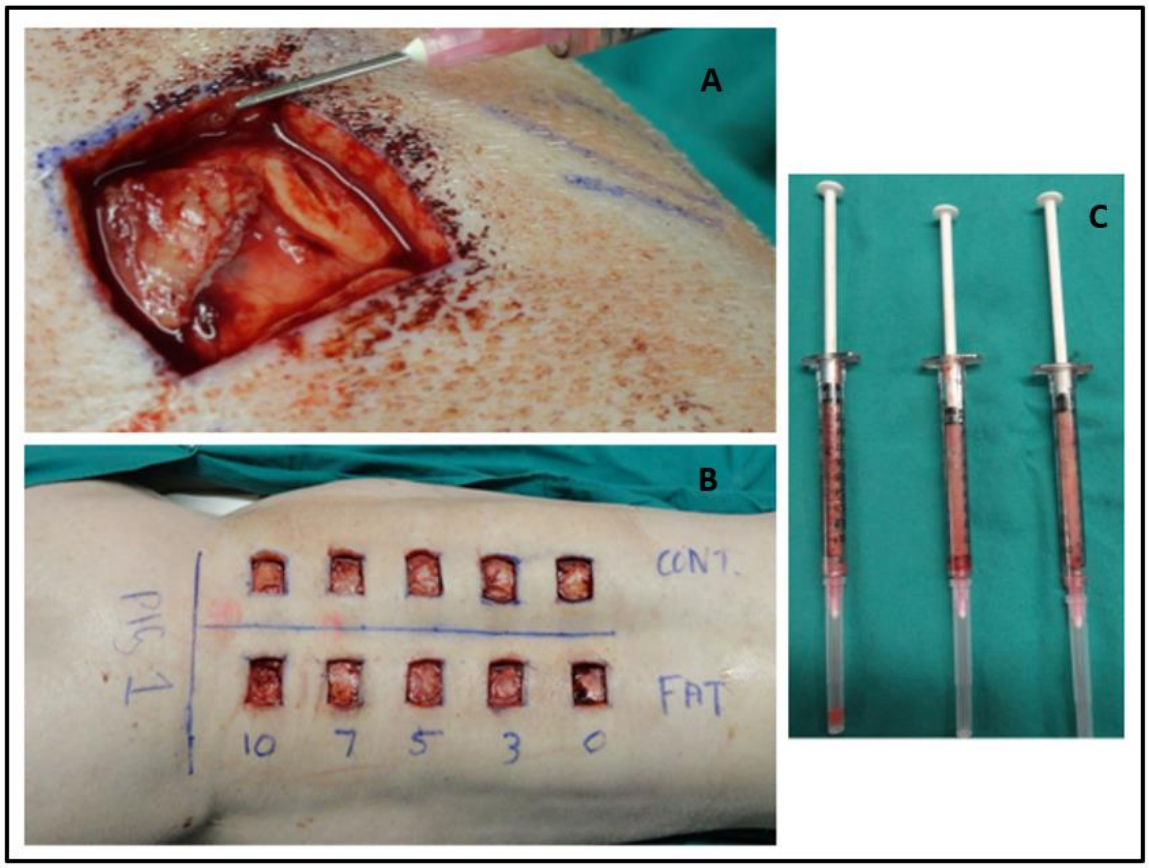


Figure 3. Five interventional and 5 control wounds on each side of the midline were created. On one side the wounds were injected with 2 ml of processed fat 0.5 ml per side. Autologous fat graft injected 0.5 ml per side **(A)**. Five interventional and five control wounds on each side of the midline **(B)**. Autologous fat graft **(C)**.

Wound surface measurements were done on Day 0, Day 3, Day 5, Day 7, and Day 10 of both the lipo-aspirate injected wounds and the control wounds.

Thus 10 wounds surface area measurement per pig, per day of investigation.

Thus a total of 60 measurements per pig for the entire study.

A total of 360 wound surface measurements taken in the study.

Histological Biopsy Specimens were taken on Day 0, Day 3, Day 5, Day 7, and Day 10 of both the lipo-aspirate injected wounds and the control wounds.

Biopsy specimens were taken by full excision of the normal skin and wound defect.

Thus 10 biopsy specimens per pig, per day of investigation.

Thus a total of 60 specimens per pig for the entire study.

A total of 360 specimens taken in the study.

The wound biopsy specimens were embedded in paraffin wax moulds by the Primary Investigator Dr M Venter, together with the help of Consultants in the Department of Anatomical Pathology, University of the Witwatersrand.

The method used were as follows:

H&E Staining for Parrafin Sections

1. Melt paraffin off slides @ 65° C for 20 minutes.

2. Treat with Xylene twice for 10 minutes each.

3. Treat with 100% EtOH twice for 5 minutes each.

4. Air dry slides

5. Stain with filtered 0.1% Mayers Hematoxylin for 10 minutes in a Coplin jar.

6. Rinse in cool running ddH2O for 5 minutes

7. Stain with Eosin (0.5% in 95%EtOH) 12 times.

8. Dip in distilled H2O until the eosin stops streaking.

9. Dip in 50% EtOH 10 times

10. Dip in 70% EtOH 10 times

11. Equilibrate in 95% EtOH for 30 seconds.

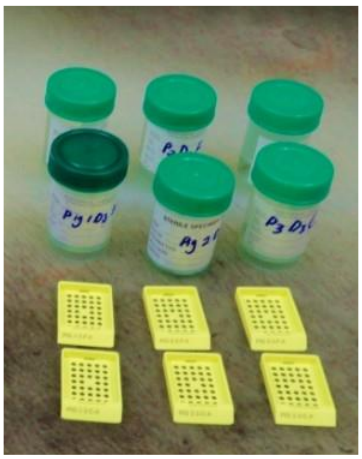
12. Equilibrate in 100% EtOH for 1 minute.

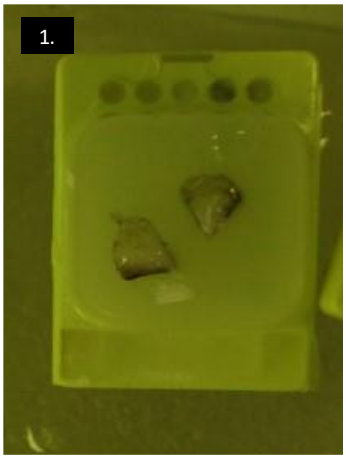
13. Dip in Xylene several times.

14. Clean slide off with a kimwipe; mount and coverslip with Cytoseal XYL

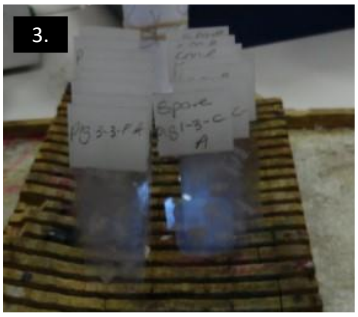
Processing of Biopsy specimens

1.) Setting in Paraffin wax blocks
2.) Microtome cutting of specimens to slides
3.) Setting of slides prior to haemotoxylin and eosin staining









The cutting and staining of the wax specimens to haematoxylin and eosin slides were done by the Primary Investigator Dr M Venter using a microtome under guidance of the Department of Anatomical Pathology, University of the Witwatersrand.

The Primary Investigator Dr M Venter sustained a laceration of her hand during the above slide cutting at which time Dr G Hariparsad, executive board member and chairman of LANCET laboratories offered assistance in the staining of the last 50 slides.

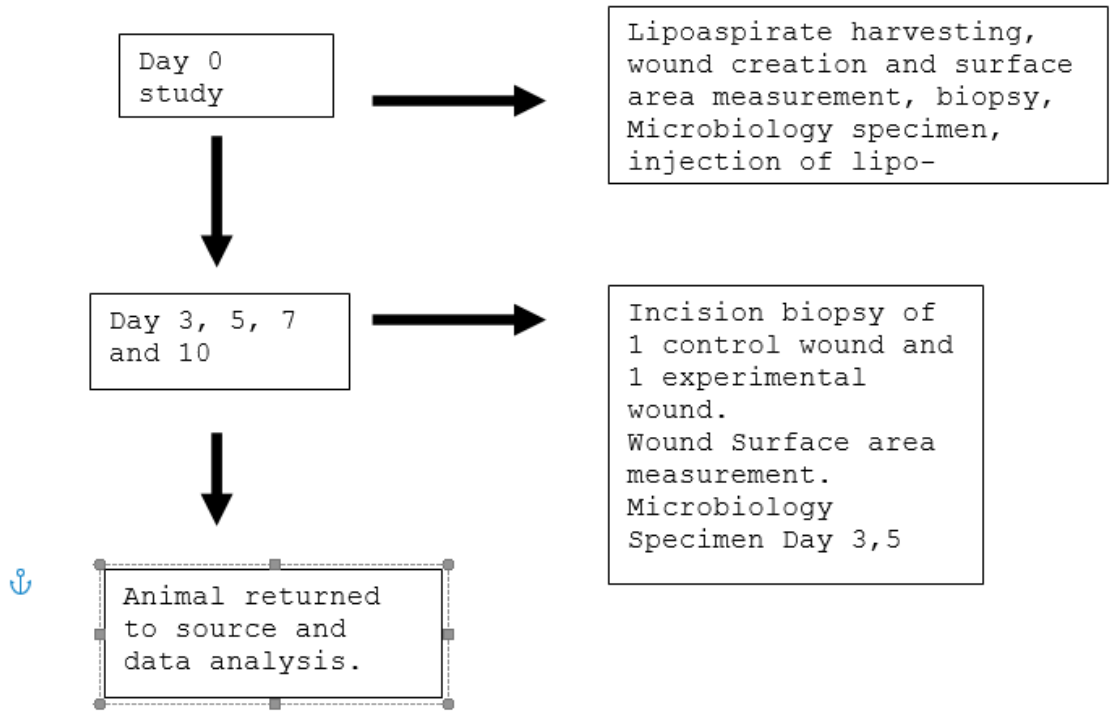
Microbiology specimens

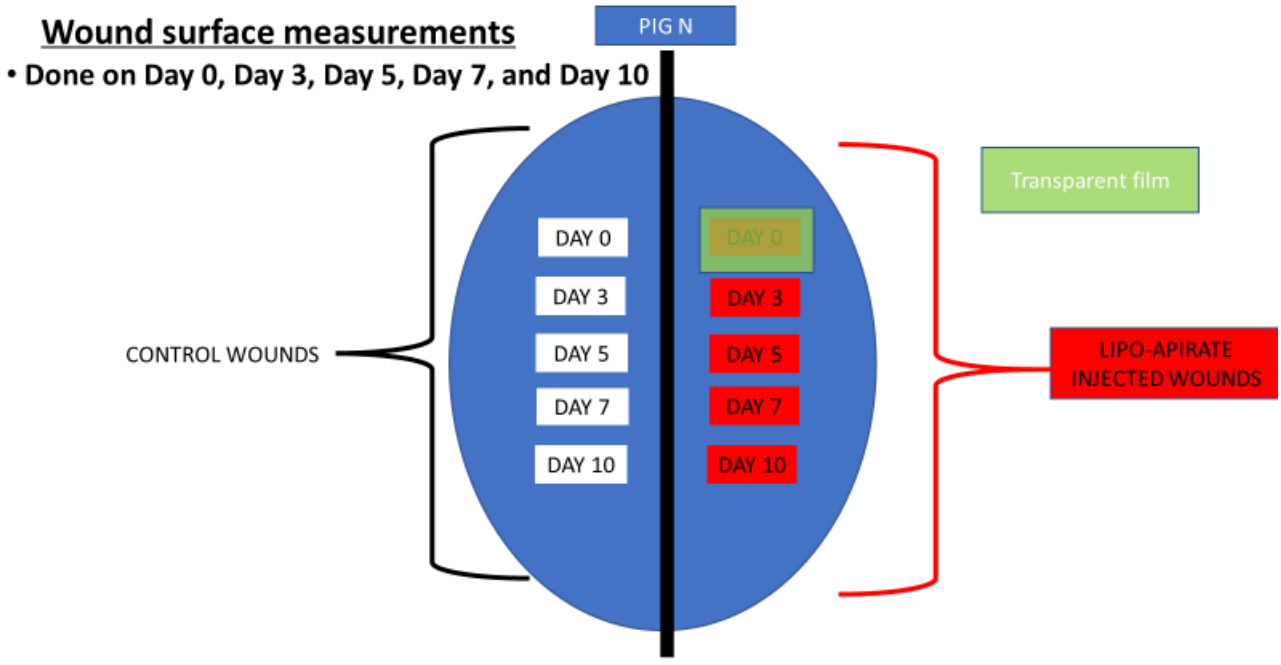
Microbiology specimens were taken on Day 0, Day 5, and Day 10. The microbiology specimens were processed for aerobic and anaerobic cultures by the department of Anatomical Pathology of the University of the Witwatersrand.

6 Microbiological specimens per pig were taken during the study.

A total of 36 microbiology specimens were taken for assessment of bacteriology.

Skin wound sequence: 6 pigs





Wound Surface Measurement

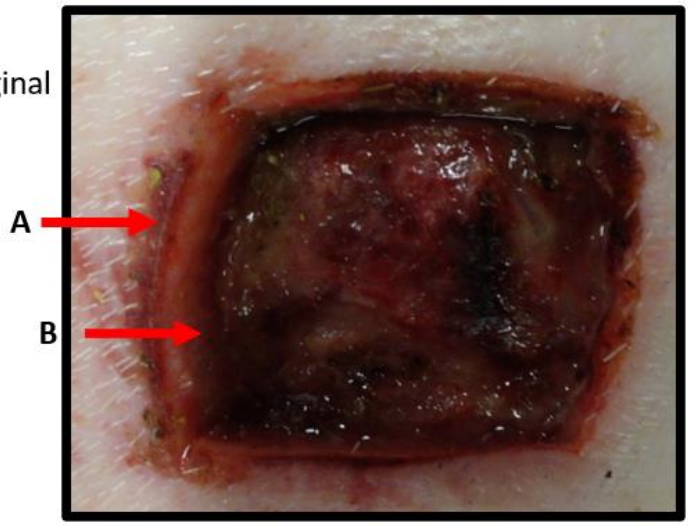
Wound surface measurements were done on day 0,3,5,7, and Day 10. Wound surface measurements were done similar to a study measuring wound surface area in a porcine wound healing model using adipose derived stem cells, Fu X et al [18]. According to the technique the surface of the wound is covered with a hyaline membrane, the edge of the wound is traced on the hyaline membrane template. After washing and drying the hyaline membrane template it is cut out and weighed for each day of wound measurement (N = day of measurement). This is the method as expained by Fu x et al [18].

$$\text{Wound Surface Area cm}^2 = \frac{\text{Weight of Hyaline membrane (g)}}{\text{Weight of the hyaline membrane per cm}^2}$$

In our study we replaced the hyaline membrane with a transparent x -ray film.

The reepithelialisation area was the area between the wound edge and newly formed epithelial edge on the day. Wounds were considered closed if no granulation tissue was apparent (Figure 4)

Figure 4. The re-epithelialization area was the area between the original wound edge **(A)** and the newly formed epithelial edge **(B)**



Histological Assessment

Incision biopsies were taken from each wound on day 0, 3, 5, 7 and 10. The initial biopsy sites were varied among the pigs to exclude bias (Figure 5). The specimens were fixed in 10% of formalin for more than 48 hours. The specimens were embedded in paraffin and cut in 5µm thick sections using a microtome. The sections were stained with haematoxylin and eosin for histopathological evaluation. Tissue sections were deparaffinized in xylene, rehydrated through graded alcohol to phosphate buffered saline (PBS), stained in haematoxylin and eosin staining (H & E) and mounted in resin. Epidermal thickness was measured from stratum basale to stratum corneum at three equidistant interfollicular sites. The mean thickness of the epidermis and epidermal length were determined in the interventional wounds that received the lipoaspirate injections and in the control wounds that did not receive the lipoaspirate injections. The slides were processed by the Lancet laboratory, Johannesburg South Africa. The results were assessed by an experienced blinded pathologist, Professor S. Nayler, Anatomical Pathologist, Drs. Gritzman &Thatcher Inc.

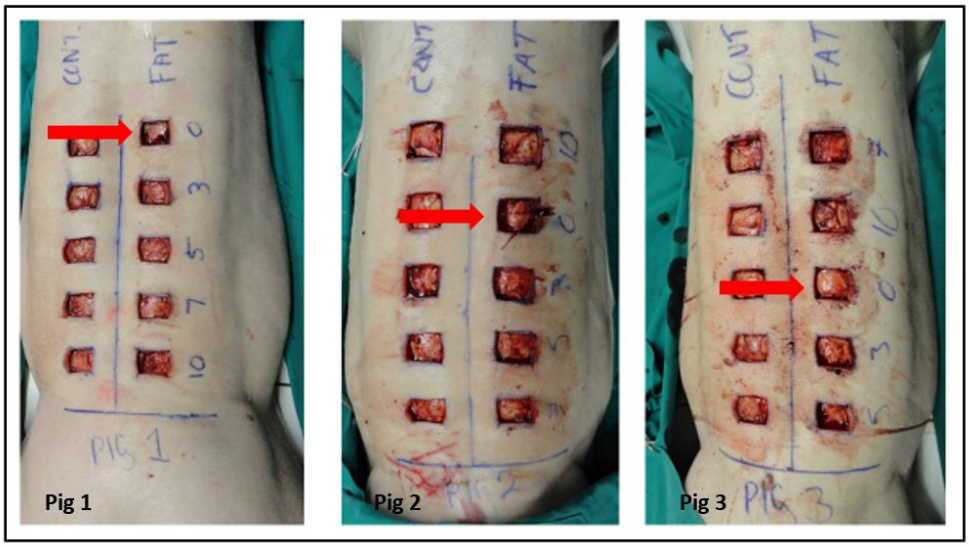


Figure 5. Initial biopsy site variability to exclude bias. Histological evaluation done on Day 0,3,5,7 and 10. Bacteriological evaluation on Day 0 ,5 and 10.

Bacteriological assessment

Biopsies were taken on day 0, 5, 10 for each of the interventional and control wounds. The aerobic plates were incubated at 37°C for 48 hours and the anaerobic plates were incubated at 37°C for 72 hours.

During the interval between sampling, the wounds were covered with a transparent film dressing and a body stocking was applied. The dressing stayed in place during the study period and was changed at each operative procedure (figure 6).

At the end of the study the pigs were handed over to the University of the Witwatersrand Animal Laboratory.



Figure 6. Transparent film dressing (A). Body stocking to protect wounds (B).

Exclusion criteria

1. Investigations rejected by the laboratory
2. Clinical wound infection
3. Adverse reaction of any animal to medication/anaesthesia

e.) Data analysis:

Statistical assistance was sought from Professor H.S Schoeman, BSc, MSc, DSc (UP)

Descriptive statistics were used to display the results of the comparative analyses on wound healing, based on measurements taken from the wounds on the Fat and Control sides of the experimental pigs' backs.

The study design implied the application of paired statistical analyses, meaning that the analyses should be performed on the differences between the paired measurements on the left (control) and right (fat) sides of the wounds, and testing the null hypothesis of no difference.

For continuous variables mean values of the differences between measurements taken from the two treatment arms were, at each point of time, compared by the paired t test, which requires the assumption that the differences are normally distributed. Normality was tested by the Shapiro-Wilk test. If normality could not be assumed the nonparametric signed ranks test was performed, using median values.

The p values that are reported in the relevant tables apply to the paired t test, unless it is indicated to refer to the signed ranks test.

Paired categorical variables were compared by the nonparametric McNemar's test.

McNemar's test is a statistical test used on paired nominal data. It is applied to 2 × 2 contingency tables with a dichotomous trait, with matched pairs of subjects, to determine

whether the row and column marginal frequencies are equal (that is, whether there is "marginal homogeneity").

The statistical analyses were performed on suite of analytics software (SAS Institute Inc, Carey, NC, USA), Release 9.4. All statistical tests were two-sided and p values ≤0.05 were considered significant.

f.) Reliability and Validity

Through the utilization of a standardized Data Collection Form the reliability and validity of the study was ensured. This form furthermore included only ordinal and categorical data and thereby the statistical objectivity, reliability and validity of the study was strengthened.

g.) Bias

Sequential sampling of interventional and control wounds was utilized using a standardised biopsy instrument thus reducing sampling bias. Performance bias was minimized through the use of objective categories to record the data. Bias in data presentation were minimized through the subject files being readily available through-out the period of their inclusion in the study for completion and review if needed. Information bias was minimized through the objective transcription of measurements onto the data collection sheet as only nominal or ordinal data. There was hence minimal measurement error as only objectively measured information was transcribed.

14.)Results

First Part of the Study:

Viable adipose derived stem cells confirmed via Flow Cytometry as explained above.

Second Part of the Study:

1. WOUND SURFACE AREA

The results from measurements of the surface areas of the wounds, reflecting the healing over time, are summarized in the following table representing 30 assessments for the Control, 30 assessments for Fat and thus 30 differences (6 pigs x 5 wounds =30).

Table 1: Wound surface area:

<u>Control:</u> Wounds not treated with lipo-aspirate

<u>Fat:</u> Wounds injected with lipo-aspirate

<u>Table 1</u>: Surface area

Treatment	Mean ±SD for surface area (cm^2) on			
	Day 3	Day 5	Day 7	Day 10
Control (n=30)	5.98 ±0.88	6.19 ±0.77	6.38 ±1.41	4.64 ±1.33
Fat (n=30)	4.71 ±0.61	4.62 ±1.00	4.22 ±1.05	2.65 ±0.83
Control – Fat (n=30)	1.27 ±0.76	1.57 ±0.85	2.16 ±1.02	1.99 ±0.93
p value	<0.001	<0.001	<0.001	<0.001*

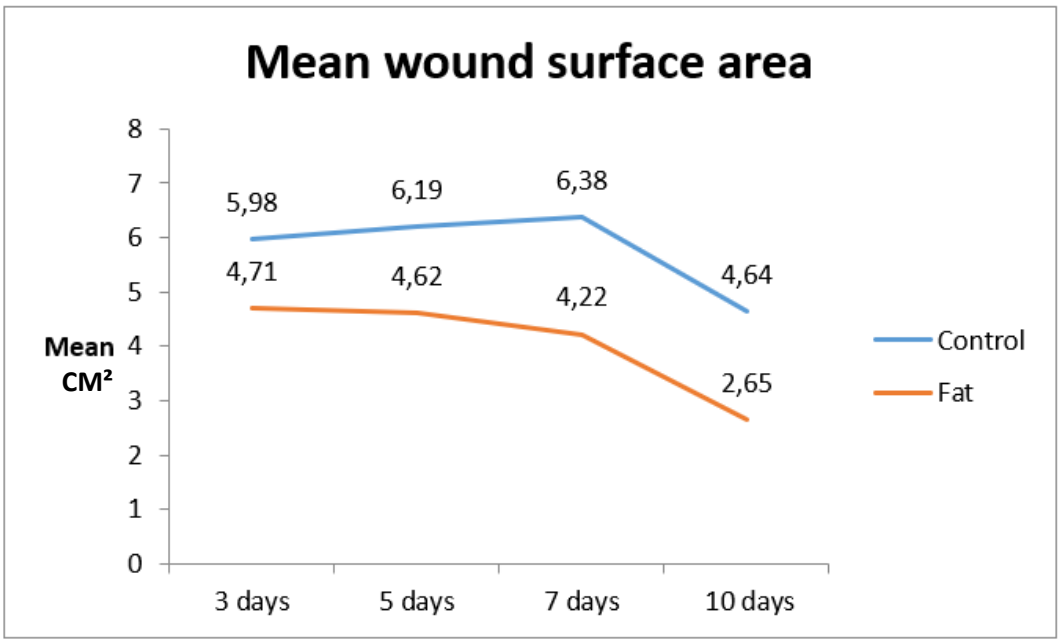
* p value from signed ranks test

* P The Wilcoxon signed rank test (also called the Wilcoxon signed rank sum test) is a non-parametric test to compare data.

The differences in the mean surface areas (Control – Fat) over time are reflected in the shaded line of the table. At each time point the mean surface area of the wounds treated with Fat were significantly smaller compared to the Control wounds, indicating faster healing with Fat.

In retrospect the t test had 99% power to detect an effect size of 2.14 (=1.99/0.93) at Day 10.

The data of the table is graphically displayed in the following figure.



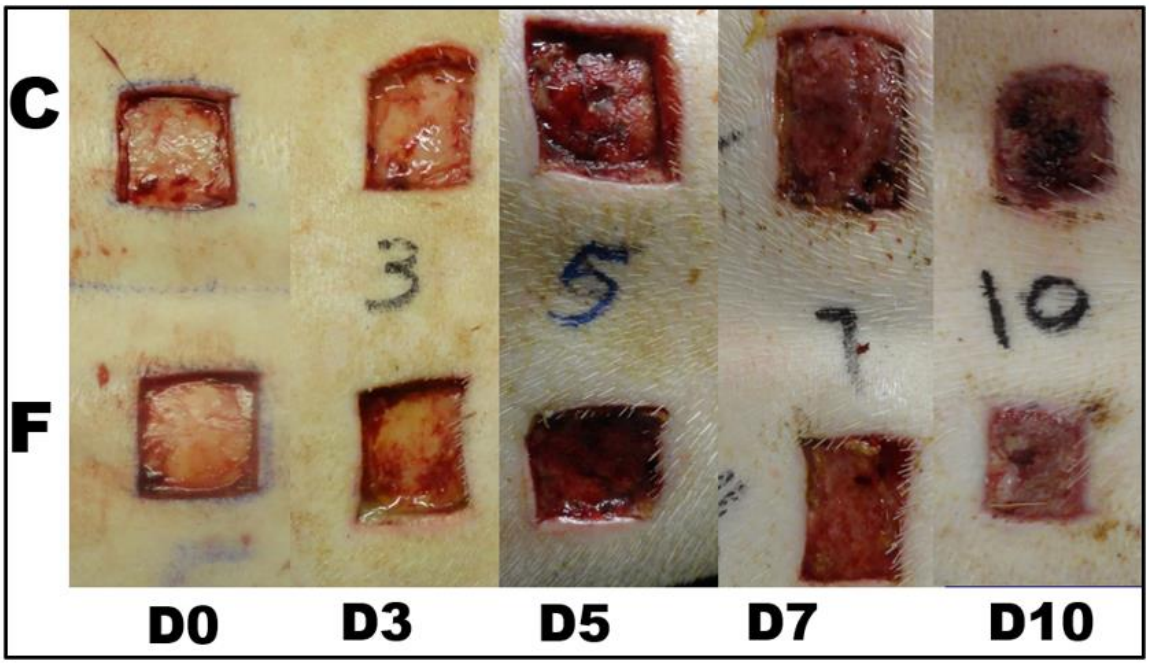


Figure 7 .Wound healing depicted per day, Day 0 , Day 3, Day 7 , Day 10 Autologous fat graft (F), control group (C)

Above see specific days as progression of wound healing – not specific to one pig.

Evidence as per progression per pig illustrated in Data Analysis.

2. NEW EPITHELIAL THICKNESS

At each day of assessment, one pair of wounds was randomly selected on each pig. Three measurements were taken of the new epithelial thickness of the wound treated with Fat and the average thickness calculated. Likewise with the Control wounds. The results are summarized in the following table.

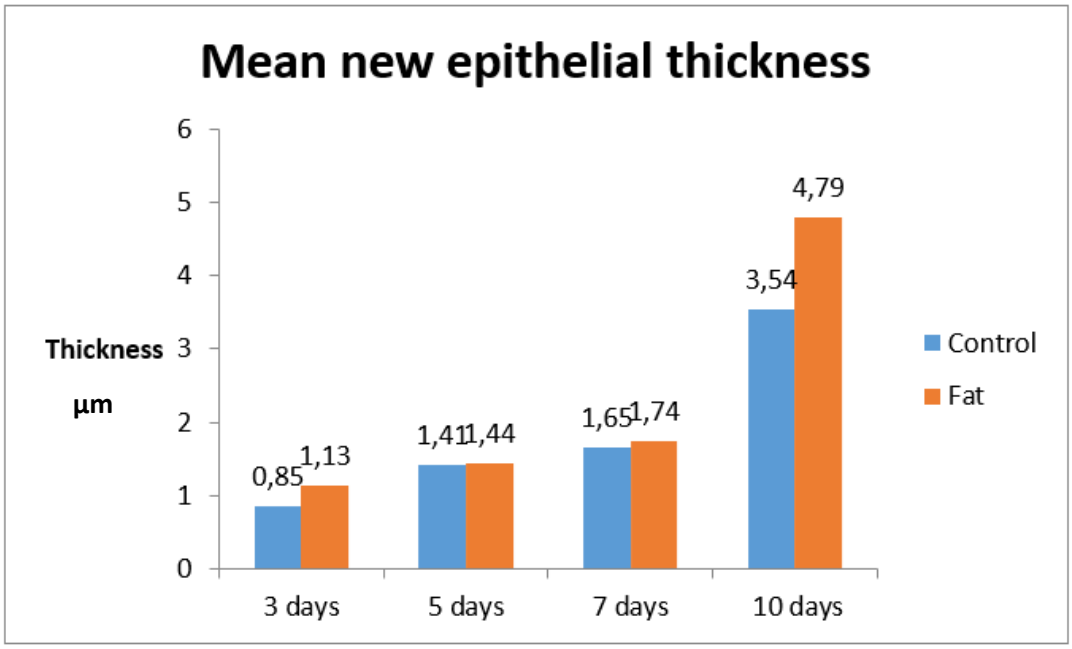
Table 2: **Average new epithelial thickness**

Treatment	Mean ±SD of the average new epithelial thickness (µm) on			
	Day 3	Day 5	Day 7	Day 10
Fat (n=6)	1.13 ±0.11	1.44 ±0.54	1.74 ±0.64	4.79 ±0.46
Control (n=6)	0.85 ±0.57	1.41 ±0.74	1.65 ±1.06	3.54 ±0.69
Fat - Control (n=6)	0.28 ±0.63	0.03 ±1.08	0.09 ±0.44	1.25±1.05
p value	0.326	0.948	0.633	0.033

The excessive mean new epithelial thickness for the wounds treated with Fat, compared to the Control wounds, are reflected in the shaded line of the table. The excess was statistically significant at Day 10, which displayed faster healing with Fat at Day 10.

In retrospect the t test had 65% power to detect an effect size of 1.19 (=1.25/1.05) at Day 10.

The data of the table is graphically displayed in the following figure.



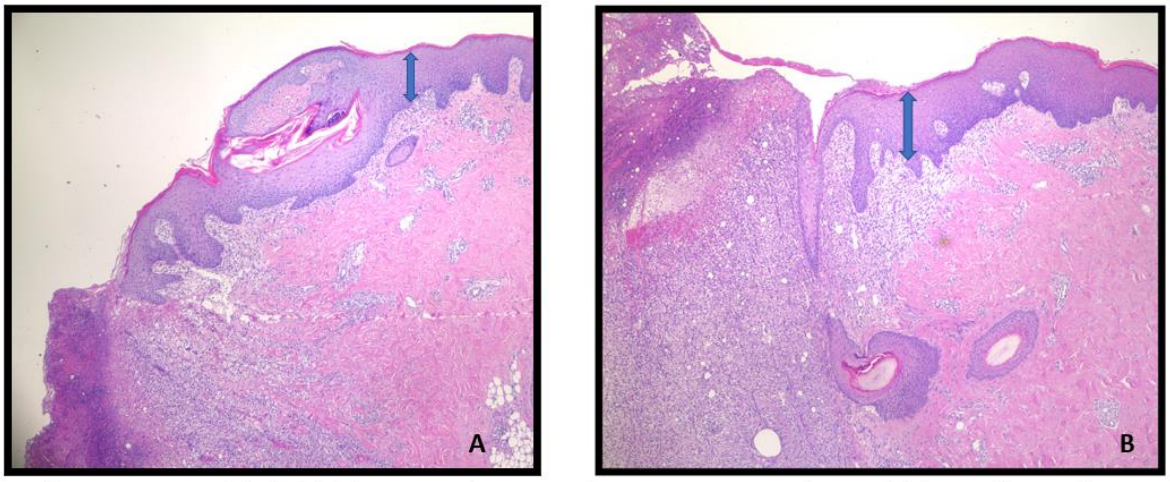


Figure 8. New epithelial thickness – Index Case: Pig 2 Day 10. Control wound (A), Autologous fat graft (B). Note the thicker epithelium in the fat grafted group.

3. NEW EPITHELIAL LENGTH

At each day of assessment, one pair of wound was randomly selected on each pig. For each wound treated with Fat the new epithelial length was measured. Likewise with the Control wounds. The results are summarized in the following table.

Table 3: New epithelial length

Treatment	Mean ±SD of the new epithelial length (μm) on			
	Day 3	Day 5	Day 7	Day 10
Fat (n=6)	1.46 ±0.75	2.97 ±0.59	3.34 ±0.64	5.31 ±1.37
Control (n=6)	1.35 ±0.57	2.25±0.82	3.20 ±0.52	4.99 ±1.50
Fat - Control (n=6)	0.11 ±0.45	0.72 ±0.42	0.14 ±0.29	0.32±0.24
p value	0.562	0.009	0.375*	0.062**

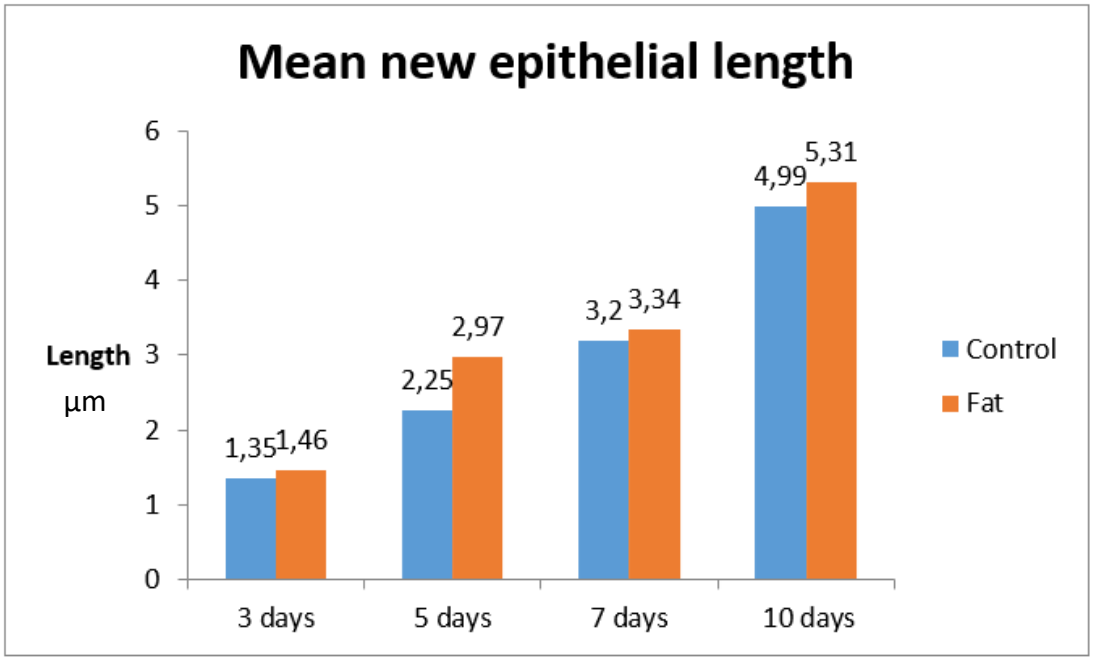
* p value from signed ranks test

** p value from signed ranks test. The p value for the t test was 0.024 which is significant but normality could not be assumed

The excessive mean new epithelial length for the wounds treated with Fat, compared to the Control wounds are reflected in the shaded line of the table. The excess displayed faster healing with Fat, which was statistically significant at Day 5, and at Day 10. P values specified in table.

In retrospect the t test had 73% power to detect an effect size of 1.33 (=0.32/0.24) at Day 10.

The data of the table is graphically displayed in the following figure.



Above Bar Graph indicating Mean New Epithelial Length

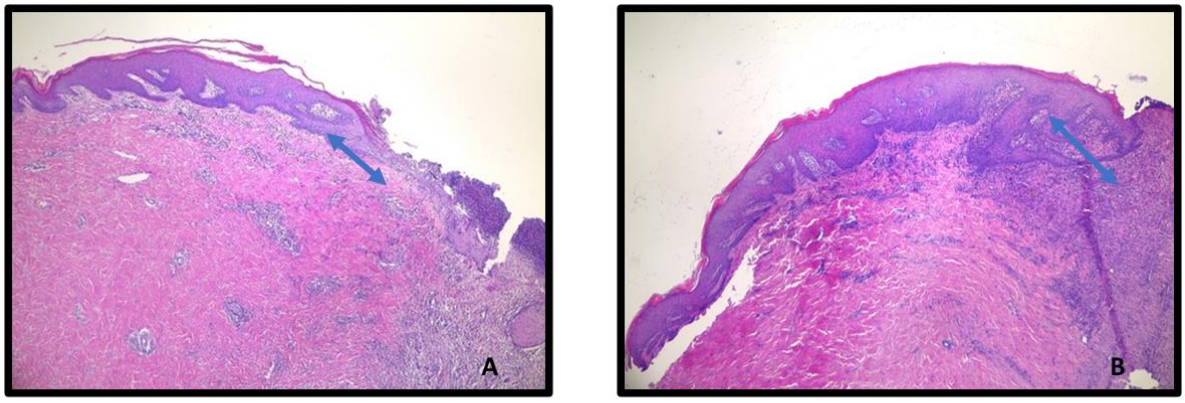


Figure 9. New epithelial Length – Index Case: Pig 1 Day 10. Control wound (A), Autologous fat graft (B). Note the longer epithelium in the fat grafted group.

4. AVERAGE NEW EPITHELIAL THICKNESS VERSUS NORMAL EPITHELIAL THICKNESS

In this section the new epithelial thickness values reported in Section 3.2 above will be compared with similarly calculated mean values of the normal epithelial thickness, done at the same time on the same wounds. The comparisons will be done separately for the wounds treated with Fat and for the Control wounds.

Finally the wounds treated with Fat will be compared with the Control wounds.

Definitions:

Normal epithelial thickness: - The thickness of epithelium in the native porcine skin prior to intervention.

New Epithelial thickness: -The newly formed epithelium post full thickness defect created.

4.1 Wounds treated with lipo-aspirate.

The results of the averages calculated for the new epithelial thickness (from Table 2 above) and for the normal epithelial thickness are summarized in the following table.

Table 4: Average new versus normal epithelial thickness for wounds treated with Fat

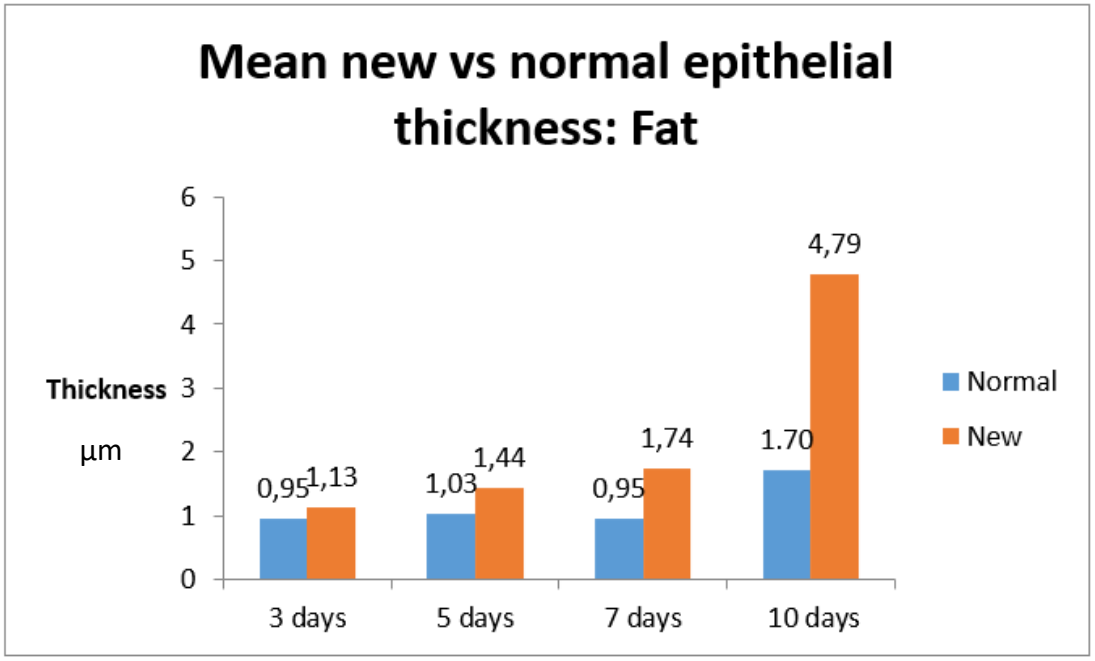
Treatment	Mean ±SD of the average new and normal epithelial thickness (µm) on			
	Day 3	Day 5	Day 7	Day 10
New thickness (n=6)	1.13 ±0.11	1.44 ±0.54	1.74 ±0.64	4.79 ±0.46
Normal thickness (n=6)	0.95 ±0.45	1.03 ±0.61	0.95 ±0.15	1.70 ±0.33
New – Normal (n=6)	0.18 ±0.49	0.41 ±1.00	0.79 ±0.56	3.09±0.75
p value	0.411	0.372	0.031*	0.031*

* p value from signed ranks test

The excessive mean new epithelial thickness values versus the normal epithelial thickness are reflected in the shaded line of the table. The excess was statistically significant at Day 7 and Day 10, which displayed faster healing with Fat at Day 7 and Day 10.

In retrospect the t test had 99% power to detect an effect size of 4.12 (=3.09/0.75) at Day 10.

The data of the table is graphically displayed in the following figure.



4.2 Control wounds

The results of the averages calculated for the new epithelial thickness (from Table 2 above) and for the normal epithelial thickness are summarized in the following table.

Table 5: Average new versus normal epithelial thickness for control wounds

Treatment	Mean ±SD of the average new and normal epithelial thickness (µm) on			
	Day 3	Day 5	Day 7	Day 10
New thickness (n=6)	0.85 ±0.57	1.41 ±0.74	1.65 ±1.06	3.54 ±0.69
Normal thickness (n=6)	1.18 ±0.60	1.79 ±1.12	1.57 ±0.38	1.89 ±0.65
New – Normal (n=6)	-0.33±0.57	-0.38±0.94	0.08 ±0.95	1.65±0.06
p value	0.218	0.360	1.000*	0.031*

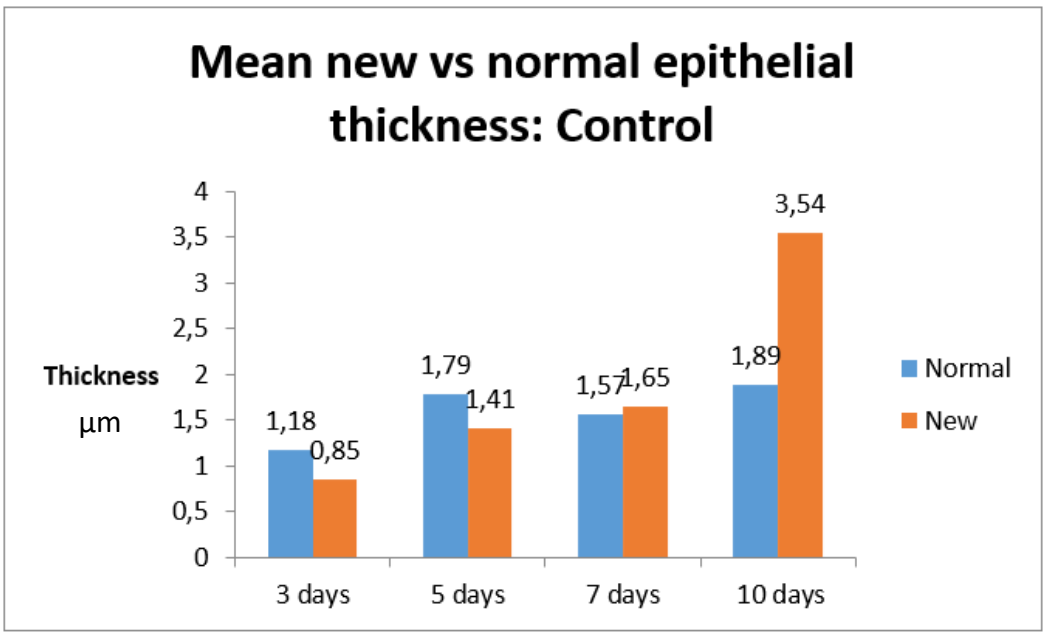
* p value from signed ranks test

The differences between the new epithelial thickness values and the normal epithelial thickness are reflected in the shaded line of the table. At Day 10 the new thickness was statistically greater than the normal thickness, which displayed faster healing of the Control wounds at Day 10.

Note that at Day 3 and Day 5 the normal thickness was better than the new thickness, which indicates that new epithelial thickness developed slow in the first 5 days.

In retrospect the t test had 99% power to detect an effect size of 27.5 (=1.65/0.06) at Day 10.

The data of the table is graphically displayed in the following figure.



4.3 Wounds treated with fat vs control wounds

In this section the differences between the new and normal epithelial thicknesses of wounds treated with Fat, will be compared with the differences between the new and normal epithelial thicknesses of the Control wounds, i.e. a comparison of the shaded line values in Table 4 with the shaded line values in Table 5. The results for the comparative analysis are summarized in the following table.

Table 6: Average new compared to normal epithelial thickness: Fat vs Control

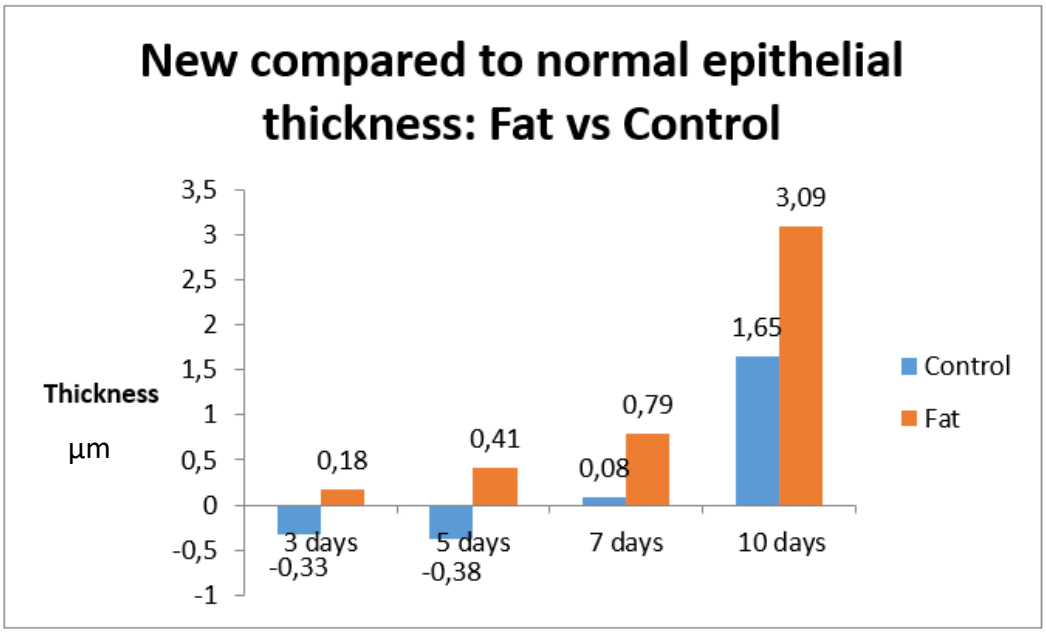
Treatment	Mean ±SD of the differences between new and normal epithelial thickness (µm) on			
	Day 3	Day 5	Day 7	Day 10
Wounds treated with Fat (n=6)	0.18 ±0.49	0.41 ±1.00	0.79 ±0.56	3.09±0.75
Control wounds (n=6)	-0.33±0.57	-0.38±0.94	0.08 ±0.95	1.65±0.06
Fat - Control (n=6)	0.51 ±0.38	0.79 ±0.46	0.71 ±0.64	1.44±0.76
p value	0.022	0.008	0.041	0.031*

* p value from signed ranks test

The differences between the Fat and Control wounds are reflected in the shaded line of the table. At each time point the mean value of the difference between new and normal epithelial thickness was significantly better for wounds treated with Fat compared to the Control wounds.

In retrospect the t test had 99% power to detect an effect size of 1.89 (=1.44/0.76) at Day 10.

The data of the table is graphically displayed in the following figure.



5. NUMBER OF INFLAMMATORY CELLS

The following table shows the paired distributions of the numbers of inflammatory cells, per high power field view, from the wounds treated with Fat versus the Control wounds. The McNamara test was applied to the data for each day to test for homogeneity of marginal frequencies.

Table 7: Inflammatory cells

Number of inflammatory cells from Control wounds	Number of inflammatory cells from wounds treated with Fat									
	Day 3		Day 5			Day 7		Day 10	
	0	1	1	2	3	1	3	2	3
0	2	1	1						
1	1	2	2	1					
2				1	1	3		2	
3							3	1	3
p value	1.000		0.392			n/a		0.317	

Inflammatory cell infiltrate represented as 1+, 2+, 3+. Indicate degree of infiltration. Could also be expressed as mild, moderate, and high, as percentage of high-power view.

The p values are all non-significant which implies that the marginal distributions of inflammatory cells for Fat and Control do not differ significantly at each day, meaning that the cells counts from the wounds treated with Fat do not differ significantly from cell counts from the Control wounds.

6. NUMBER OF COLLAGEN BUNDLES

The following table shows the paired distributions of the numbers of collagen bundles, per high power field view, from the wounds treated with Fat versus the Control wounds. The McNamara test was applied to the data for each day to test for homogeneity of marginal frequencies.

Table 8: Collagen bundles

Number of collagen bundles from Control wounds	Number of collagen bundles from wounds treated with Fat								
	Day 3	Day 5		Day 7			Day 10		
	0	0	1	0	1	2	1	2	
0	6	2	1		3				
1		2	1	2			3		
2						1	1	2	
p value	n/a	0.564		0.978			0.317		

Collagen bundle formation: represented as 1+, 2+, 3+, indicate degree of formation. Could also be expressed as mild, moderate, and high, as percentage of high-power electron microscope view.

The p values are all non-significant which implies that the marginal distributions of the collagen bundle counts for Fat and Control do not differ significantly at each day, meaning that the bundle counts from the wounds treated with Fat do not differ significantly from the bundle counts of the Control wounds.

7. MICROBIOLOGY

Microbiology was assessed at Day 0, Day 5 and Day 10. Anaerobic and aerobic cultures did not indicate a significant bacterial growth in either the lipo-aspirate injected or the control groups. This is an important finding as it has been thought previously that he lipo-aspirate may induce wound infection.

A summary of the results is presented in the following table.

Table 9: Microbiology

Microbiology	Frequency			
	Day 5		Day 10*	
	Fat	Control	Fat	Control
Anaerobic				
Negative	6	6	5	5
Aerobic				
1+	4	6	4	4
2+			1	1
Negative	2			
Organism				
E coli			1	1
Staph Aureus	4	6	4	4
Organism 2				
E coli	1	2	3	1
Strep dysgalactia		1		1
Klebsiella		1		1
Organism 3				
Acitenobacter				1

*Only 5 pigs

15.) Discussion

In our study the application of autologous fat graft resulted in a highly significantly reduced wound surface area compared to the control wounds at 3 days, 5 days, 7 days, and 10 days ($p<0.001$).

This study is the first to conclusively show the ability of uncultured and unexpanded adipose tissue to reduces wound surface area. In addition no bacterial infection was induced by injecting lipo-aspirate into iatrogenic wounds.

A study by Ebrahimiam et al, which comprised a rodent model showed that the application of autologous adipose grafted tissue enhanced by growth factors initiated tissue regeneration and resulted in accelerated decreased wound surface area [10]. Our study comprises a porcine model, and therefore cannot be directly compared to the result in this rodent model, however we report a similar finding. Fu X al. in their study, which is comparable utilized a porcine animal model where fat was harvested from the posterior cervical region of miniature pigs, adipose cells populated and applied to interventional wounds inflicted in the same animal. In Fu X al 's study, during the 1st three days, the wounds become significantly smaller [18]. Our study finding is in keeping with the findings of Fu X al.

In our study, with regards new epithelial thickness, no significance was demonstrated between the interventional wound and the control wound at 3 days ($p=0.32$), 5 days ($p=0.98$), or 7 days ($p=0.63$). At day 10 a significant difference was demonstrated in the interventional wound compared to the control wound, favouring the interventional wound which demonstrated increased new epithelial thickness ($p=0.03$). A similar porcine study done by Fu X al. found a significant difference in new epithelial thickness favouring the wounds to which autologous fat graft was applied [18]. Our study finding is in keeping with the finding in this

study. Blanton et al utilized a porcine model, and specifically used new epithelial thickness as an outcome measure, showed that autologous fat grafting resulted in a significant difference between well vascularised interventional wounds compared to well vascularised control wound, favouring well vascularised interventional wound which demonstrated increased new epithelial thickness. In Blanton's study no significant result in poorly vascularised wounds was seen unless platelet derived growth factor was added [12]. Our study comprised wounds inflicted in a healthy pig model which were well vascularised. The finding in the above study regarding well vascularised wounds can be compared to the finding in the well vascularised wounds in our study. Our study finding supports the finding in well vascularised cohort in this study.

Regarding new epithelial length our study finding was a significant increase in new epithelial length for wounds treated with autologous fat grafting compared to a control wound at day 5 ($p=0.009$). Lim et al. looked at a rodent model and utilized adipose derived stem cells, not autologous fat grafting, demonstrated a significant increase in re-epithelization in wounds on day 7,10 and 14. The same study further reported that the control wounds demonstrated a significantly slower rate of epithelization at the same study endpoints [22]. Although Lim et Al.'s study was a rodent model and utilized adipose derived stem cells, not a porcine model utilizing autologous fat grafting as was performed in our study and is therefore not directly comparable, the results in this study are in line with the results of our study. Fu X al. mentioned previously used a porcine model assessed the significance of adipose derived stem cells, fibroblast growth factor, epidermal growth factor and normal saline, in wound healing. In their study no new epithelial length was demonstrated during the first 3 days but at day 7 and day 14 there was a significant difference in the new epithelial length between wounds treated with adipose derived stem cells compared to the control wounds treated with saline

[18]. Our study finding was that autologous fat grafting significantly increased new epithelium length on day 5 and hence, although Fu et al. utilized adipose derived stem cells and not autologous fat grafting it demonstrates the shared efficacy of adipose derived stem cells and autologous fat grafting on increased new epithelial length on day 5 compared to control wounds.

In our study no significant difference was demonstrated between the numbers of inflammatory cells per high power field view, in the wounds treated with autologous fat grafting versus the control wounds, at any of our study end points. Our study finding hence demonstrates that the injection of autologous fat graft does not induce an inflammatory response. Huss et al. looked specifically at inflammation in a human model, post the injection of autologous fat graft into burn wounds, demonstrated that despite the hypothesis that the fat cells wound die and induce an inflammatory reaction, a significant proportion of the fat cells in fact survived and replicated with an insignificant inflammatory response [19]. While our study is a porcine, and not a human study, our study finding supports the findings of Huss et al. This is supported by another human study done by Tabit et al. that found that that autologous fat grafts induced angiogenesis and even reduced the inflammatory response [13]. Our porcine model study finding is not directly comparable with this human study although we report similar findings. The porcine study of Fu X et al. looking at the application of adipose derived stem cells in a wound healing model reported that no harmful inflammatory response occurred in wounds treated with adipose derived stem cells [18]. Our study is again not directly comparable with this study, as we used autologous fat grafting and not adipose derived stem cells, although our study finding supports the findings.

In our study the collagen bundles were evaluate by immunohistochemical staining. In our study no significant difference was demonstrated between the amount of collagen bundles in wounds injected with autologous fat graft and the control wounds. Wang yet al notes that the correct way to evaluate collagen content is by using Picrosirius red-haematoxylin staining to determine the presence of type I and III collagen [24]. We did not employ the ideal technique, due to financial constraints at the time of our study in the laboratory used, our study finding is methodologically flawed, and our result is hence inconclusive. By inference our study finding of a statistically significant increase in the epidermal thickness in wounds treated with autologous fat grafting versus control wounds demonstrates that increased collagen bundles did occur.

Regarding bacteriology our study tissue was sent for aerobic and anaerobic culture on day 5 and day 10. Our study result demonstrated no statistically significant bacterial count between the autologous fat grafted group versus the control group. Our study finding is supported by the result of a human study which demonstrated that autologous fat grafting did not lead to an increased bacterial count nor infection rate [19].

While wound healing has been looked at in multiple studies there are no directly comparable outcome measures nor end points. A limitation in our study is the study end point, greater significance would have been demonstrated if the study were extended until full wound closure was achieved. Please see figure 8 below showing complete wound closure in the fat injected wounds (A) vs the non-fat injected wounds (B)

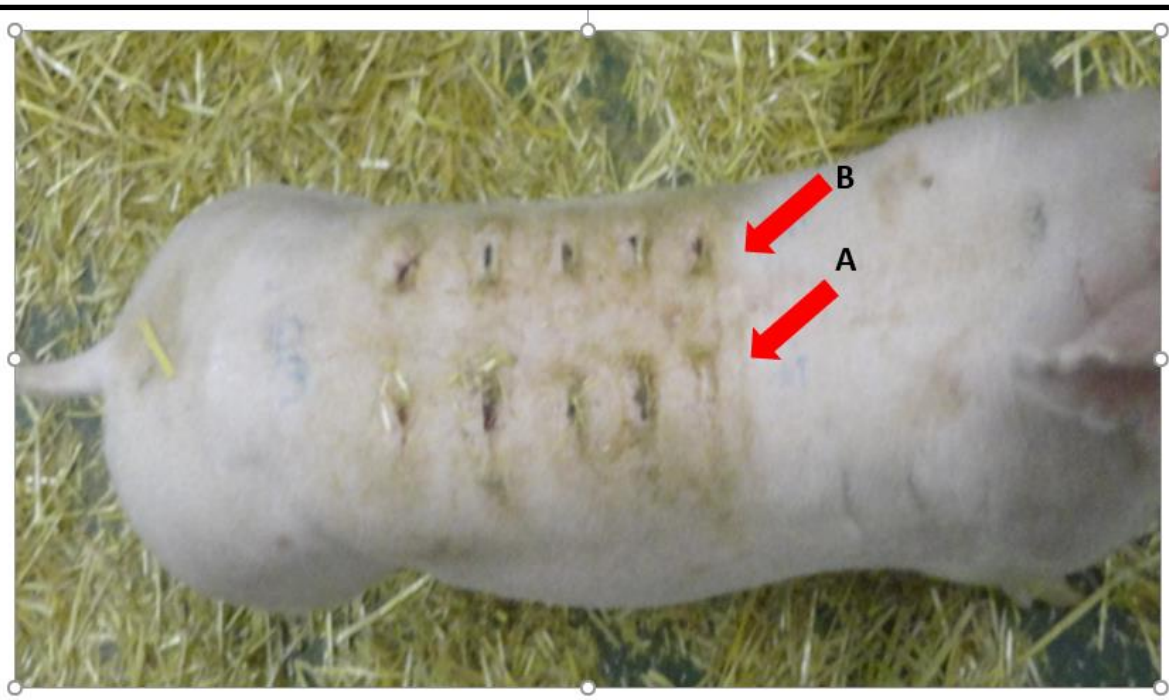


Figure 8. Porcine wound healing model. Picture indicating smaller wounds in autologous fat graft group **A)** compared to control group **B)** at day 21.

16.)Conclusion

In our porcine model, our study has demonstrated that autologous fat grafting, without the isolation culture and expansion of adipose derived stem cells, significantly reduces wound surface area, increases new epithelial thickness, and new epithelial length. Our study further demonstrated that autologous fat grafting does not induce an inflammatory response nor an increased bacterial count. We further noted autologous fat grafting to be inexpensive and easy to perform. Through the results of our study we recommend autologous fat grafting to be added as a tool in the armamentarium of Plastic and Reconstructive Surgery in the management of wounds.

17.) References

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18.)Appendices

A: Study Participant Data Collection Sheet

B: Excel spreadsheet data summary sheet

C: WITS Ethics certificate

D: Informed Consent

A: Study Participant Data Collection Sheet

Study participant number:

……..

Wound surface area measurements (g)

Day 0: Control: ……. Interventional: …….

Day 3: Control: ……. Interventional: …….

Day 5: Control: ……. Interventional: …….

Day 7: Control: ……. Interventional: ……

Day 10: Control: ……. Interventional: …….

……

New epithelial thickness (µm)

Day 0: Control: ……. Interventional: …….

Day 3: Control: ……. Interventional: …….

Day 5: Control: ……. Interventional: …….

Day 7: Control: ……. Interventional: ……

Day 10: Control: ……. Interventional: …….

……..

New epithelial length (µm)

Day 0: Control: ……. Interventional: …….

Day 3: Control: ……. Interventional: …….

Day 5: Control: ……. Interventional: …….

Day 7: Control: ……. Interventional: ……

Day 10: Control: ……. Interventional: …….

……..

Number of inflammatory cells (per high power field view)

Day 0: Control: ……. Interventional: …….

Day 3: Control: ……. Interventional: …….

Day 5: Control: ……. Interventional: …….

Day 7: Control: ……. Interventional: ……

Day 10: Control: ……. Interventional: …….

……

Number of collagen bundles (per high power field view)

Day 0: Control: ……. Interventional: …….

Day 3: Control: ……. Interventional: …….

Day 5: Control: ……. Interventional: …….

Day 7: Control: ……. Interventional: ……

Day 10: Control: ……. Interventional: …….

……

Bacteriology (control)

Day 0

Aerobic: Positive (Organism:) / Negative

Anaerobic: Positive (Organism:) / Negative

Day 5

Aerobic: Positive (Organism:) / Negative

Anaerobic: Positive (Organism:) / Negative

Day 10

Aerobic: Positive (Organism:) / Negative

Anaerobic: Positive (Organism:) / Negative

Bacteriology (interventional)

Day 0

Aerobic: Positive (Organism:) / Negative

Anaerobic: Positive (Organism:) / Negative

Day 5

Aerobic: Positive (Organism:) / Negative

Anaerobic: Positive (Organism:) / Negative

Day 10

Aerobic: Positive (Organism: / Negative

Anaerobic: Positive (Organism:) / Negative

B: Excel spreadsheet data summary sheet

Statistician: Professor H.S Schoeman, BSc, MSc, DSc(UP); clinstat@telkomsa.net;

Wound Surface Measurements

Pig	Wound		Control_3	Fat_3	Con_Fat_3		Control_5	Fat_5	Con_Fat_5		Control_7	Fat_7	Con_Fat_7		Control_10	Fat_10	Con_Fat_10
1	wound 1		7.1	4.0	3.1		6.8	5.2	1.6		8.0	4.8	3.2		6.0	4.2	1.8
1	wound 2		6.1	4.6	1.5		6.1	3.8	2.3		6.9	4.0	2.9		5.8	2.5	3.3
1	wound 3		6.7	5.0	1.7		7.7	4.4	3.3		8.0	5.0	3.0		6.8	3.2	3.6
1	wound 4		7.6	5.6	2.0		7.9	5.2	2.7		10.0	5.6	4.4		8.0	3.6	4.4
1	wound 5		6.4	5.2	1.2		6.0	4.8	1.2		5.6	5.0	0.6		4.1	2.9	1.2
2	wound 1		8.0	5.4	2.6		6.7	5.6	1.1		6.4	5.3	1.1		5.2	3.5	1.7
2	wound 2		6.9	5.4	1.5		6.7	5.4	1.3		7.9	5.7	2.2		6.1	3.7	2.4
2	wound 3		6.2	6.0	0.2		7.1	7.2	-0.1		8.4	6.0	2.4		6.2	4.8	1.4
2	wound 4		5.7	4.6	1.1		6.8	4.8	2.0		6.7	4.2	2.5		5.4	3.3	2.1
2	wound 5		6.7	5.6	1.1		6.0	5.6	0.4		6.8	4.8	2.0		3.8	1.9	1.9
3	wound 1		6.0	5.2	0.8		7.0	7.2	-0.2		6.7	5.2	1.5		4.4	2.5	1.9
3	wound 2		7.2	5.4	1.8		6.5	5.4	1.1		8.4	5.8	2.6		6.2	4.0	2.2
3	wound 3		6.7	5.4	1.3		6.4	5.0	1.4		6.9	4.0	2.9		4.0	2.1	1.9
3	wound 4		5.6	4.8	0.8		4.8	3.3	1.5		5.0	3.6	1.4		3.6	2.0	1.6
3	wound 5		5.4	5.2	0.2		5.4	5.0	0.4		4.4	5.0	-0.6		4.2	2.9	1.3
4	wound 1		5.8	4.4	1.4		5.8	3.4	2.4		6.2	2.6	3.6		3.8	1.5	2.3
4	wound 2		5.8	4.2	1.6		5.4	3.8	1.6		4.6	3.2	1.4		4.2	2.7	1.5
4	wound 3		5.2	4.2	1.0		5.6	3.6	2.0		4.4	2.7	1.7		2.4	1.5	0.9
4	wound 4		4.2	4.2	0.0		5.4	3.8	1.6		5.4	3.5	1.9		3.8	1.9	1.9
4	wound 5		5.8	4.4	1.4		5.8	3.8	2.0		5.8	3.5	2.3		3.6	2.3	1.3
5	wound 1		5.0	3.9	1.1		6.4	4.8	1.6		6.0	4.4	1.6		3.3	2.3	1.0
5	wound 2		5.2	4.2	1.0		5.8	4.8	1.0		7.3	5.7	1.6		5.0	2.7	2.3
5	wound 3		5.0	4.4	0.6		6.2	4.4	1.8		7.3	4.4	2.9		6.9	2.3	4.6
5	wound 4		5.4	3.8	1.6		6.4	5.2	1.2		6.9	3.2	3.7		4.2	2.6	1.6
5	wound 5		5.6	4.2	1.4		6.9	4.2	2.7		6.1	3.8	2.3		4.2	2.0	2.2
6	wound 1		5.0	4.4	0.6		5.4	3.8	1.6		4.2	3.5	0.7		3.1	2.0	1.1
6	wound 2		5.2	4.2	1.0		5.4	3.6	1.8		4.4	3.1	1.3		3.5	2.8	0.7
6	wound 3		5.0	5.2	-0.2		4.8	4.2	0.6		5.4	3.2	2.2		3.2	1.5	1.7
6	wound 4		6.7	4.2	2.5		6.8	3.8	3.1		5.4	2.7	2.7		4.2	2.0	2.2
6	wound 5		6.2	4.0	2.2		5.8	3.6	2.2		5.8	3.1	2.7		4.0	2.3	1.7

Histological Measurements

Day_C	Pig	Day	C_No_Inf_Cells	C_Collagen	C_New_Epi_length	C_Average_NEWET	C_Average_NORET	Day_F	F_No_Inf_Cells	F_Collagen	F_New_Epi_length	F_Average_NEWET	F_Average_NORET
P1D0_C			0	0	0.00	0.00	0.68	P1D0_F	0	0	0.00	0.00	0.97
P1D3_C	1	3	1	0	1.86	0.63	1.93	P1D3_F	1	0	1.72	1.05	1.81
P1D5_C	1	5	1	0	2.10	0.70	2.19	P1D5_F	2	1	2.51	1.11	2.23
P1D7_C	1	7	2	0	3.09	0.70	1.28	P1D7_F	1	1	2.96	1.16	0.88
P1D10_C	1	10	3	1	3.64	3.03	1.39	P1D10_F	3	1	4.06	5.20	1.42
													3.00
P2D0_C			0	0	0.00	0.00	0.94	P2D0_F	0	0	0.00	0.00	0.61
P2D3_C	2	3	1	0	1.41	0.49	0.98	P2D3_F	0	0	0.92	1.28	1.03
P2D5_C	2	5	0	0	2.16	1.40	3.00	P2D5_F	1	0	3.34	0.84	1.09
P2D7_C	2	7	3	1	2.84	2.81	1.53	P2D7_F	3	0	3.26	2.37	1.13
P2D10_C	2	10	2	2	6.17	3.68	2.08	P2D10_F	2	2	6.59	4.30	1.88
P3D0_C			0	0	0.00	0.00	1.09	P3D0_F	0	0	0.00	0.00	0.81
P3D3_C	3	3	0	0	0.78	1.89	1.77	P3D3_F	1	0	0.83	1.01	0.87
P3D5_C	3	5	2	1	2.05	2.80	3.12	P3D5_F	3	1	2.80	0.98	0.85
P3D7_C	3	7	3	2	4.22	2.16	2.32	P3D7_F	3	2	4.61	2.21	0.78
P3D10_C	3	10	3	2	6.70	4.78	3.03	P3D10_F	2	1	6.52	4.56	2.19
P4D0_C			0	0	0.00	0.00	0.81	P4D0_F	0	0	0.00	0.00	0.81
P4D3_C	4	3	0	0	1.89	0.46	0.28	P4D3_F	0	0	2.73	1.08	0.58
P4D5_C	4	5	1	1	3.68	1.34	0.89	P4D5_F	1	0	3.73	1.56	0.73
P4D7_C	4	7	2	0	3.09	0.70	1.38	P4D7_F	1	1	2.97	1.16	0.88
P4D10_C	4	10	3	1	3.64	3.03	1.39	P4D10_F	3	1	4.06	5.20	1.42
P5D0_C			0	0	0.00	0.00	0.95	P5D0_F	0	0	0.00	0.00	0.61
P5D3-C	5	3	1	0	1.61	0.52	1.04	P5D3_F	1	0	1.72	1.22	0.64
P5D5_C	5	5	2	1	2.36	0.89	0.78	P5D5_F	2	0	3.28	2.11	0.70
P5D7_C	5	7	3	1	2.84	2.81	1.53	P5D7_F	3	0	3.26	2.37	1.13
P5D10_C	5	10	2	2	6.17	3.68	2.08	P5D10_F	2	2	6.59	4.30	1.88
P6D0_C			0	0	0.00	0.00	0.72	P6D0_F	0	0	0.00	0.00	0.72
P6D3_C	6	3	0	0	0.54	1.13	1.08	P6D3_F	0	0	0.85	1.16	0.80
P6D5_C	6	5	1	0	1.13	1.30	0.78	P6D5_F	1	0	2.13	2.01	0.60
P6D7_C	6	7	2	0	3.09	0.70	1.38	P6_D7_F	1	1	2.96	1.16	0.88
P6D10_C	6	10	3	1	3.64	3.03	1.39	P6D10_F	3	1	4.06	5.20	1.42

C: University of the Witwatersrand Ethics certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2011/39/04

APPLICANT: **Dr M Venter**

DEPARTMENT: **Plastic Surgery**

PROJECT TITLE: **The Effects of Adipose Extract on Wound and Bone Healing**

Number and Species

8 White pigs (6 pigs to assess skin wound healing and 2 to assess bone wound healing)

Approval was given for to the use of animals for the project described above at an AESC meeting held on **06 September 2011**. This approval remains valid until **05 September 2013**.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Condition 1 - A pilot study being conducted in 2 pigs to assess the effects of lipo-aspirate on the healing of bone defects.

Condition 2 - Before the aforementioned pilot study is conducted that evidence of stem cells in the lipo-aspirate is provided or evidence is provided from the scientific literature that lipo-aspirate may improve bone healing without the presence of stem cells.

Conditions3 - That in the pilot study being conducted on the aforementioned 2 pigs, a single bone defect (16 mm in diameter) is created in one leg of one pig first and if the animal tolerates this, then a second defect may be created in the opposite leg. If the animal tolerates these approaches, then in the second pig, bone defects may be created in both legs simultaneously.

Signed: _____ (Chairperson, AESC) Date: 20/09/2011

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 21/09/2011

cc: Supervisor:
Director: CAS

D: Informed Consent

I, Dr Marisse Venter, understand that I am the data collector for the study titled:

The effect of autologous fat grafting on wound healing- an animal study

I appreciate the fact that participating in this study will expose me to information that is strictly confidential. I will therefore according to the rules of the WITS Ethics office and ethical practice keep this information confidential and will at no point provide this information to an outside party. Should it arise that I need to deal with some information that requires me to do so, I will consult with Professor Elias Ndobe (Research supervisor) as well as the WITS Ethics Committee regarding how the matter should be handled and together we will decide on the way forward.