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# The effect of all-trans retinoic acid on cell proliferation and migration during wound healing: an in vitro study

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# THE EFFECT OF *ALL-TRANS* RETINOIC ACID ON CELL PROLIFERATION AND MIGRATION DURING WOUND HEALING: AN *IN VITRO* STUDY

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree

of

Master of Science

May, 2011

## DECLARATION

I, Oladiran Ibukunolu Olateju declare that this dissertation is my work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……

…… day of May, 2011

**DEDICATION**

To Jehovah

(My Wonderful guidance)

And

To My family,

'The OLATEJUs'

## ABSTRACT

Wound healing in skin is a complex process involving inflammatory responses, cell proliferation and migration, and extracellular matrix deposition. While, *all-trans* retinoic acid (ATRA) is believed to promote wound healing in skin, there are contradictory reports on its effect in both *in vivo* and *in vitro* studies. This study aimed at investigating the effects of ATRA at a concentration of 1μM (in DMSO) on cell migration and proliferation in 'wound' closure. A HaCaT and a HDF cell line as well as a co-culture of both cell lines were utilized. The cultures were maintained in DMEM supplemented with 5% fetal bovine serum incubated at 37ºC in a 5% $CO_2$ in air humidified incubator. Scratch 'wounding' of the HaCaT culture and the co-culture were carried out prior to treatment with ATRA or its controls [DMSO (vehicle control) or DMEM (untreated control)].

ATRA did not have a significant effect on cell proliferation in either the HaCaT or HDF cultures or in the co-cultures. DMSO inhibited proliferation in the HDF cultures and in the co-cultures, while there was no effect on the HaCaT cultures. In addition, ATRA had no significant effect on 'cell migration' during 'wound' closure in both the HaCaT culture and the co-culture. However, DMSO appeared to be inhibitory to migration of cells in both cultures as there was a significant decrease in migration in cultures grown in DMSO when compared to ATRA treatment. The failure of ATRA to promote cell migration and proliferation during 'wound' closure in the HaCaT culture and the co-culture would seem to suggest that the activity of ATRA was compromised by DMSO.

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## TABLE OF CONTENTS


| | | Page |
|---|---|---|
| | DECLARATION | ii |
| | DEDICATION | iii |
| | ABSTRACT | iv |
| | ACKNOWLEDGEMENTS | v |
| | TABLE OF CONTENTS | vi-x |
| | LIST OF FIGURES | xi-xviii |
| | LIST OF TABLES | xix |
| **1.0** | **INTRODUCTION** | **1** |
| **2.0** | **MATERIALS AND METHODS** | **15** |
| 2.1 | Culturing of cell lines | 15 |

| | | |
|---|---|---|
| 2.2 | *All-trans* retinoic acid (ATRA) | 16 |
| 2.3 | Cell proliferation assay | 17 |
| 2.3.1 | Preparation of cultures for ATRA treatment and evaluation of cell proliferative activity | 17 |
| 2.3.2 | Evaluation of cell proliferation using the CellTiter 96 Aqueous® reagent | 18 |
| 2.4 | Scratch assay | 20 |
| 2.4.1 | Preparation of HaCaT cultures for scratch 'wound' assay and ATRA treatment | 20 |
| 2.4.2 | Preparation of co-cultures for scratch 'wound' assay and ATRA treatment | 21 |
| 2.4.3 | Analysis of 'wound' closures in the HaCaT cells and in the co-cultures | 22 |
| 2.5 | Ki-67 immunolabelling assays | 25 |

| | | |
|---|---|---|
| 2.5.1 | Preparation of HaCaT cells for immunocytochemistry | 25 |
| 2.5.2 | Preparation of cells in the co-cultures of HaCaT and HDF cells for immunocytochemistry | 26 |
| 2.5.3 | Ki-67 immunolabelling using the Avidin-Biotin method | 27 |
| 2.5.4 | Estimation of the number of Ki-67 positively immunolabelled nuclei around the 'wound' regions | 29 |
| **3.0** | **RESULTS** | **31** |
| 3.1 | General observation of the cultures | 31 |
| 3.2 | Effect of ATRA on proliferation of the HaCaT and the HDF cell lines | 35 |
| 3.3 | General observations in the 'wounded' cultures | 38 |
| 3.4 | Effect of ATRA on 'wound' closure | 46 |
| 3.4.1 | Comparison of 'wound' closure in the HaCaT cultures with that in the co-cultures | 50 |

| | | |
|---|---|---|
| 3.5 | Cell proliferation | 54 |
| 3.5.1 | Effect of ATRA on the number of actively proliferating HaCaT cells | 58 |
| 3.5.2 | Effect of ATRA on the number of actively proliferating cells in the co-cultures | 60 |
| 3.5.3 | Comparison of number of actively proliferating cells in the HaCaT cultures with the co-cultures | 62 |
| **4.0** | **CHAPTER FOUR: DISCUSSION** | **65** |
| Appendix I | Preparation of 1μM ATRA | 77 |
| Appendix II | Counting of cells using a haemocytometer chamber | 79 |
| Appendix III | Preparation of calcium and magnesium-free phosphate buffered saline (CMF-PBS) | 80 |
| Appendix IV | Preparation of reagents for immunocytochemistry | 81 |

| | | |
|---|---|---|
| Appendix V | Statistical tests for colorimetric cell proliferation assays | 82 |
| Appendix VI | Statistical tests for ‘wound’ closure analyses | 83 |
| Appendix VII | Statistical test of the comparison of ‘wound’ closure between the HaCaT cultures and the co-cultures | 85 |
| Appendix VIII | Statistical test of the comparison between two independent counts of actively proliferating cells in the HaCaT cultures and the co-cultures | 86 |
| Appendix IX | Statistical tests for the number of actively proliferating cells in the HaCaT cultures and the co-cultures | 87 |
| Appendix X | Statistical test of the comparison of the number of actively proliferating cells between the HaCaT cultures and the co-cultures | 89 |
| | **REFERENCES** | **90** |

## LIST OF FIGURES


| | | |
|---|---|---|
| Figure 1 | a) Diagrammatic illustrations of the scratch region at the time of scratching (0 hour) and different time periods (*t* hours) illustrating how the measurements of the width of the scratch at five different regions of the 'wound' were taken; and b) a representative photomicrograph of the scratch region at 8 hours following scratching illustrating the irregular margin of the scratch region. Phase contrast microscopy X4. | 24 |
| Figure 2 | Representative photomicrographs of cultures of a) HaCaT and b) HDF cell lines after 72 hours of seeding. Phase contrast microscopy, X10. | 32 |
| Figure 3 | Representative photomicrographs of a co-culture of HaCaT and HDF cells on the a) first and b) second day after seeding. Phase contrast microscopy, X10. | 33 |

Figure 4 Representative photomicrographs of a co-culture of HaCaT and HDF cells on the a) fourth day after seeding and b) at confluence in which the HDF cells were no longer easily visible in the co-culture. Phase contrast microscopy, X10. 34

Figure 5 A graphical illustration of the relationships of ATRA treatment, DMSO (vehicle control) and DMEM (untreated control) on cell proliferation of the HaCaT cell cultures. There was no statistically significant difference in cell proliferation when the three treatment groups were compared (n=6). 36

Figure 6 A graphical illustration of the relationships of ATRA treatment, DMSO (vehicle control) and DMEM (untreated control) on cell proliferation of the HDF cell cultures. There was a significant difference in cell proliferation when ATRA treatment and vehicle control or vehicle control and untreated controls were compared (n=6, **p<0.01). 37

| | | |
|---|---|---|
| Figure 7 | Representative photomicrographs showing 'wound' closures in a HaCaT culture immunolabelled with Ki-67 primary antibody. Phase contrast microscopy, a) X10 b) X40. | 39 |
| Figure 8 | Representative photomicrographs of the same 'wounded' region of a HaCaT culture with ATRA treatment at different time periods. Phase contrast microscopy, X4. | 40 |
| Figure 9 | Representative photomicrographs of the same 'wounded' region of an untreated HaCaT culture at different time periods. Phase contrast microscopy, X4. | 41 |
| Figure 10 | Representative photomicrographs of the same 'wounded' region of a HaCaT culture grown in vehicle control medium at different time periods. Phase contrast microscopy, X4. | 42 |
| Figure 11 | Representative photomicrographs of the same 'wounded' region of a co-culture of HaCaT and HDF cells with ATRA treatment at different time periods. | 43 |

Phase contrast microscopy, X4.

Figure 12 Representative photomicrographs of the same 'wounded' region of an untreated co-culture of HaCaT and HDF cells at different time periods. Phase contrast microscopy, X4. 44

Figure 13 Representative photomicrographs of the same 'wounded' region of a co-culture of HaCaT and HDF cells grown in vehicle control medium at different time periods. Phase contrast microscopy, X4. 45

Figure 14 A graphical illustration of the effects of ATRA treatment, DMSO (vehicle control) and DMEM (untreated control) on 'wound' closure in the HaCaT cultures at different time periods. By 10 and 24 hours after 'wounding', 'wound' closure was significantly different when the ATRA treatment and the vehicle control were compared (n=12, *p<0.05). 47

Figure 15 A graphical illustration of the effects of ATRA treatment, DMSO (vehicle control) and DMEM (untreated control) on 'wound' closure in the co- 49

cultures of HaCaT and HDF cells at different time periods. By 24 hours after wounding, wound closure was significantly different when the ATRA treatment and the vehicle control were compared (n=12, *p<0.05).

Figure 16 A graphical illustration of ‘wound’ closure in response to ATRA treatment when HaCaT cultures were compared with co-cultures of HaCaT and HDF cells at different time periods. There was a significant difference in ‘wound’ closure when the cultures were compared by 10 hours after ‘wounding’ (n=12, *p<0.05). 51

Figure 17 A graphical illustration of ‘wound’ closure in untreated HaCaT cultures compared with untreated co-cultures of HaCaT and HDF cells at different time periods. ‘Wound’ closure was significantly different when the cultures were compared by 24 hours after ‘wounding’ (n=12, *p<0.05). 52

Figure 18 A graphical illustration of ‘wound’ closure in HaCaT cultures grown in vehicle control medium compared 53

with co-cultures of HaCaT and HDF cells grown in a similar medium at different time periods. There was no statistically significant difference in 'wound' closure when the cultures at different time periods were compared (n=12).

Figure 19 Photographic representations of HaCaT cultures and co-cultures of HaCaT and HDF cells immunolabelled with Ki-67 primary antibody. Photographs $a_1$ - $a_2$ represent the cultures with positive Ki-67 immunolabelled nuclei while $b_1$ – $b_2$ represent the cultures in which the Ki-67 primary antibody was omitted (negative control). Phase contrast microscopy, X40. 55

Figure 20 Photographic representations of the HaCaT cultures immunolabelled with Ki-67 antibody (nuclei labelled dark brown) for the untreated cultures at 0 hour, cultures with ATRA treatment at 24 hours, untreated control cultures at 24 hours and cultures grown in vehicle control medium also at 24 hours. Phase contrast microscopy, X10. 56

Figure 21 Photographic representations of the co-cultures of HaCaT and HDF cells immunolabelled with Ki-67 antibody (nuclei labelled dark brown) for the untreated cultures at 0 hour, cultures with ATRA treatment at 24 hours, untreated control cultures at 24 hours and cultures grown in vehicle control medium at 24 hours. Phase contrast microscopy, X10. 57

Figure 22 A graphical illustration of the number of actively proliferating HaCaT cells at 0 hour and at 24 hours under different treatment regimes. The number of actively proliferating cells significantly increased from 0 hour to 24 hours in the ATRA treated and in the vehicle control cultures. There was no significant difference in the number of actively proliferating cells in the HaCaT cultures at 24 hours under the three treatment regimes (n=4, **p<0.01). 59

Figure 23 A graphical illustration of the number of actively proliferating cells in the co-cultures of HaCaT and HDF cells under different treatment regimes at 0 hour and 24 hours. The number of actively proliferating cells was 61

significantly increased from 0 hour to 24 hours. The number of actively proliferating cells was significantly different when the ATRA treatment and the vehicle control or the untreated control and the vehicle control were compared at 24 hours after the start of the experiment (n=4' **p<0.01).

Figure 24 A graphical illustration of the comparison of the number of cells positive for Ki-67 immunolabelling in the HaCaT cultures and in the co-cultures of HaCaT and HDF cells at 0 hour and at 24 hours under different treatment regimes. The number of actively proliferating cells was significantly different when the HaCaT cultures and the co-cultures were compared for the ATRA treatment and the untreated control respectively at 24 hours (n=4, **p<0.01). 64

## LIST OF TABLES


| | | |
|---|---|---|
| Table 1 | Number of actively proliferating cells in the HaCaT cultures at 0 hour and 24 hours compared with the co-cultures of HaCaT and HDF cells at the same time period. | 63 |

# CHAPTER ONE

## INTRODUCTION

The skin is the largest organ by weight and one of the most complex organs of the human body. It is composed of the epidermis and the dermis. The epidermis is mainly made up of keratinocytes (Kanitakis, 2002). The other cells present in the epidermis are Langerhans cells, melanocytes, Merkel cells and lymphocytes (Kanitakis, 2002; Kerr, 2010). The keratinocytes are arranged in stratified layers named the *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* from the basement membrane to the external surface of the epidermis (Singer and Clark, 1999; Kanitakis, 2002; Kerr, 2010). The keratinocytes migrate from the basal layer to the surface during which they differentiate and acquire the ability to produce keratin (Schaffer and Nanney, 1996, Kanitakis, 2002), flatten and are eventually shed from the surface of the skin (Kanitakis, 2002).

The dermis is a vascularized, supportive and contractible layer of the skin (Kanitakis, 2002; Kerr, 2010). It has elastic connective tissue providing support and protection for the epidermis. The dermis contains several nerve fibres running through it and innervating the epidermis. Fibroblasts are the main cell type found in the dermis. Other cells found in the dermis include mast cells and macrophages (Kanitakis, 2002). In addition, the dermis also contains many fibres (mainly collagen types I and III) and ground substance (proteoglycans and glycoproteins) (Kanitakis,

2002) which are synthesized mainly by fibroblasts (Singer and Clark, 1999; Kanitakis, 2002; Metcalfe and Ferguson, 2006).

The epidermal layer is separated from the dermis by a basement membrane at the epidermal-dermal junction (Kanitakis, 2002; Kerr, 2010). The basement membrane is a complex supportive tissue which is synthesized by both the keratinocytes and the dermal fibroblasts (Kanitakis, 2002). The basement membrane regulates the exchange of metabolic products between the layers of the skin as well as providing support for the migration of cells (lymphocytes, macrophages, fibroblasts) during wound healing (Kanitakis, 2002; Metcalfe and Ferguson, 2006).

The human skin has several functions, most importantly, protection of the body from factors such as radiation, chemical burns, biological or physical injuries. Loss of function can, however, lead to disability or death (Singer and Clark, 1999). The vulnerability of the skin to injuries has led to many studies into the factors and mediators that influence the biological processes of wound healing in skin (Singer and Clark, 1999; Metcalfe and Ferguson, 2006; Sweitzer *et al.*, 2006).

Wound healing in skin is a complex and event-driven process which starts immediately after injury. The process of wound healing is initiated by the release of chemical signals usually from both the traumatized cells and the uninjured cells in the wound area (Metcalfe and Ferguson, 2006; Sweitzer *et al.*, 2006). This initiates a well organized healing process comprising of inflammation, proliferation, migration and maturation (tissue remodeling) phases (Singer and Clark, 1999; Metcalfe and Ferguson, 2006; Sweitzer *et al.*, 2006). These phases are influenced by complex

biological interactions between the cells present in the skin, the infiltrating cells into the wound region including blood cells and the extracellular matrix (Singer and Clark, 1999; Metcalfe and Ferguson, 2006; Sweitzer *et al.*, 2006; Kondo, 2007).

The inflammatory phase begins when a blood vessel is severed. The exudation of blood triggers a haemostatic response to halt blood loss. This is characterized by the formation of a platelet plug and vascular spasm (Singer and Clark, 1999; Metcalfe and Ferguson, 2006). Concurrently, an anti-inflammatory response is triggered which is characterized by the continuous infiltration of neutrophils and lymphocytes into the wound region in order to reduce inflammation (Chettibi and Ferguson, 1999; Singer and Clark, 1999; Kondo, 2007).

Proliferation and migration phases as well as angiogenesis (Metcalfe and Ferguson, 2006) overlap with inflammation and clot formation. These phases are characterized by the migration of keratinocytes and fibroblasts from the dermis and the infiltration of cells such as macrophages and neutrophils (Singer and Clark, 1999; Metcalfe and Ferguson, 2006). The proliferation of these cells and the biological interactions between them contribute to the regeneration of the epidermis and dermis (Singer and Clark, 1999; Metcalfe and Ferguson, 2006; Eming *et. al.,* 2007; Kondo, 2007). The interactions between the cells resident in skin and the infiltrating cells trigger the release of cytokines and growth factors such as transforming growth factor-α (TGF- α), epidermal growth factor, keratinocyte growth factor and vascular endothelial growth factor (Singer and Clark, 1999; Metcalfe and Ferguson, 2006; Eming *et. al.,* 2007; Kondo, 2007).

The final process of wound healing after the regeneration of the epidermis and the deposition of new granulation tissue in the dermis, is the maturation phase. This phase can take several months for completion in human skin (Singer and Clark, 1999; Metcalfe and Ferguson, 2006). The maturation phase is characterized by a well organized relationship between the cells of the skin, the newly formed extracellular matrix and numerous secretions of cytokines (Chettibi and Ferguson, 1999; Singer and Clark, 1999; Metcalfe and Ferguson, 2006). During this phase, fibroblasts differentiate into myofibroblasts which contribute to wound contraction and the formation of the extracellular matrix (Singer and Clark, 1999; Kanitakis, 2002). Upon the regeneration of the epidermis and formation and contraction of formed extracellular matrix, the barrier properties of the skin are believed to be re-established (Singer and Clark, 1999; Metcalfe and Ferguson, 2006). However, the repaired skin never reverts to the same tensile strength as the uninjured human skin (Singer and Clark, 1999).

In addition to the influence of numerous cytokines, growth factors and mediators, external factors have been reported to affect the healing process as well. One such factor is retinoic acid. Retinoic acid belongs to the family of retinoids which are derived from vitamin A (Arukwe and Nordbo, 2008). Vitamin A is present in animal tissues as retinyl esters and retinoid-like retinol and its derivative (Fisher and Voorhees, 1996). Vitamin A is not synthesized by animals for their growth and development, but rather it is synthesized from dietary intake (Fisher and Voorhees, 1996). Therefore, retinoids are usually referred to as ‘dietary hormones’ but lack

endogenic origin (Fisher and Voorhees, 1996; Simms and Ross, 2000; Bastien and Rochette-Egly, 2004; Arukwe and Nordbo, 2008).

During digestion in vertebrates, the retinyl ester is converted to retinol which is later re-esterified to retinyl esters (Fisher and Voorhees, 1996; Harrison and Hussain, 2001). The retinyl ester is released into the blood circulation and then transported to the liver or in a lesser amount, to adipose tissue for storage (Harrison and Hussain, 2001). In the case of insufficient levels of retinol in the blood, the stored retinyl ester is converted back into retinol before being released into the blood plasma (Fisher and Voorhees, 1996).

Retinol serves as a precursor for the biosynthesis of at least two important retinoids: retinal, a vision-related compound and retinoic acid, an active metabolite (Fisher and Voorhees, 1996; Dawson, 2000; Arukwe and Nordbo, 2008; Bremner and McCaffery, 2008). Retinoic acid adopts three primary isomers *viz*: *all-trans* retinoic acid (ATRA), 9-*cis* retinoic acid and 13-*cis* retinoic acid (Alsop *et al.*, 2003; Marill *et al.*, 2003). It is believed that most of the functions of vitamin A such as regulation of gene expression (Bremner and McCaffery, 2008), as well as differentiation of epidermal keratinocytes (Rendl *et al.,* 2002; Eckhart *et al.*, 2008) are performed by its active metabolic component, ATRA (Fisher and Voorhees, 1996; Arukwe and Nordbo, 2008). Interestingly, ATRA has also been found to modulate its own synthesis in the epidermis through its unique ability to regulate the enzymes responsible for ATRA production (Lee *et al.*, 2009).

The ability of retinoic acid to regulate gene expression, differentiation and growth is dependent on retinoic acid binding to two nuclear hormone receptor families: the retinoic acid receptors (RAR) and retinoid X receptors (RXR) (Fisher and Voorhees, 1996; Dawson, 2000; Ross *et al.*, 2000; Bremner and McCaffery, 2008). In addition, a number of receptors known as ligand-receptors have also been implicated in the regulation of the retinoid response (Blumberg and Evans, 1998) and activation of genes responsible for cell morphogenesis, differentiation and proliferation (Alsop *et al.*, 2003).

Ulland *et al.* (1997) in a study on wound healing in male Sprague-Dawley rats with subcutaneous corticosteroid pellet-implants indicated that ATRA had a positive effect on impaired wounds by promoting the metabolism of depressed arginine to ornithine. The metabolism of depressed arginine to ornithine is important for reducing inflammation and in stimulating collagen accumulation and fibroplasias (Ulland *et al.*, 1997). Similarly, Wicke *et al.* (2000) reported that ATRA reversed the non-healing effects of corticosteroid on dorsal skin wounds in the male Sprague-Dawley rats. Paquette *et al.* (2001) reported that biopsies taken from non-healing leg ulcers of patients treated daily with 'short-contact' ATRA resulted in increased deposition of granulation tissue and deposition of collagen, as well as the appearance of new capillaries.

Popp *et al.* (1995) reported that the skin biopsies from severely photo-damaged human-male forearms which were initially treated with topical ATRA cream (Retin A® cream) revealed a considerable network of capillaries, a compact stratum

corneum and increased dermal cells when compared to the control. There was an increase in wound closure and a facilitated repair of the epithelium from the start of the experiment in the treated group when compared to the control (Popp *et al.*, 1995). In a study of the effect of ATRA on full-thickness wounds in Wistar-Albino rats, Basak *et al.* (2002) reported a significant improvement in healing, characterized by increased synthesis of collagen, appearance of blood vessels and new granulation tissue around the wound regions.

Retinoic acid has also been said to promote healing of impaired wounds (non-healing wounds). Tom *et al.* (2005) in a study on healing in non-infected foot ulcers of diabetic male patients reported that topical application of an ATRA solution promoted healing of the ulcers. This was evidenced by a significant reduction in the size of wounds and mild wound healing which are common to diabetic patients such as delayed wound healing, peripheral vascular disease, decreased and disrupted collagen formation and most importantly chronic inflammation compared to the control (Tom *et al*., 2005).

There are however several reports in the literature that contradict the beneficial effect of retinoic acid on wound healing. Hung *et al.* (1989) in a study of the effect of ATRA on pig skin found that daily treatment with ATRA for ten days prior to partial-thickness wounding promoted healing of the wound. However, continued treatment with ATRA post wounding inhibited re-epithelization by increasing inflammation and dermal fibroplasia. Hung *et al.* (1989) suggested that the retardation of wound

healing in the pig model by ATRA treatment could have either been directly linked to the continuity of treatment or was the result of continuous dermal inflammation.

In addition, Meuhlberger *et al.* (2005) in a study on sutured incision wounds on male CD-1 mice reported that topical application of ATRA for one week post wounding, significantly reduced the breaking strength of skin when compared to that of the vehicle control or untreated control groups. However, there was no significant difference in the breaking strength of the skin after two or three weeks of treatment post wounding (Meulhberger *et al.*, 2005). Interestingly, Meulhberger *et al.* (2005) reported that treatment of wounds with ATRA for one week post wounding showed significant disturbance of the epidermal barrier as well as an increased accumulation of neutrophils and increased proliferation of fibroblasts when compared to that of the wounds treated with ATRA for two or three weeks post wounding. Disruption of the dermis was also enhanced in the wounds treated with ATRA for one week when compared to other treatment groups (Meulhberger *et al.*, 2005). Despite an increase in proliferation of the fibroblasts within the first week compared to the vehicle or untreated groups, the least amount of collagen was produced in the wounds treated with ATRA when compared to other treatment groups for the same treatment duration (Meulhberger *et al.*, 2005). It was thus suggested that ATRA did not have an effect on unimpaired wounds but might rather be beneficial to patients with impaired vitamin A metabolism or patients with chronic non-healing wounds such as diabetes (Meulhberger *et al.*, 2005).

Kitano *et al.* (2001) in a study on the effect of ATRA on wound healing in diabetic and in non-diabetic female mice reported that pretreatment for two days had minimal effect on the wound closure in normal (non-diabetic) mice, while there was a significant promotion of wound closure in the diabetic mice. It was suggested that ATRA may possess a unique ability to reverse the healing of impaired wounds in diabetic animals and that pretreatment may facilitate wound healing (Kitano *et al.*, 2001).

The contradictions in the reports on the effect of ATRA on wound healing may be due to the differences in the animal models, experimental methods and conditions used in the studies. For example, several different animals are used including mice (Kitano *et al.*, 2001; Meuhlberger *et al.*, 2005); rats (Ulland *et al.*, 1997; Basak *et al.*, 2002) pigs (Hung *et al.*, 1989) and humans (Popp *et al.*, 1995; Tom *et al.*, 2005). Similarly, studies have used animal models with different disease states such as in diabetes mellitus (Kitano *et al.*, 2001; Tom *et al.*, 2005) or in conditions that impair healing such as the use of corticosteroid implants (Ulland *et al.*, 1997). Despite the controversies, retinoic acid is approved by the U.S. Food and Drug Administration (FDA) and is still widely used for the treatment of acne and photo-aging (Lee *et al.*, 2005; Bremner and McCaffery, 2008) and as pretreatment against skin injuries before laser therapy (McDonald *et al.*, 1999; Paquette *et al.*, 2001).

Interestingly, the contradictions in the effect of ATRA on wound healing are not only peculiar to *in vivo* studies but also to *in vitro* studies. Nelson and Balian (1984) reported a significant reduction in cell proliferation and in the synthesis of

procollagen by human dermal fibroblasts in a manner directly related to an increase in ATRA concentration from $10^{-11}$M to $10^{-6}$M. Kono *et al.* (1991) in a study on mouse 3T3 fibroblasts embedded in a three-dimensional collagen gel reported that ATRA at concentrations of 1μM and 0.1μM resulted in increased contraction of the collagen gels when compared with the untreated control cultures. In the same study, it was reported that cell proliferation of the 3T3 fibroblasts in the collagen gel was significantly inhibited by ATRA at both concentrations (Kono *et al.*, 1991). Interestingly, it was found that ATRA at a concentration of 1μM and 0.1μM had no effect on the contraction of the collagen gel and on cell proliferation of two transformed fibroblast cell lines (3T12-3 and 3T3-B-SV40) used in the same study (Kono *et al.*, 1991). It was thus concluded that ATRA promoted contraction of collagen but it was uncertain whether ATRA promoted cell proliferation (Kono *et al.*, 1991).

In addition to the reports on the effect of ATRA in *in vitro* studies, it has been found that ATRA at a concentration of 10μM has the ability to up-regulate the plasminogen activator system in normal human epidermal keratinocytes (Braungart *et al.*, 2001). The ability of ATRA to promote the plasminogen activator system was suggested to be one of the mechanisms through which ATRA may promote re-epithelization during wound healing (Braungart *et al.*, 2001).

Considering the contradictions on the effect of ATRA on wound healing, it is possible that the bio-molecular mode of action of retinoic acid could be complex and not yet fully understood (Takami *et al.*, 2005). In addition, ATRA is said to possess

pleiotrophic effects (Watson *et al.*, 2004; Takami *et al.*, 2005). One of the diverse, yet unknown, effects may possibly be indirect stimulation or release of biological mediators which could be responsible for promoting or inhibiting any of the phases of wound healing (Davies *et al.*, 1988; Watson *et al.*, 2004; Takami *et al.*, 2005). A possible interaction of ATRA with other co-activators which are released by cells or whose release is regulated by ATRA could similarly have an influence on the retinoic acid receptors (Goyette *et al.*, 2000). At the same time, the co-activators could augment or diminish the effects of ATRA (Lee *et al.*, 2009). It is also possible that ATRA may elicit numerous biological functions that could be difficult to link to a specific gene transcription (Elder *et al.*, 1991; Stoll and Elder, 1998). The possible interference of co-activators or the diverse effect of ATRA on biological functions could be the reasons for the contradictions in the reports on the effect of ATRA.

In addition, it has been reported that the *in vitro* effect of ATRA on the differentiation of keratinocytes was directly opposite to the *in vivo* effect (Fisher and Voorhees, 1996). To re-emphasize, differentiation is important during the maturation phase of wound healing in which the keratinocyte acquires the ability to produce keratin (Schaffer and Nanney, 1996). Fisher and Voorhees (1996) compared reports on the effect of ATRA on keratinocytes and concluded that ATRA promoted proliferation of keratinocytes in the basal layer of the epidermis *in vivo*. Following this, the basal cells migrated to the suprabasal layer before terminally differentiating and losing their ability to proliferate. *In vitro* however, differentiation of the keratinocytes resulted in the arrest of cell proliferation (Fisher and Voorhees, 1996) or the cells undergo functional differentiation characterized by the expression of markers for

differentiation (Jetten, 1990; Fisher and Voorhees, 1996). ATRA however, had a more significant effect in inhibiting the expression of markers for differentiation in keratinocytes than preventing the arrest of proliferation of keratinocytes (Jetten, 1990; Fisher and Voorhees, 1996).

ATRA inhibits the expression of markers of differentiation in keratinocytes, *in vitro*, at a concentration of 1μM (Fisher and Voorhees, 1996; Rendl *et al.*, 2002; Eckhart *et al.*, 2008; Lee et al., 2009). For this reason, Eckhart *et al.* (2008) regarded a concentration of 1μM of ATRA as a 'physiologically relevant concentration'. This is thus the concentration of ATRA frequently utilized in several studies on skin cell types (Tsou *et al.*, 1994; Weninger *et al.*, 1998; Diaz *et al.,* 2000; Lee *et al.*, 2009).

The present study utilized an *in vitro* situation to investigate the effects of ATRA on cell migration and proliferation during wound healing. Two individual cell lines derived from human skin, an immortalized non-tumorigenic human keratinocyte (HaCaT) cell line (Boukamp *et al.*, 1988) and the human dermal fibroblast (HDF) cell line were utilized. It was envisaged that an *in vitro* situation would be more effective in monitoring the effects of ATRA on wound healing compared to the use of an *in vivo* condition, as other biological factors (co-activators) could be present in an *in vivo* situation.

In addition, wound healing capabilities of ATRA were investigated on an *in vitro* co-culture system of HaCaT and HDF cell lines which was used as a mimic of intact skin. It was envisaged that the biological functions during wound healing in skin as they relate to or are affected by ATRA could be monitored more effectively in an *in*

*vitro* condition using a co-culture (Maas-Szabowski *et al.*, 2004). It was envisaged also that the co-culture system could possibly provide data on the effect of ATRA on wound healing which could be comparable to 'intact' skin as described in the literature.

It is worthy to state that no completely functional *in vitro* skin model that truly represents the anatomical, physiological and biological stability of skin has been reported (Lim *et al.*, 2002; Suhonen *et al.*, 2003; Maas-Szabowski *et al.*, 2004; Metcalfe and Ferguson, 2006). Boyce and Waden (2002) in a review of principles and practices for the treatment of wounds with cultured skin substitutes, emphasized the importance of a complete 'recapitulation of ontogenesis' of skin. While complete regeneration is important for a functional skin, this recreation is yet to be achieved under *in vitro* conditions (Boyce and Waden, 2002). In addition, vascularization has never been achieved in an *in vitro* skin model (Metcalfe and Ferguson, 2006). Thus, an organotypic culture with a developed permeability barrier cannot be compared to a more complex and highly interactive basement membrane as found in healthy skin (Lim *et al.*, 2002; Suhonen *et al.*, 2003; Maas-Szabowski *et al.*, 2004; Metcalfe and Ferguson, 2006). Instead, attempts at developing a functional skin are directed towards mimicking the conditions found in normal skin, such as the presence of an extracellular matrix and the presence of two or more types of skin cells which basically mimic the cellular interactions of skin (Lim *et al.*, 2002; Suhonen *et al.*, 2003; Metcalfe and Ferguson, 2006).

*In vitro* studies of the skin have been attempted with the use of the different types of skin cells cultured alone, for example: fibroblasts (Lee *et al.*, 1998; Ohgoda *et al.*, 1998; Dewor *et al.*, 2007) or keratinocytes (Weninger *et al.*, 1998; Rendl *et al.*, 2002; Eckhart *et al.*, 2008). In addition, *in vitro* studies of skin have been conducted on co-cultures of keratinocytes and fibroblasts without an extracellular matrix (Lee *et al.*, 2006) or with an extracellular matrix (Maas-Szabowski *et al.*, 2004; Lee *et al.*, 2005). There are reports in the literature that the dermis of skin consists predominantly of fibroblasts which are responsible for synthesizing collagen fibres for repair of the dermis (Singer and Clark, 1999; Moulin *et al.*, 2000; Kanitakis, 2002; Lee *et al.*, 2006; Metcalfe and Ferguson, 2006) as well as secreting growth factors which are stimulatory to epithelial proliferation and cell motility (Lawrence and Diegelmann, 1994; Goulet *et al.*, 1996; Singer and Clark, 1999; Lee *et al.*, 2006) during wound healing.

In order to assess the effect of ATRA on 'wound' closure *in vitro*, the study aimed to:

i. determine the effect of ATRA on cell proliferation of a HaCaT cell line and a HDF cell line

ii. evaluate the effect of ATRA on cell migration during 'wound' closure in the HaCaT cultures and in the co-cultures of HaCaT and HDF cell lines, and

iii. investigate the effect of ATRA on cell proliferation during 'wound' closure in the HaCaT cultures and in the co-cultures of HaCaT and HDF cell lines.

# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 Culturing of cell lines

Two cell lines were used in the experiments:

a. an immortalized non-tumorigenic human keratinocyte (HaCaT) cell line derived from normal adult human trunk skin (Boukamp *et al.*, 1988). These cells are capable of stratification (Maas-Szabowski, *et al.*, 2004; Deyrieux and Wilson, 2007). The cell line was donated by the German Cancer Research Centre. The HaCaT cell line was seeded into a 75 ml Nunc® culture flask and maintained in Dulbecco's Modified Eagle's medium (DMEM) (Lonza, South Africa) supplemented with 10% fetal bovine serum (FBS) (Lonza, South Africa) and a 0.1% mixture of streptomycin and penicillin in phosphate buffered saline (PBS) (Sigma, South Africa) at 37ºC in a 5% $CO_2$ in air humidified incubator. The culture medium was changed every two to three days as necessary.

b. a human dermal fibroblast (HDF) cell line derived from adult skin was obtained from American Type Culture Collection, ATCC (CCD-1140Sk). The HDF cell line was seeded into a 25 ml Nunc® culture flask. The cultures of the HDF cell line were maintained in Iscove's Modified Dulbecco's Medium

(IMDM) (Lonza, South Africa) supplemented with 10% FBS and a 0.1% mixture of streptomycin and penicillin in PBS at 37ºC in a 5% $CO_2$ in air humidified incubator. The culture medium was changed every four to five days as required.

The cultures of the HaCaT and the HDF cell lines were allowed to reach 80-90% confluence before sub-culturing. Unlike the HaCaT cell line which is theoretically capable of unlimited growth and number of passages, the HDF cell line is only capable of undergoing about 42 population doublings (ATCC technical Bulletin, 2007) without phenotypic changes. The HDF cell line was supplied at passage two and in this study, was used at the fourth to seventh passage.

### 2.2 *All-trans* retinoic acid (ATRA)

ATRA ($C_{20}H_{26}O_2$) is a yellow powder (molecular weight of 300.44) supplied in a light protected vial (Sigma, South Africa). It was stored at -20ºC. According to the accompanying Sigma product information sheet (Product no: R2625), the ATRA powder is virtually insoluble in water, only slightly soluble in alcohol but highly soluble in dimethylsulphoxide (DMSO). DMSO is thus often used as a diluent for ATRA (Muehlberger *et al.*, 2005; Bianchi *et al.,* 2008; Eckhart, *et al.*, 2008). For this study, a stock solution of ATRA (0.0667M) was obtained by dissolving 100mg ATRA powder in 5ml 100% DMSO (Appendix I). The ATRA stock solution was stored in a light protected vial at -4ºC because of its high sensitivity to ultra-violet light, heat and air, especially when in solution (Sigma product information, product no R2625). Serial dilutions of ATRA were prepared fresh under subdued light.

In the present study, ATRA at a concentration of 1μM was used in all the experiments as it is regarded as a 'physiologically relevant' concentration at which terminal differentiation of keratinocytes is inhibited (Jetten, 1990; Fisher and Voorhees, 1996; Rendl *et al.*, 2002; Eckhart *et al.*, 2008). This concentration is also frequently utilized in several studies on skin cell types such as keratinocytes, fibroblasts and melanocytes (Tsou *et al.*, 1994; Weninger *et al.*, 1998; Diaz *et al.,* 2000; Lee *et al.*, 2009). The concentration of DMSO (vehicle control) used was 0.0015% which is equivalent to the DMSO concentration in 1μM ATRA (Appendix I). In this study, except where stated otherwise, 'ATRA treatment' refers to 1μM ATRA (reconstituted in DMSO) in DMEM. Controls utilizing DMSO in DMEM or DMEM alone will be referred to as 'vehicle control' or 'untreated control' respectively. All the medium for the ATRA treatment, vehicle control and the untreated control were supplemented with 10% FBS.

### 2.3 Cell proliferation assay

The cell proliferative effect of ATRA at a concentration of 1μM was investigated on the HaCaT and the HDF cell lines as described in section 2.3.1.

#### 2.3.1 Preparation of cultures for ATRA treatment and evaluation of cell proliferative activity

In this analysis, the culturing and the treatment of the HaCaT cell line was performed in DMEM while culturing and treatment of the HDF cell line was done in IMDM. The experiments utilizing each of these two cell lines were performed in

separate 96-well microplates in triplicate. For each of these cell lines, one 96-well microplate was prepared for the ATRA treatment and the vehicle control. The wells at the periphery of the 96-well microplate contained only sterile PBS in order to prevent drying out of the content of the wells in the plate. The HaCaT or HDF cells were counted using a haemocytometer (Appendix II) and seeded at a concentration of $1X10^4$ cells per ml per well into all wells of the plate except for one column which served as the blank for spectrophotometry. The blank served as the control for the colour intensity of the medium used and contained only media (DMEM or IMDM supplemented with 10% FBS) and no cells.

The medium for the untreated control cultures was DMEM or IMDM alone. The medium for the treated cultures was ATRA at a concentration of 1µM (Appendix I) while in the medium for the vehicle control cultures contained 0.0015% DMSO in DMEM or IMDM. The solution in each of the wells in the microplate had a final volume of 100µl. The cultures were incubated at 37ºC in a 5% $CO_2$ in air humidified incubator for 24 hours. The experiment was repeated and thus the total numbers of samples for the ATRA treatment, the vehicle control and the untreated control (DMEM or IMDM alone) were six samples each.

### 2.3.2 Evaluation of cell proliferation using the CellTiter 96 Aqueous® reagent

CellTiter 96® aqueous reagent is a 'one solution' reagent used as a cell proliferation assay. It utilizes a colorimetric method for determining the number of viable proliferating cells or of evaluating the cytotoxicity of agents in monolayer cultures (Promega technical bulletin: Part no TB245). The CellTiter 96® aqueous reagent

contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and an electron coupling reagent, phenazine ethosulfate (PES). The PES enhances chemical stability by allowing it to form a stable solution with the tetrazolium compound (Promega technical bulletin: Part no TB245). This tetrazolium compound is biologically reduced by proliferating cells into a coloured soluble formazan product in tissue culture media (Promega technical bulletin: Part no TB245).

After treating the cells for 24 hours as described in section 2.3.1, 20µl of CellTiter 96 Aqueous® reagent was added to each well. The cultures were incubated at 37ºC in a 5% $CO_2$ in air humidified incubator for three hours. According to the manufacturer's information, the colour intensity of the solution in each well should indicate the amount of soluble formazan product produced by the cellular reduction of the tetrazolium compound. Following incubation, absorbance was measured at 490nm (Promega technical bulletin: Part no TB245) with an Anthos 2010 spectrophotometer.

The average absorbance value of the blank control was subtracted from the individual absorbance readings. A graph of the absorbance readings, which gives an indication of cell proliferation expressed as a percentage of the untreated control, for both the HaCaT and the HDF cell line was plotted against the ATRA treatment and the vehicle control. Evaluation of a statistically significant difference in cell proliferation in the three treatment groups (ATRA treatment, vehicle control and untreated control) was performed using a Kruskal-Wallis one way analysis of

variance (ANOVA) test (a non-parametric test for three or more independent samples) at a significant level of 5%.

### 2.4. Scratch assay

In an *in-vitro* study of the factors influencing the healing of a wound, a scratch assay is often used (Conant *et al.*, 2010). The effect of a therapeutic agent on cell motility or cell proliferation, as well as molecular activities such as cell signaling, the inflammatory response and tissue remodeling, which are associated with wound healing, can be monitored using a scratch assay (Conant *et al.*, 2010). The 'wound' is made by drawing the tip of a pipette or pin through a confluent culture (Buth *et al.*, 2007; Dewor *et al.*, 2007; Cheng *et al.*, 2008; Fronza *et al.*, 2009). This deliberate damage to the monolayer of a confluent cell culture leaves a cell-free region ('wound') within the culture (Conant *et al.*, 2010). The 'wound' that is created by using this method mimics to a certain extent, the biological phenomena of an injury to normal human skin (Yarrow *et al.,* 2004; Conant *et al.*, 2010).

#### 2.4.1 Preparation of HaCaT cultures for scratch 'wound' assay and ATRA treatment

The HaCaT cell line was seeded at a concentration of $1X10^6$ cells per ml in a 6-well culture plate. Two 6-well culture plates were designated for each of the three treatment groups (ATRA treatment, vehicle control and untreated control). The cultures were incubated at 37ºC in a 5% $CO_2$ in air humidified incubator until they had reached 100% confluence usually in five days following seeding. Prior to

seeding of the cells, two parallel lines (5mm apart) were drawn on the undersurface of the plastic well at the centre of the wells using a waterproof marker (Liang *et al.*, 2007). This was used to demarcate the region at which repeated photographs would be taken at different time periods.

On reaching confluence, the wells were washed with calcium and magnesium-free PBS (CMF-PBS) (Appendix III). The CMF-PBS was used to reduce cell membrane adhesion (Buth *et al.*, 2007). After the CMF-PBS was aspirated, the cultures were 'wounded' by making a scratch at right angles to the marked parallel lines in each well with the tip of a 200µl pipette tip held in an upright position. The wells were washed again after scratching with CMF-PBS in order to remove dislodged cells. Then, 2ml of either 1µM ATRA treated medium (n=12), untreated control medium (n=12) or vehicle control medium (n=12) were added into the respective wells of the culture plates.

### 2.4.2 Preparation of co-cultures for scratch 'wound' assay and ATRA treatment

Two 6-well culture plates were designated for each of the three treatment groups (ATRA treatment, vehicle control and untreated control). Prior to seeding of the cells, the demarcated region on the 6-well culture plates were prepared as described in section 2.4.1. Initially, the HDF cell line was seeded at a concentration of 1 X $10^4$ cells per ml of IMDM supplemented with 10% FBS into a 6-well culture plate and incubated at 37ºC in a 5% $CO_2$ in air humidified incubator for five days. After five days, the medium was aspirated and then the HaCaT cells, at a

concentration of 1 X $10^6$ cells per ml of DMEM supplemented with 10% FBS, was carefully seeded on to the HDF cultures and then incubated at 37ºC in a 5% $CO_2$ in air humidified incubator until both cell lines had reached 100% confluence. On reaching confluence, the co-cultures were 'wounded' as described in section 2.4.1. Then, 2ml of solution of either the 1μM ATRA treated medium (n=12), the vehicle control medium (n=12) or the untreated control medium (n=12) was added into the respective designated culture plates.

### 2.4.3. Analysis of 'wound' closures in the HaCaT cells and in the co-cultures

Following 'wounding', the demarcated region in each well was immediately photographed on an inverted phase contrast microscope with an attached Olympus digital camera. A diagrammatic representation of the wound region is depicted in Figure 1a. All the photographs for the scratch-wound analysis were taken at the same magnification, light intensity, camera resolution and camera zoom. The time period immediately after the scratching was designated as "0"-hour of wound closure. The photographs of the same region of each culture were taken at 0, 2, 4, 6, 8, 10 and 24 hours following 'wounding'.

The images thus obtained were analyzed by overlaying with a grid from ImageJ 1.42q software from National Institutes of Health (NIH) in the United States. The width of the scratch region was measured at five different regions along the length of the scratch as illustrated in figure 1b. These measurements were taken by tracing the margins of the scratch on the y-coordinate of the grid. In this way, the individual width of the cell-free region ('wound' closure) of the scratch in each of the cultures

was obtained (Appendix VI). The average width of the scratch at time 0 hour (X) and at each of experiment time points, *t* (2, 4, 6, 8, 10 and 24 hours) (Y) was also determined for each experimental group. The change in the width of the scratch was then calculated and compared (i.e. X-Y), as illustrated in Figure 1b, for each experimental group for both the HaCaT cell monolayers and the co-cultures. It is important to note that the actual margin of scratch was not straight (Fig. 1b). The change in the width of the scratch was used as an indication of 'cell migration' after the start of experiment under the different treatment regimes (Dewor *et al.*, 2007). However, the measurements actually reflect 'wound' closure which may be a combination of cell proliferation and migration (Dewor *et al.*, 2007).

A statistically significant difference in 'wound' closure in the three treatment groups (ATRA treatment, vehicle control and untreated control) at different time periods was evaluated using a Kruskal-Wallis one-way ANOVA test (a non-parametric test for three or more independent samples) at a significant level of 5%. A Mann-Whitney (a non-parametric test for two independent samples) was used to evaluate a statistical difference in wound closure between the HaCaT cultures and the co-cultures for either ATRA treatment or vehicle control or untreated control.

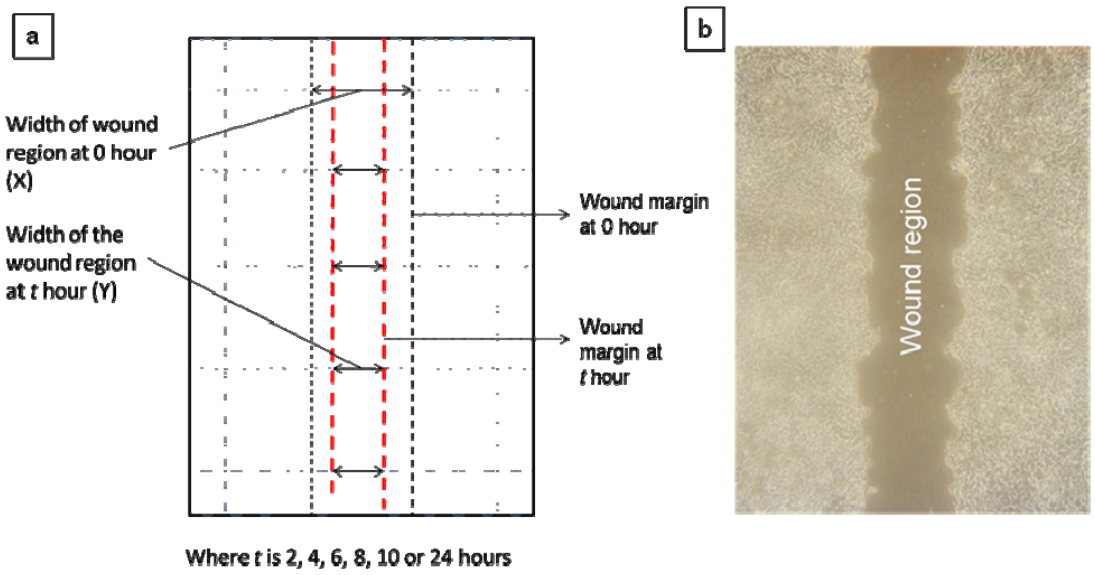



Figure 1: a) Diagrammatic illustrations of the scratch region at the time of scratching (0 hour) and different time periods (*t* hours) illustrating how the measurements of the width of the scratch at five different regions of the 'wound' were taken; and b) a representative photomicrograph of the scratch region at 8 hours following scratching illustrating the irregular margin of the scratch region. Phase contrast microscopy X4.

## 2.5 Ki-67 immunolabelling assays

Ki-67, a non-histone protein (395 kDa), is a nuclear antigen that is exclusively expressed in the proliferative stages (late G1, S1, G2 and M phases) of the cell cycle (Fisher *et al.*, 2002; Potemski *et al.*, 2006; Faratzis *et al.*, 2009). Ki-67 gene is located on chromosome 10. There is down-regulation of Ki-67 protein when cells exit the cell cycle which makes Ki-67 protein an excellent marker for proliferating cells (Fisher *et al.*, 2002; Potemski *et al.*, 2006; Faratzis *et al.*, 2009). For this reason, actively proliferating cells are immunolabelled or identified usually by their characteristic dark staining of the nucleus when a monoclonal antibody to Ki-67 is utilized (Fisher *et al.*, 2002).

### 2.5.1 Preparation of HaCaT cells for immunocytochemistry

Prior to the seeding of the HaCaT cell line, sterilized glass cover slip (22mm X 22mm) was placed in each of the wells of a 6-well culture plate. The cover slips were rinsed briefly with sterile PBS before the cells were seeded on to them at a concentration of $1X10^6$ cells per ml. A 6-well culture plate was designated each for 0 and 24 hour treatment durations. Only two wells in the 6-well culture plate designated for 0 hour contained the cultured HaCaT cells while the other wells contained PBS alone. For the culture plate designated for 24 hour treatment duration, two wells of the 6-well culture plate were assigned for each of the three treatment groups (ATRA treatment, vehicle control and untreated control). The experiment was repeated once. After seeding, the cells were incubated at 37ºC in a 5% $CO_2$ in air humidified incubator until they had reached 100% confluence.

On reaching confluence, the HaCaT cultures were 'wounded' as described in section 2.4.1. After 'wounding', the cells in the wells of the culture plate designated for 0 hour were immediately rinsed with PBS and the cells on the cover slips were fixed in 3ml of 3% formaldehyde in PBS. Into the wells of culture plate designated for 24 hour, 2 ml of solution of either the 1μM ATRA treated medium (n=4) or the untreated control medium (n=4) or the vehicle control medium (n=4) was added into each well.

At 24 hours, the liquid content of the wells was aspirated and 3ml of 3% formaldehyde in PBS was added into each well. The fixation was performed for 24 hours before processing for immunocytochemistry as described in section 2.5.3.

### 2.5.2 Preparation of cells in the co-cultures of HaCaT and HDF cells for immunocytochemistry

A 6-well culture plate containing sterilized cover slips was designated each for 0 and 24 hour treatment durations as described in section 2.5.1. The HDF cells were initially seeded on to the sterilized cover slips at a concentration of 1 X $10^4$ cells per ml of IMDM supplemented in 10% FBS and incubated at 37ºC in a 5% $CO_2$ in air humidified incubator for five days. Following this, the medium was aspirated and then the HaCaT cells, at a concentration of 1X$10^6$ cells per ml of DMEM supplemented with 10% FBS, were carefully seeded on to the HDF cell line. The co-cultures of HaCaT and HDF cells were further incubated at 37ºC in a 5% $CO_2$ in air humidified incubator until they had reached 100% confluence.

After reaching confluence, the cells in the culture plates designated for the 0 and 24 hour were wounded as described in section 2.5.1. After 'wounding', the cells in the wells of the culture plate designated for 0 hour were immediately washed in sterile PBS before fixing in 3ml of 3% formaldehyde in PBS. Then, 2ml of solution of either the 1μM ATRA treated medium (n=4), the vehicle control medium (n=4) or the untreated control medium (n=4) was added into each well of the culture plate. Following 24 hours of incubation, the liquid content of the wells was removed and 3ml of 3% formaldehyde in PBS was added to each well in order to fix the cells. The cells were fixed for 24 hours before processing for immunocytochemistry as described in section 2.5.3.

### 2.5.3 Ki-67 immunolabelling using the Avidin-Biotin method

The Ki-67 immunolabelling for the HaCaT cell line and the co-cultures were carried out by adding the different reagents for immunocytochemistry (Appendix IV) directly into each of the wells containing the fixed cultures. After fixing for 24 hours, the fixative was aspirated and the wells were washed once with Tris-Buffered Saline (TBS). The TBS was completely aspirated and the cells permeabilized with 0.2% Triton X-100 (from stock of 20% Triton in PBS) for five minutes at room temperature before quenching in fresh 0.1% sodium borohydride in TBS for another five minutes.

The solution was replaced with TBS before 1ml of blocking buffer [5.5% normal goat serum in TBS/Triton (0.1% Triton X-100 in TBS)] was added to each well for one hour at room temperature. The cells were washed once again with 1ml TBS and then aspirated. The cells were incubated overnight at 4ºC with a mouse monoclonal

anti-proliferating cell protein Ki-67 primary antibody (Sigma, South Africa) in 3% bovine serum albumin in TBS (1:800). In the wells for the negative control, the Ki-67 antibody was omitted and TBS alone was added.

After the overnight incubation, the cells were washed twice for five minutes each with 1ml TBS/Triton and then washed once with TBS. The cells were subsequently incubated with a polyclonal goat anti-mouse biotinylated secondary antibody (Dako, South Africa) diluted in 3% bovine serum albumin in TBS (1:500) for one hour at room temperature. This solution was aspirated, and the cells washed three times for five minutes each with 1ml TBS/Triton and then once for five minutes with 1ml TBS. Following this, the cultures were incubated for exactly 30 minutes in 0.6% hydrogen peroxide in TBS at room temperature. The solution was aspirated, and the cells were once again washed three times for five minutes each with 1ml TBS/Triton. Further washing in one change of TBS was carried out before the cells were incubated for one hour with 800µl Vectastain ABC Kit (Dako, South Africa) at room temperature.

The solution was completely aspirated, and the cells washed twice for five minutes each with 1ml TBS. Then, 1ml diaminobenzidine tetrahydrochloride (DAB) reagent (Sigma, South Africa) was added into each well. This reaction was monitored using a microscope in order to reduce background effect. The reaction was terminated by quickly adding exactly 1ml distilled $H_2O$ into each well. This solution was aspirated and the cells washed once again with 1ml distilled $H_2O$. The cover slips were dipped twice each in a graded series of alcohol and then in two changes of xylene before

being mounted with Entellan (Merck, South Africa) on to glass slides (1mm X 76mm X 26mm).

### 2.5.4 Estimation of the number of Ki-67 positively immunolabelled nuclei around the 'wound' regions

The estimation of the number of the Ki-67 positively immunolabelled nuclei in both the HaCaT cultures and the co-cultures for 0 hour and 24 hour (for the ATRA treatment, untreated control and vehicle control) treatment durations was determined by counting these actively proliferating nuclei located around the 'wound' region (Dewor *et al.*, 2007). For ease of counting and validation of the counted cells, five photographs were taken in series at every 3-mm interval around the margin of the 'wound' region with X10 objective of a Zeiss inverted microscope attached to a digital camera. Thus the area of field was standard in all the photographed regions. The regular interval was achieved by moving the calibrated stage of the microscope.

A total of 10 photographs (five photographs from each side of the wound) were taken per culture. The Ki-67 positively immunolabelled nuclei on each of the photographs were manually counted with the use of a grid and a cell counter using the ImageJ software from the NIH in the United States. The actively proliferating nuclei in the photographs were counted again after a two week interval to obviate bias.

The estimated number of the Ki-67 positively immunolabelled nuclei at 0 hour and 24 hours (for the ATRA treatment, the untreated control and the vehicle control) for both the HaCaT cultures and the co-culture were compared. Initially, a Mann-Whitney test (a non-parametric test for two independent samples) was used to evaluate the significant difference between the two independent counts of the Ki-67 positively immunolabelled nuclei for each sample. Then, a Kruskal-Wallis one way ANOVA test (a non-parametric test for three or more independent samples) was performed to evaluate whether a statistically significant difference occurred in the number of actively proliferating cells between the groups of data (0 hour and 24 hours: ATRA treatment, untreated control, vehicle control). A significance level of 5% was used as an indicator of significance difference for both statistical analyses.

# CHAPTER THREE

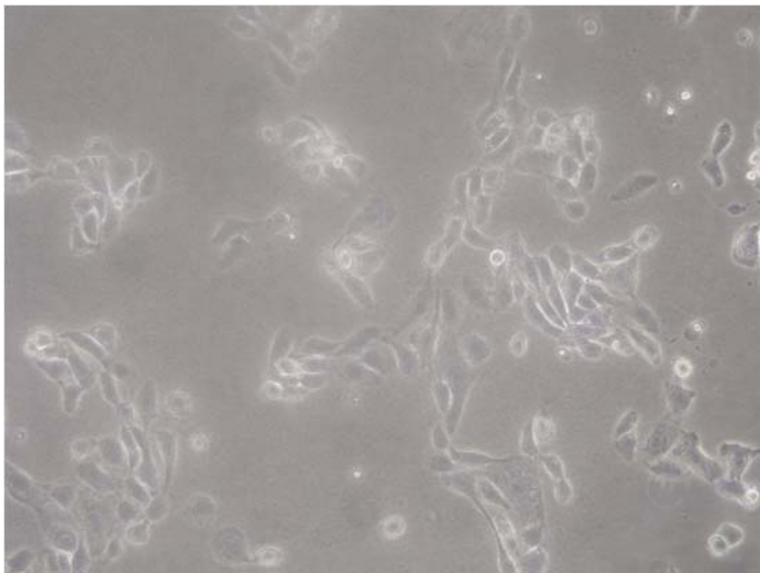
# RESULTS

## 3.1 General observations of the cultures

The HaCaT cells were polygonal in shape (Fig. 2a). They attached within 24 hours of seeding. Similar to the HaCaT cells, the HDF cells also attached within 24 hours. The HDF cells were spindle shaped and did not exhibit any obvious characteristics of senescence such as increased cytoplasmic processes and slowed or halted cell growth (Fig. 2b).

In the co-cultures of the HaCaT and HDF cell lines, the HDF cells were visible prior to confluence (Fig. 3 and 4a). The co-cultures usually reached confluence on day five after seeding the HaCaT cells on to the cultures of HDF cells. However, when the HaCaT cells or the HDF cells were cultured alone, confluence was usually reached at day five and day eight respectively after seeding. In the confluent co-culture, the HaCaT cells grew over the HDF cells and thus the HDF cells were no longer easily visible (Fig. 4b).

a)

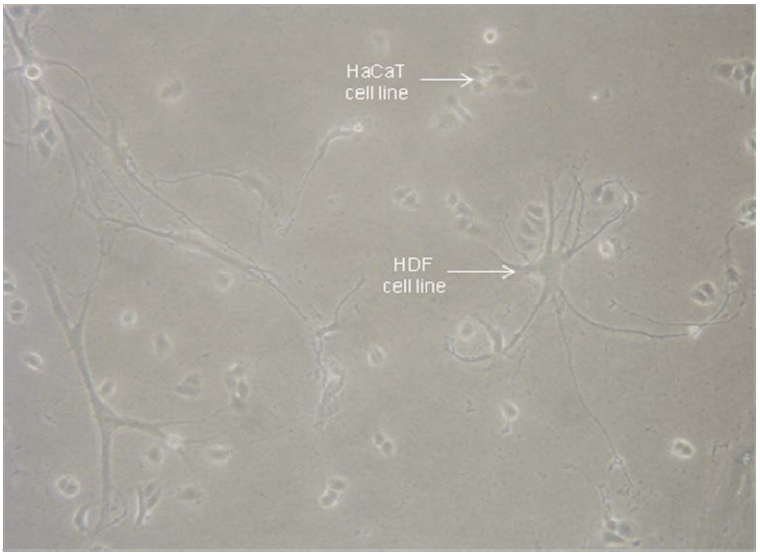


b)



Figure 2: Representative photomicrographs of cultures of a) HaCaT and b) HDF cell lines after 72 hours of seeding. Phase contrast microscopy, X10.

a)




b)

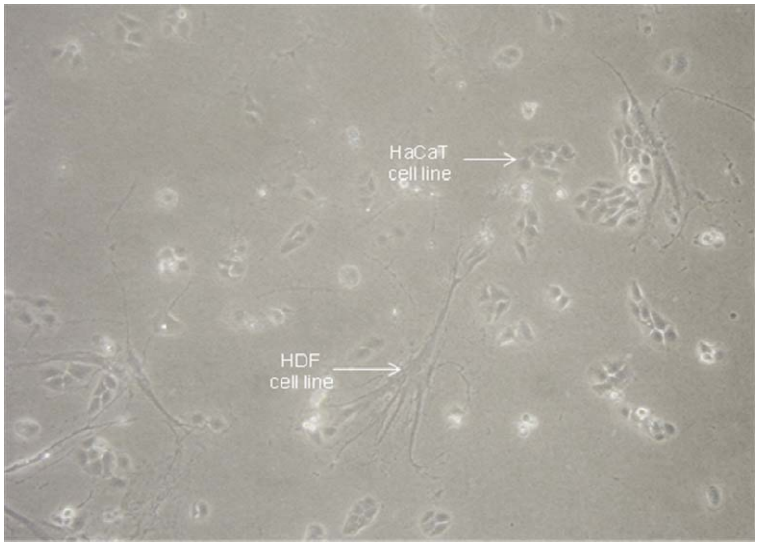
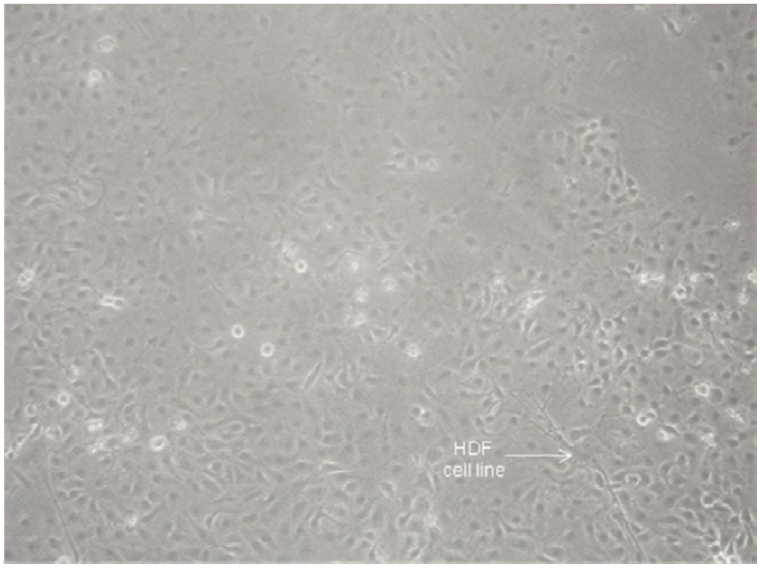



Figure 3: Representative photomicrographs of a co-culture of HaCaT and HDF cells on the a) first and b) second day after seeding. Phase contrast microscopy, X10.

a)




b)

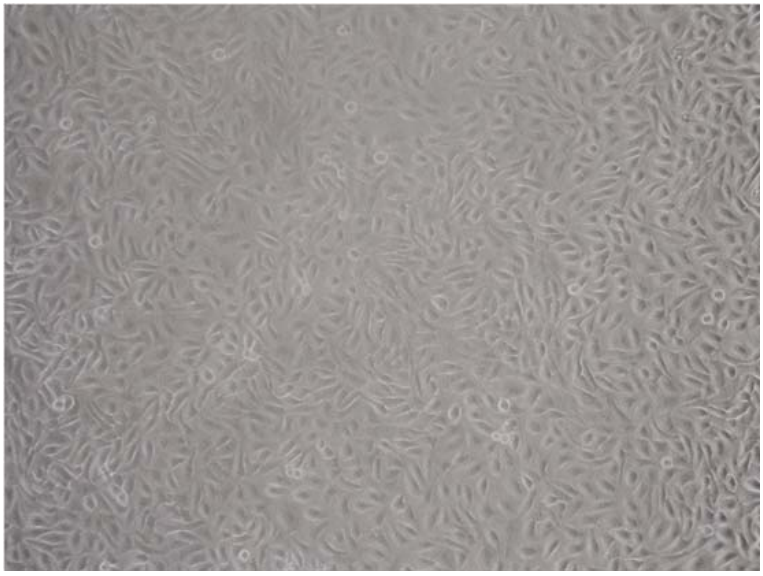


Figure 4: Representative photomicrographs of a co-culture of HaCaT and HDF cells on the a) fourth day after seeding and b) at confluence in which the HDF cells were no longer easily visible in the co-culture. Phase contrast microscopy, X10

### 3.2 Effect of ATRA on proliferation of the HaCaT and the HDF cell lines

Cell proliferation analysis using the colorimetric assay on the HaCaT cell cultures showed no significant difference in cell proliferation of the cultures treated with 1μM ATRA compared to the untreated control cultures ($p=0.2980$) or the vehicle control cultures ($p=0.1735$) (Fig. 5). There was similarly no significant difference in cell proliferation when the vehicle control cultures were compared with the untreated control cultures ($p=0.8102$) (Appendix V).

In the HDF cell cultures (Fig. 6), cell proliferation was decreased in the cultures treated with ATRA but this decrease was not significantly different when compared with the untreated controls ($p=0.1495$). However, both the HDF cultures treated with ATRA and the untreated control cultures showed a significant increase in cell proliferation when compared to the vehicle control cultures ($p=0.0051$ and $p=0.0051$ respectively).

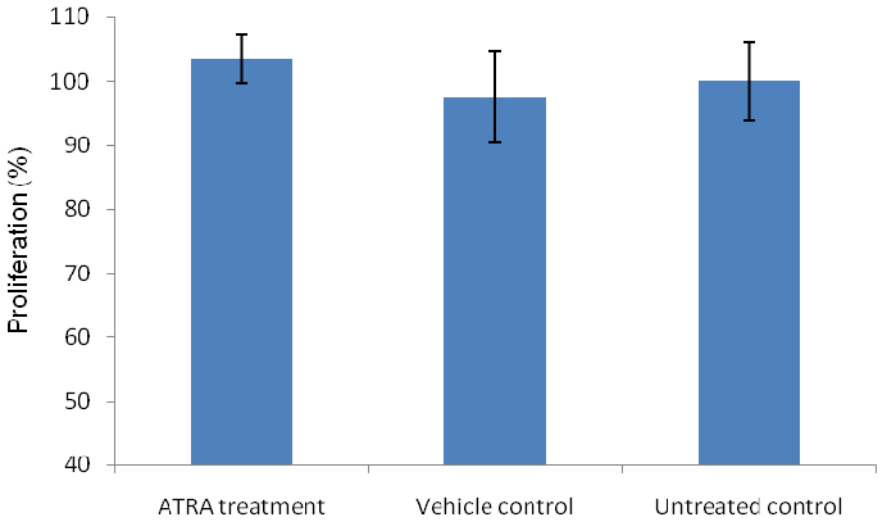



Figure 5: A graphical illustration of the relationships of ATRA treatment, DMSO (vehicle control) and DMEM (untreated control) on cell proliferation of the HaCaT cell cultures. There was no statistically significant difference in cell proliferation when the three treatment groups were compared (n=6).

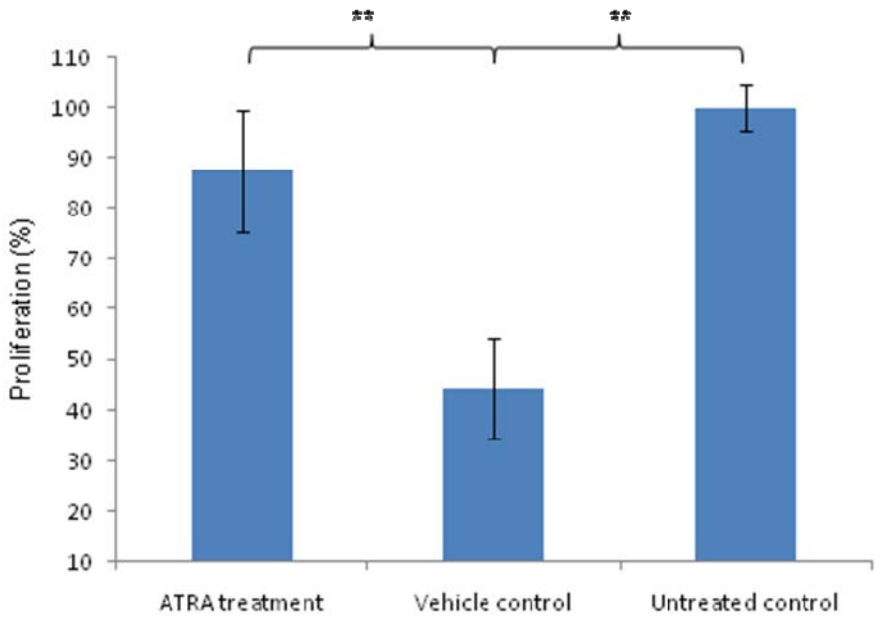
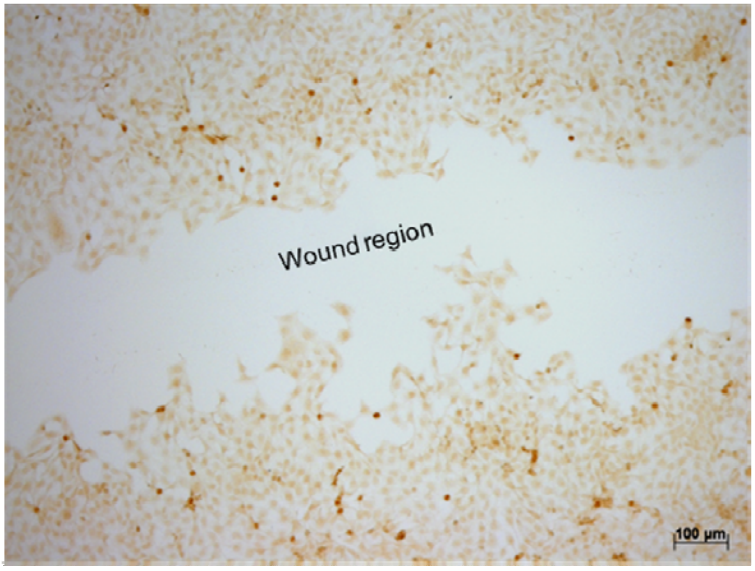



Figure 6: A graphical illustration of the relationships of ATRA treatment, DMSO (vehicle control) and DMEM (untreated control) on cell proliferation of the HDF cell cultures. There was a significant difference in cell proliferation when ATRA treatment and vehicle control or vehicle control and untreated controls were compared (n=6, **p<0.01).

### 3.3 General observations in the ‘wounded’ cultures

The score regions of the HaCaT cultures and the co-cultures were relatively clear of any cellular debris, following scratching. Score closure in the HaCaT cultures and in the co-cultures occurred progressively. The closure of the ‘wound’ appeared to be the result of migration of cells at the score edge and the proliferation of cells around the ‘wound’ region (Fig. 7). Representative photomicrographs of the ‘wounded’ regions in the HaCaT cultures and the co-cultures for the ATRA treatment, the untreated control and the vehicle control at different time periods are shown in figures 8 – 13.

a)




b)

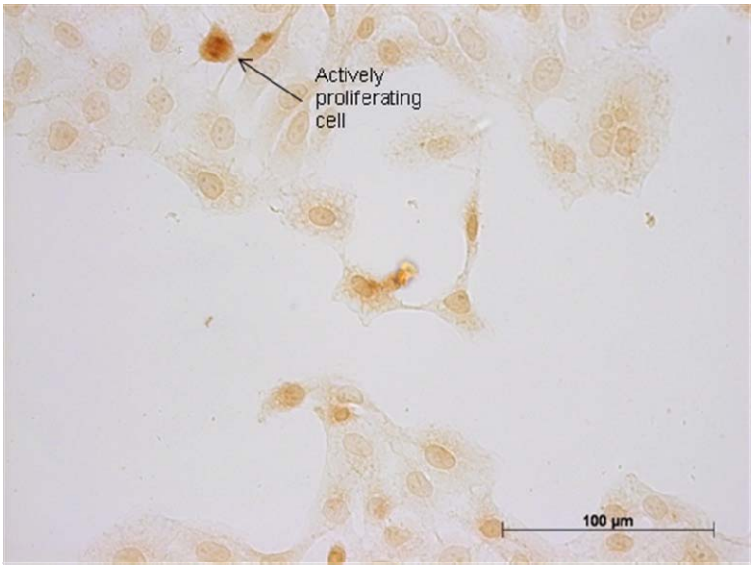



Figure 7: Representative photomicrographs showing ‘wound’ closures in a HaCaT culture immunolabelled with Ki-67 primary antibody. Phase contrast microscopy, a) X10 b) X40.

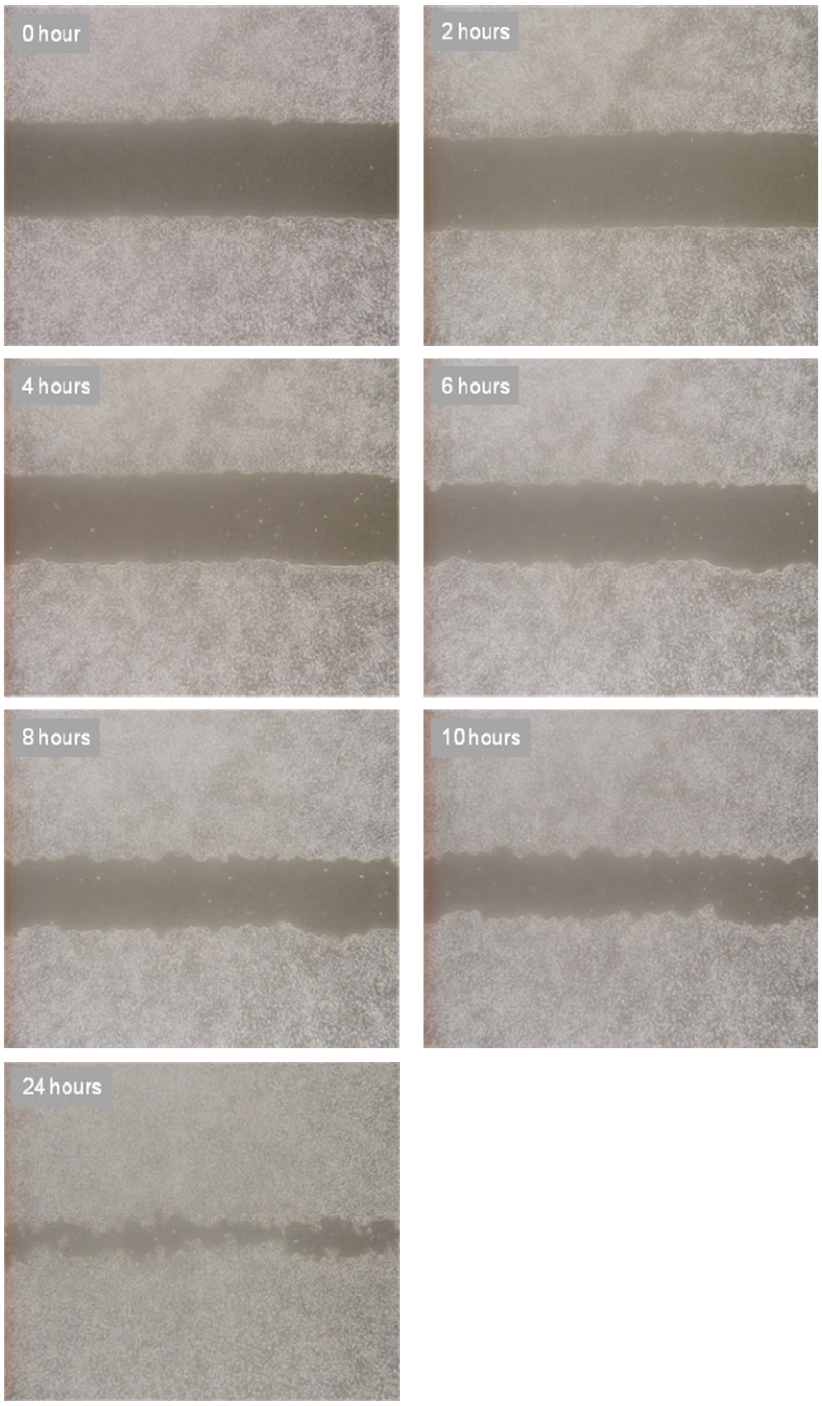



Figure 8: Representative photomicrographs of the same ‘wounded’ region of a HaCaT culture with ATRA treatment at different time periods. Phase contrast microscopy, X4.

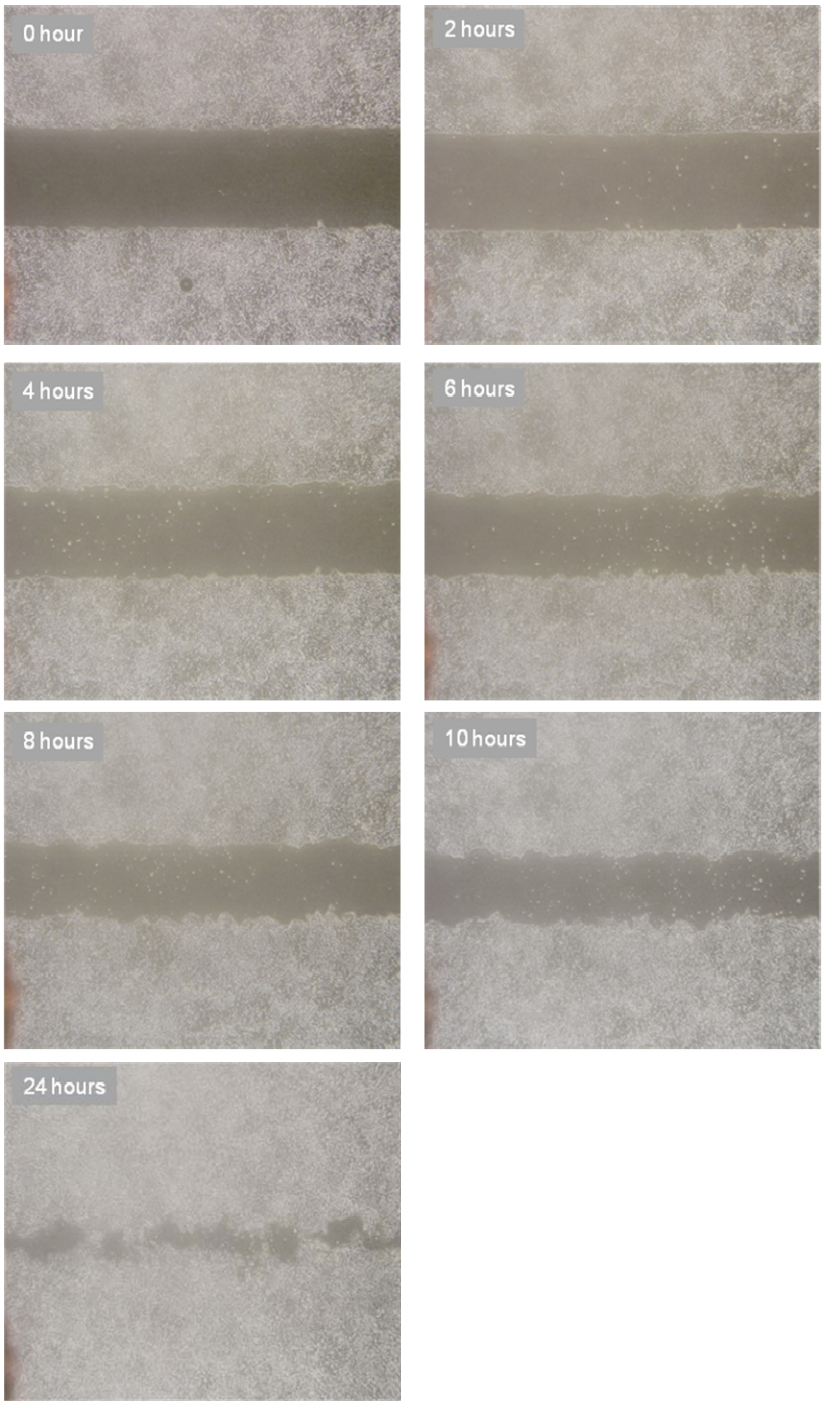



Figure 9: Representative photomicrographs of the same ‘wounded’ region of an untreated HaCaT culture at different time periods. Phase contrast microscopy, X4.

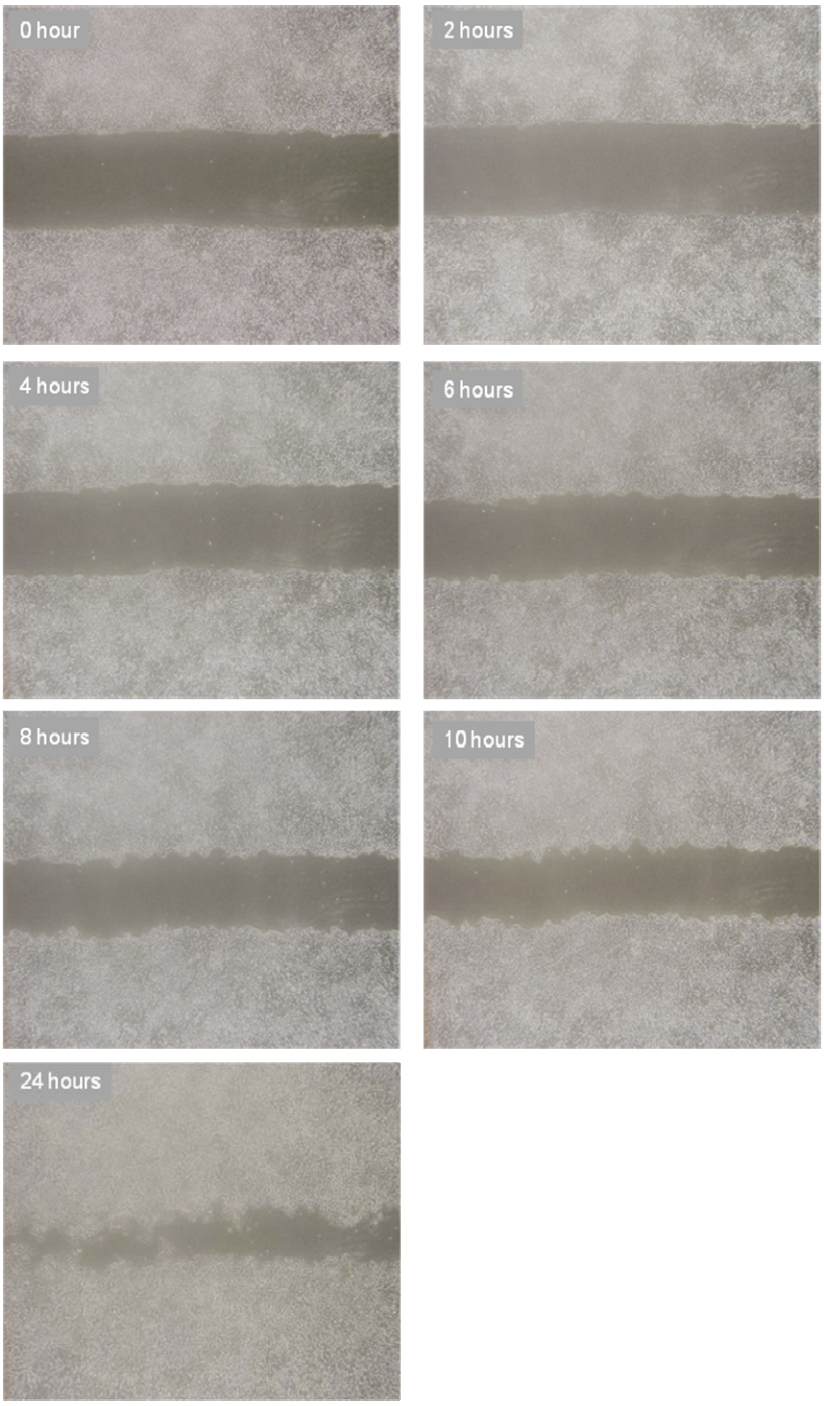



Figure 10: Representative photomicrographs of the same ‘wounded’ region of a HaCaT culture grown in vehicle control medium at different time periods. Phase contrast microscopy, X4.

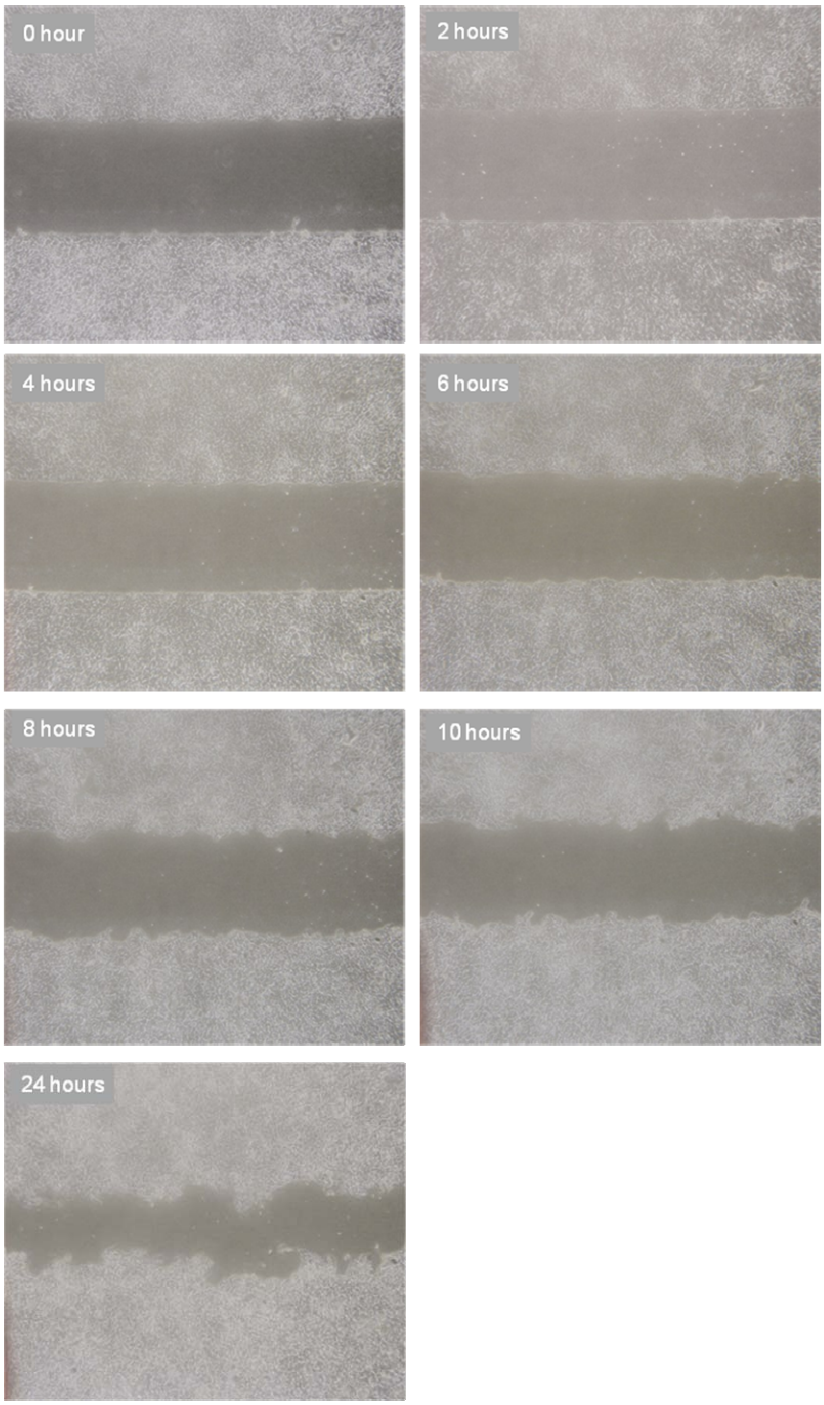



Figure 11: Representative photomicrographs of the same ‘wounded’ region of a co-culture of HaCaT and HDF cells with ATRA treatment at different time periods. Phase contrast microscopy, X4.

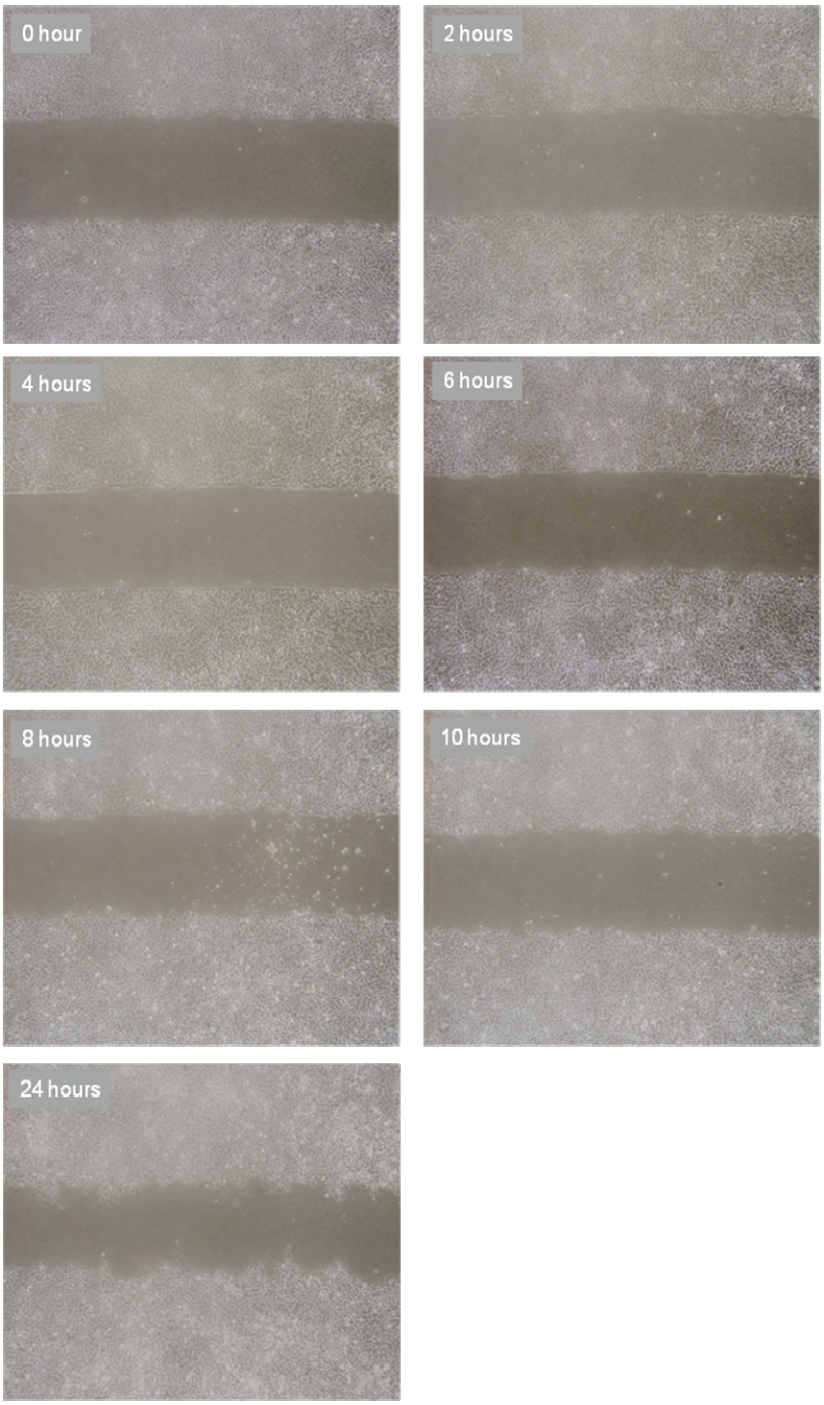



Figure 12: Representative photomicrographs of the same ‘wounded’ region of an untreated co-culture of HaCaT and HDF cells at different time periods. Phase contrast microscopy, X4.

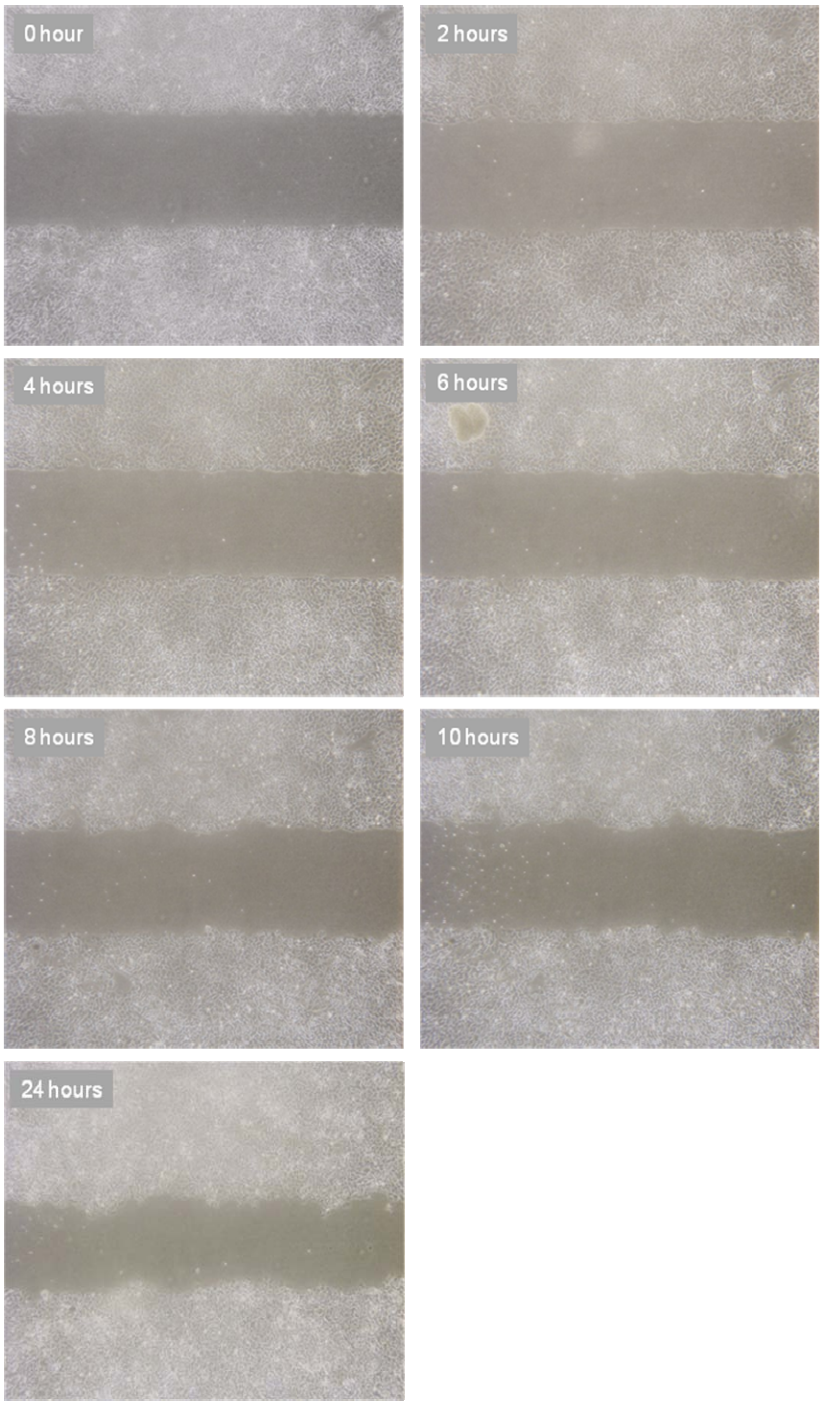



Figure 13: Representative photomicrographs of the same ‘wounded’ region of a co-culture of HaCaT and HDF cells grown in vehicle control medium at different time periods. Phase contrast microscopy, X4.

### 3.4 Effect of ATRA on ‘wound’ closure

#### HaCaT cultures

The ‘wounds’ treated with ATRA were reduced in width compared to those in the untreated control cultures and the vehicle control cultures at the same time periods i.e. 6 to 24 hours, while ‘wound’ closure was increased in the untreated control cultures compared to the vehicle control cultures (Fig. 14). There was no statistically significant difference in score closure when the cultures treated with ATRA or the untreated controls or the vehicle controls were compared at different time periods after the start of score closure (Appendix VI). However by 10 hours and 24 hours respectively (Fig. 14), ‘wound’ closure was significantly increased in the cultures treated with ATRA when compared to the vehicle control cultures (10 hours: $p=0.0166$; 24 hours: $p=0.0404$).

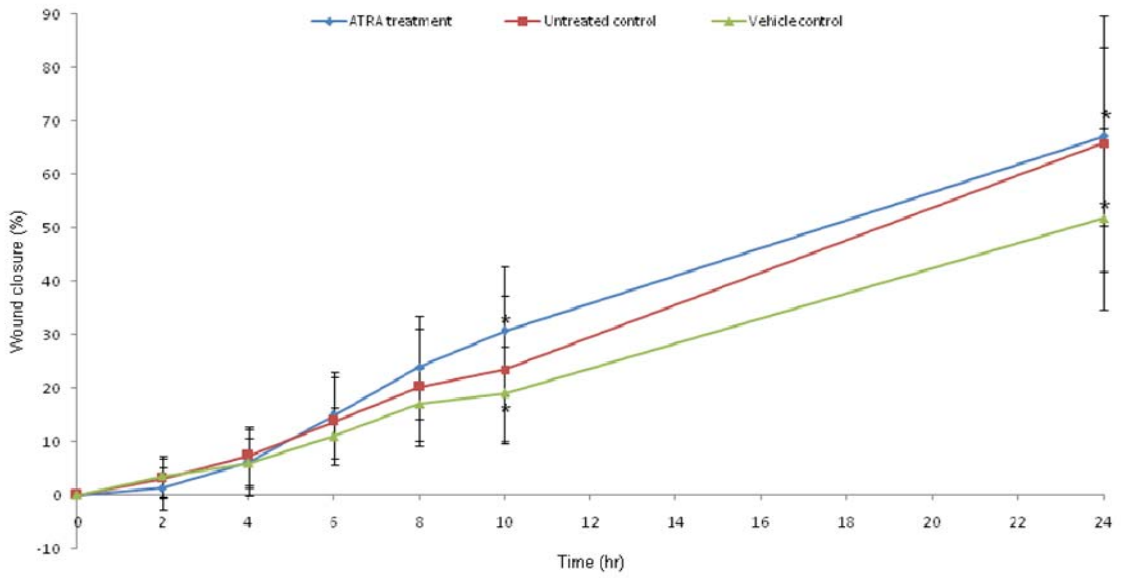



Figure 14: A graphical illustration of the effects of ATRA treatment, DMSO (vehicle control) and DMEM (untreated control) on 'wound' closure in the HaCaT cultures at different time periods. By 10 and 24 hours after 'wounding', 'wound' closure was significantly different when the ATRA treatment and the vehicle control were compared (n=12, *p<0.05).

**Co-cultures**

Similar to ‘wound’ closure in the HaCaT cultures, the ‘wounds’ treated with ATRA were reduced in width compared to those in the untreated control cultures and the vehicle control cultures at the same time periods i.e. 4 to 24 hours (Fig. 15). At the different time periods after the start of score closure, there was no statistically significant difference in ‘wound’ closure when the co-cultures with ATRA treatment or the untreated co-cultures or the co-cultures grown in the vehicle control medium were compared (Appendix VI). By 24 hours however, score closure was significantly increased in the co-cultures treated with ATRA when compared to the co-cultures grown in vehicle control medium ($p=0.0226$).

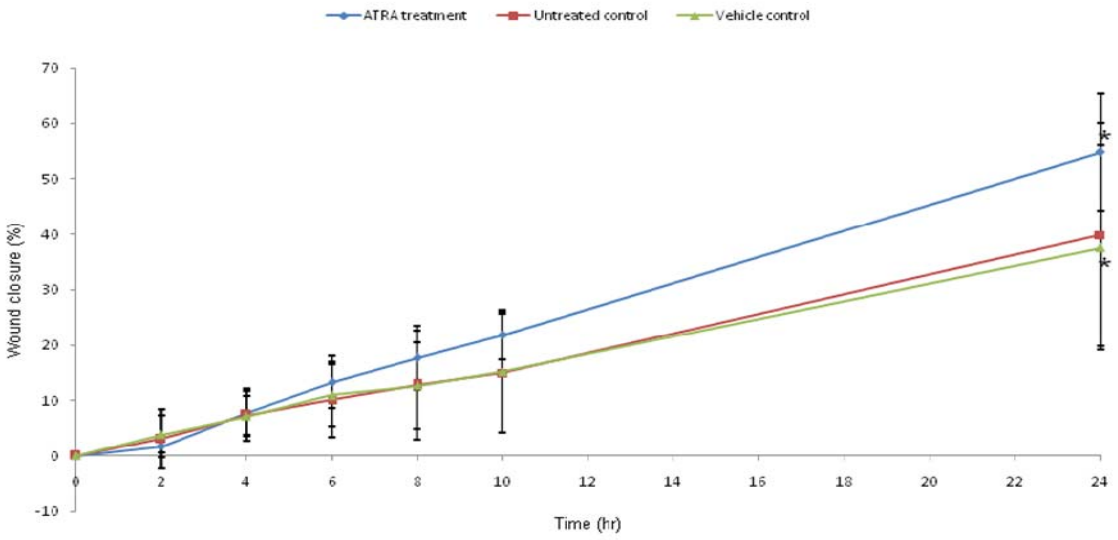



Figure 15: A graphical illustration of the effects of ATRA treatment, DMSO (vehicle control) and DMEM (untreated control) on 'wound' closure in the co-cultures of HaCaT and HDF cells at different time periods. By 24 hours after 'wounding', 'wound' closure was significantly different when the ATRA treatment and the vehicle control were compared (n=12, *$p<0.05$).

### 3.4.1 Comparison of 'wound' closure in the HaCaT cultures with that in the co-cultures

In all the treatment groups (ATRA treatment, untreated control or vehicle control), 'wound' closure appeared to be more pronounced in the HaCaT cultures than in the co-cultures. In the cultures treated with ATRA (Fig. 16), score closure was significantly increased in the HaCaT cultures when compared to the co-cultures ($p=0.0173$) by 10 hours post-wounding. By 24 hours post-wounding however, there was a slight increase in score closure in the HaCaT cultures, which was not significantly different from the co-cultures ($p=0.0519$) (Appendix VII).

In the untreated cultures (Fig. 17), 'wound' closure in the HaCaT cultures was significantly increased when compared to that of the co-cultures ($p=0.0173$) by 24 hours post-wounding. In contrast, in the experiments using the vehicle control medium, there was an increase in score closure in the HaCaT cultures, but this was not significantly different when compared to that of the co-cultures over all experiment time points (Fig. 18).

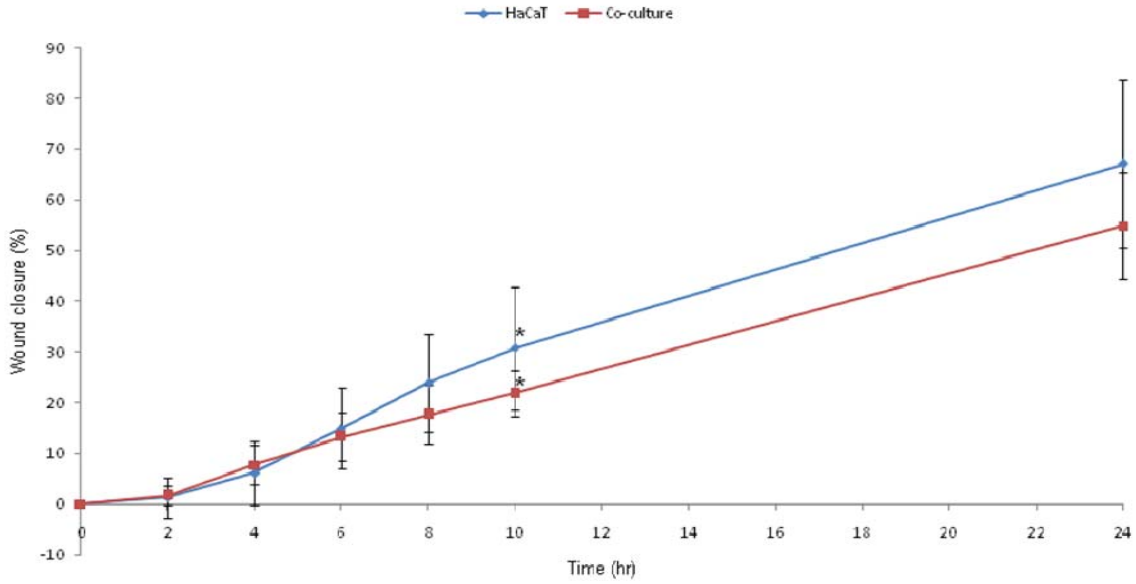



Figure 16: A graphical illustration of ‘wound closure’ in response to ATRA treatment when HaCaT cultures were compared with co-cultures of HaCaT and HDF cells at different time periods. There was a significant difference in ‘wound’ closure when the cultures were compared by 10 hours after ‘wounding’ (n=12, *$p<0.05$).

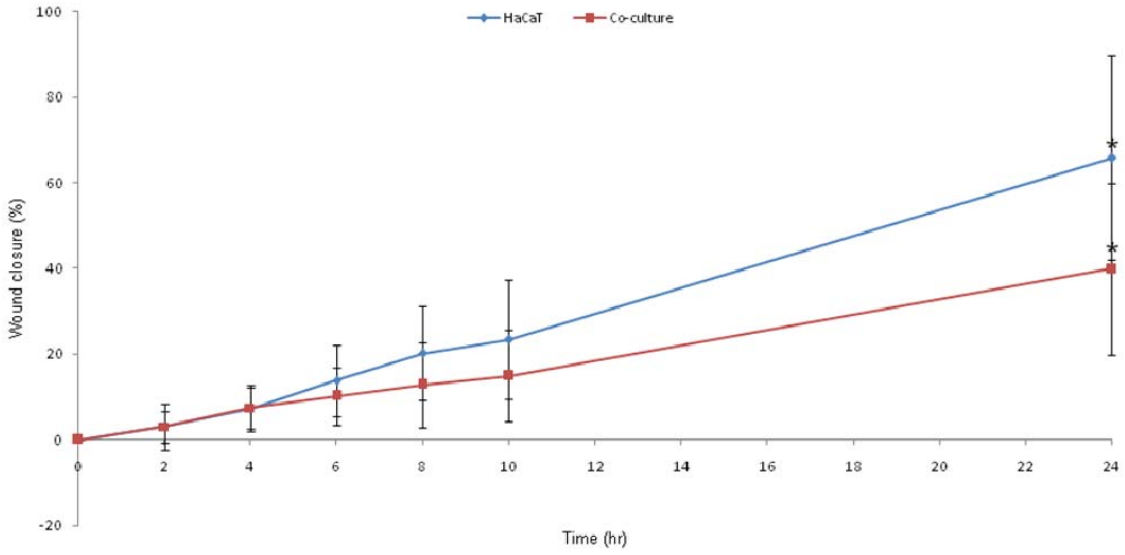



Figure 17: A graphical illustration of 'wound' closure in untreated HaCaT cultures compared with untreated co-cultures of HaCaT and HDF cells at different time periods. 'Wound' closure was significantly different when the cultures were compared by 24 hours after 'wounding' (n=12, *$p<0.05$).

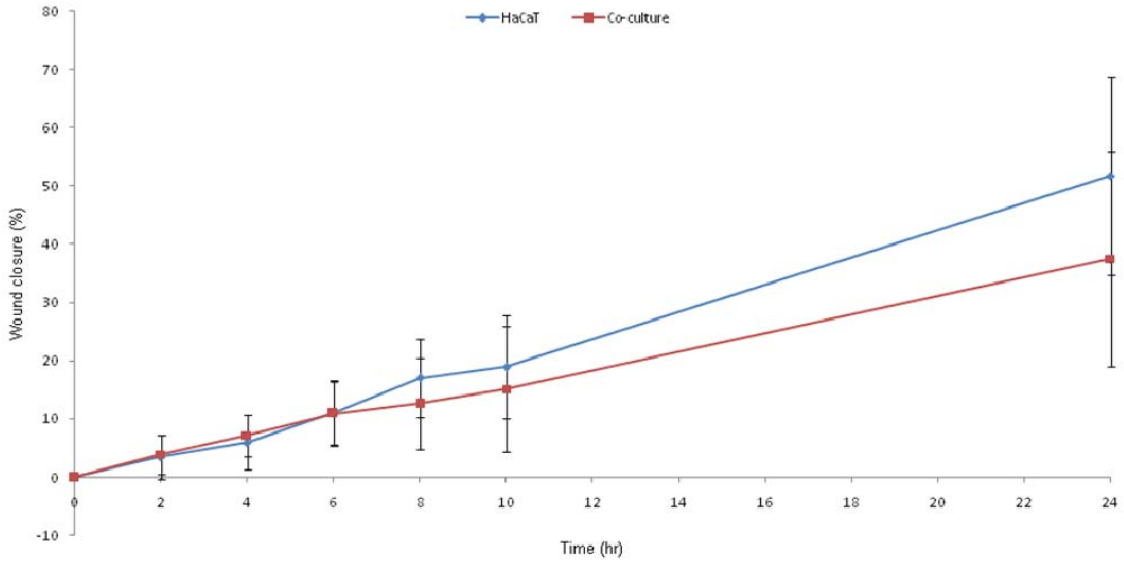



Figure 18: A graphical illustration of ‘wound’ closure in HaCaT cultures grown in vehicle control medium compared with co-cultures of HaCaT and HDF cells grown in a similar medium at different time periods. There was no statistically significant difference in ‘wound’ closure when the cultures at different time periods were compared (n=12).

### 3.5 Cell proliferation

Ki-67 immuno-staining labels nuclei in the $G_1$, S, $G_2$ or mitotic phases of the cell cycle (Fisher *et al.*, 2002; Potemski *et al.*, 2006; Faratzis *et al.*, 2009). Positively Ki-67 immunolabelled nuclei were seen as darkly labeled nuclei in both the HaCaT cultures and the co-cultures (Fig. 19$a_1$ - $a_2$). No cells with immunolabelled nuclei were found when the Ki-67 primary antibody was omitted as a negative control (Fig. 19$b_1$ – $b_2$). Representative photomicrographs of the HaCaT cultures and the co-cultures immunolabelled with Ki-67 antibody for the different treatment groups at different time periods are depicted in Figure 20 and 21 respectively.

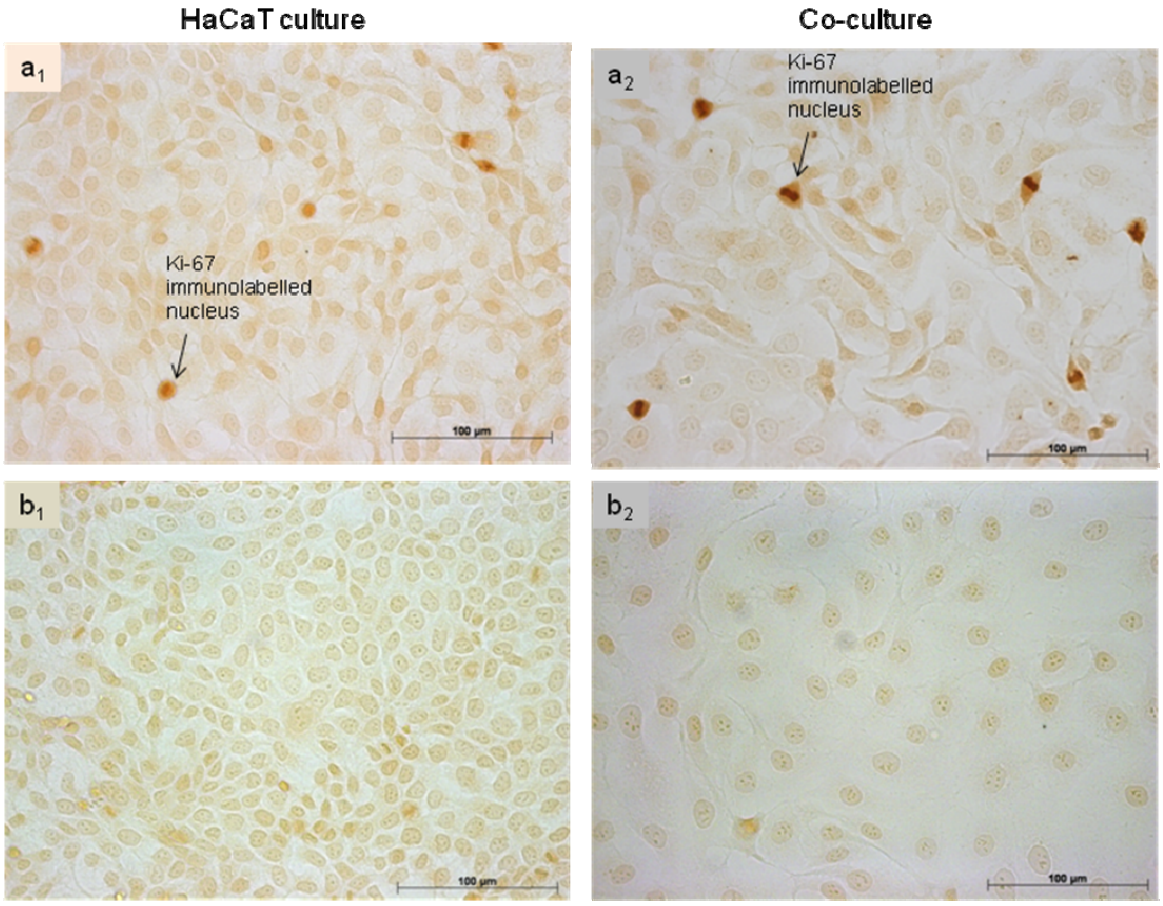



Figure 19: Photographic representations of HaCaT cultures and co-cultures of HaCaT and HDF cells immunolabelled with Ki-67 primary antibody. Photographs $a_1$ - $a_2$ represent the cultures with positive Ki-67 immunolabelled nuclei while $b_1$ – $b_2$ represent the cultures in which the Ki-67 primary antibody was omitted (negative control). Phase contrast microscopy, X40.

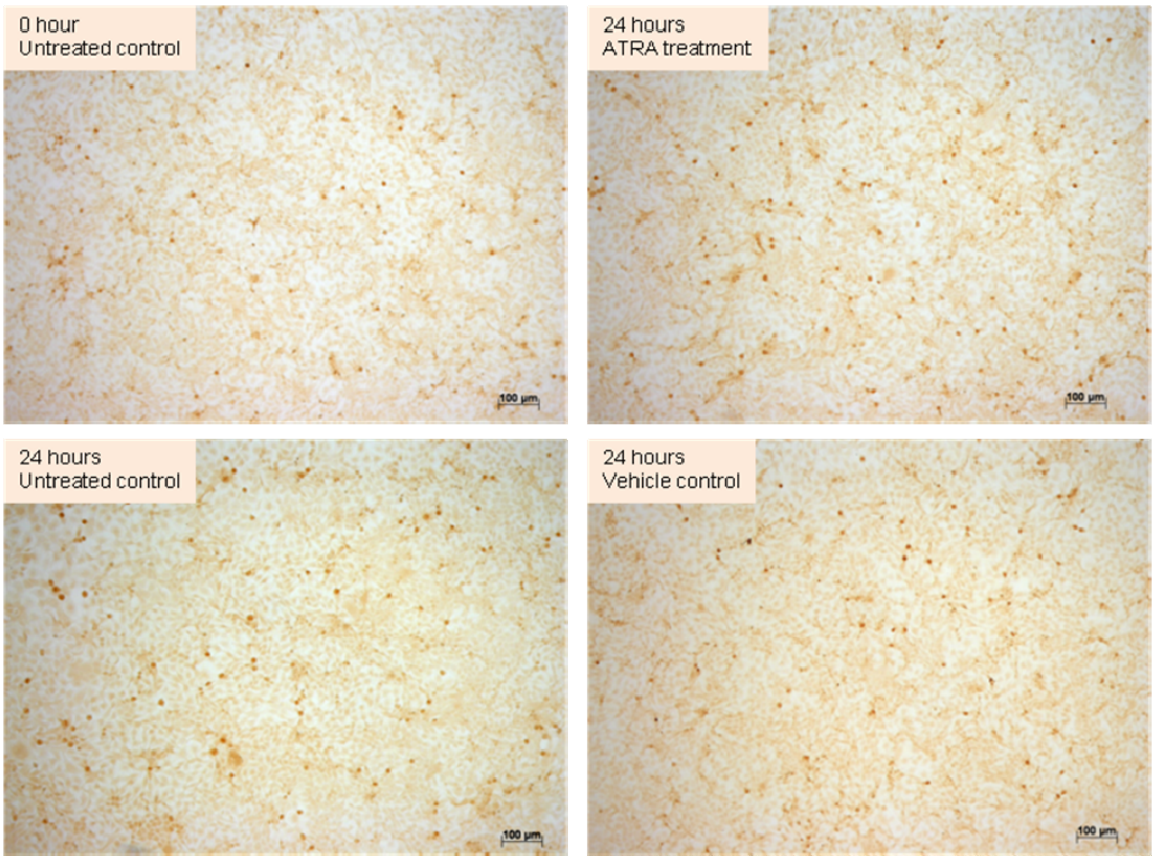



Figure 20: Photographic representations of the HaCaT cultures immunolabelled with Ki-67 antibody (nuclei labelled dark brown) for the untreated cultures at 0 hour, cultures with ATRA treatment at 24 hours, untreated control cultures at 24 hours and cultures grown in vehicle control medium also at 24 hours. Phase contrast microscopy, X10.

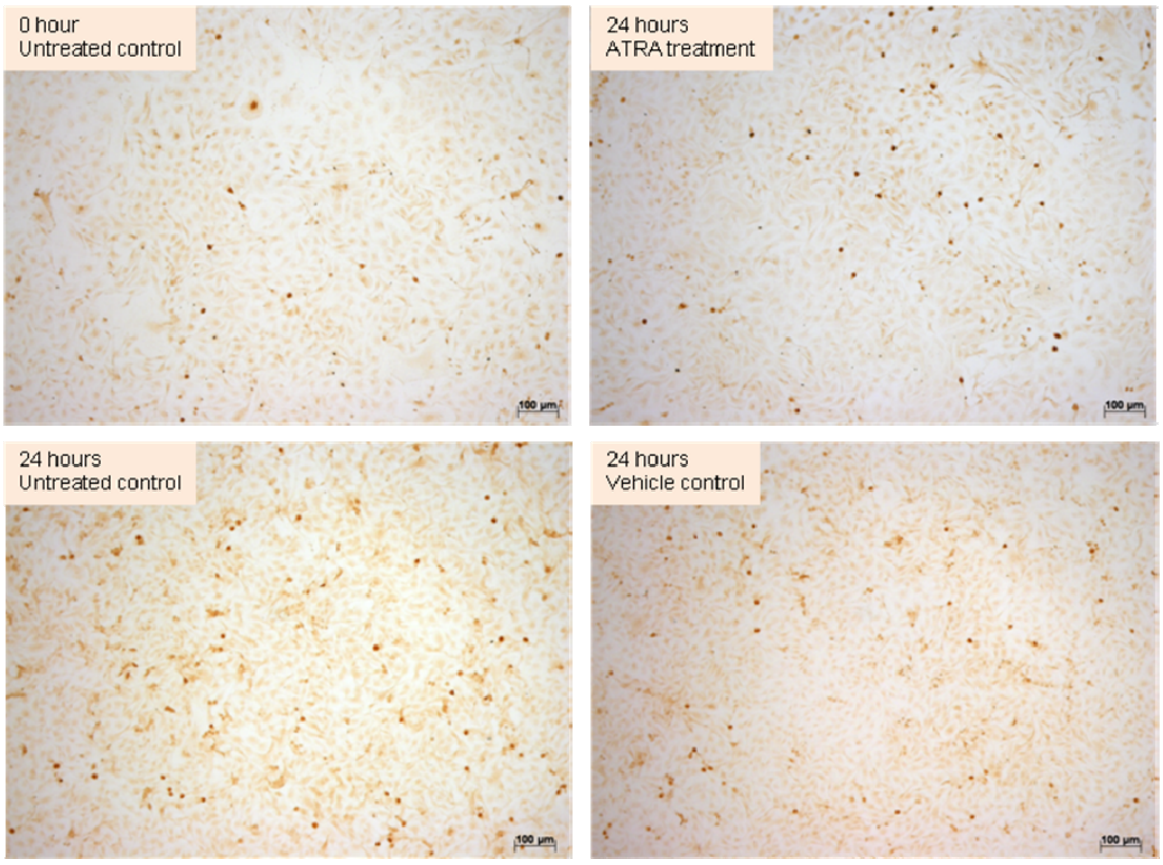



Figure 21: Photographic representations of the co-cultures of HaCaT and HDF cells immunolabelled with Ki-67 antibody (nuclei labelled dark brown) for the untreated cultures at 0 hour, cultures with ATRA treatment at 24 hours, untreated control cultures at 24 hours and cultures grown in vehicle control medium at 24 hours. Phase contrast microscopy, X10.

### 3.5.1 Effect of ATRA on the number of actively proliferating HaCaT cells

There was no statistically significant difference in the two independent counts of the number of actively proliferating cells in each of the samples (Appendix VIII) for 0 hour and 24 hours under the different treatment regimes (ATRA treatment, vehicle control and untreated control).

When the number of actively proliferating cells around the 'wound' region in the untreated HaCaT cultures (Appendix IX) at 0 hour was compared to the HaCaT cultures treated with ATRA ($p=0.0026$) at 24 hours or the HaCaT cultures grown in vehicle control medium ($p=0.0018$) at 24 hours (Fig. 22), there was a statistically significant increase in the number of proliferating cells. Despite a slight increase in the number of actively proliferating cells, there was no significant difference when the untreated HaCaT cultures at 0 hour was compared to the untreated HaCaT cultures at 24 hours ($p=0.0555$).

At 24 hours (Fig. 22), the number of actively proliferating cells around the 'wound' region in the HaCaT cultures treated with ATRA was not significantly different when compared to the untreated control cultures ($p=0.2423$) or cultures grown in vehicle control medium ($p=0.8174$). In addition, there was no significant difference in the number of actively proliferating cells in the untreated control cultures when compared with the cultures grown in vehicle control medium ($p=0.1113$).

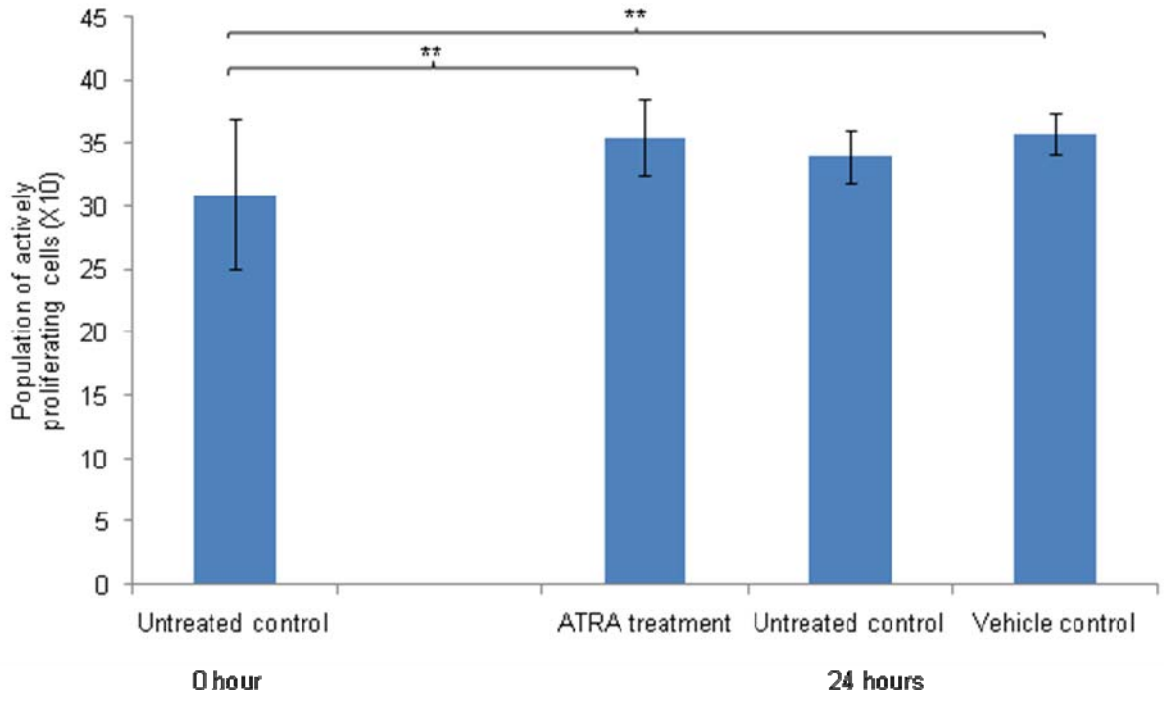



Figure 22: A graphical illustration of the number of actively proliferating HaCaT cells at 0 hour and at 24 hours under different treatment regimes. The number of actively proliferating cells significantly increased from 0 hour to 24 hours in the ATRA treated and in the vehicle control cultures. There was no significant difference in the number of actively proliferating cells in the HaCaT cultures at 24 hours under the three treatment regimes (n=4, **p<0.01).

### 3.5.2 Effect of ATRA on the number of actively proliferating cells in the co-cultures

There was no statistically significant difference in the two independent counts of the number of actively proliferating cells in each of the samples (Appendix VIII) for 0 hour and 24 hours (for the ATRA treatment, vehicle control and untreated control).

There was a statistically significant increase in the number of actively proliferating cells around the ‘wound’ region in the untreated co-cultures at 0 hour when compared with the co-cultures treated with ATRA (p=1.27E-07) at 24 hours or the untreated co-cultures (p=0.0036) at 24 hours or the co-cultures grown in vehicle control medium (p=3.67E-09) at 24 hours (Appendix X).

At 24 hours (Fig. 23), there was a decrease in the number of actively proliferating cells around the ‘wound’ region in the co-cultures treated with ATRA when compared to the untreated control but this decrease was not significantly different (p=0.6033). However, there was a significant decrease in the number of actively proliferating cells in the co-cultures grown in the vehicle control medium when compared to the co-cultures treated with ATRA or the untreated co-cultures (p=0.0020 or p=2.11E-05 respectively).

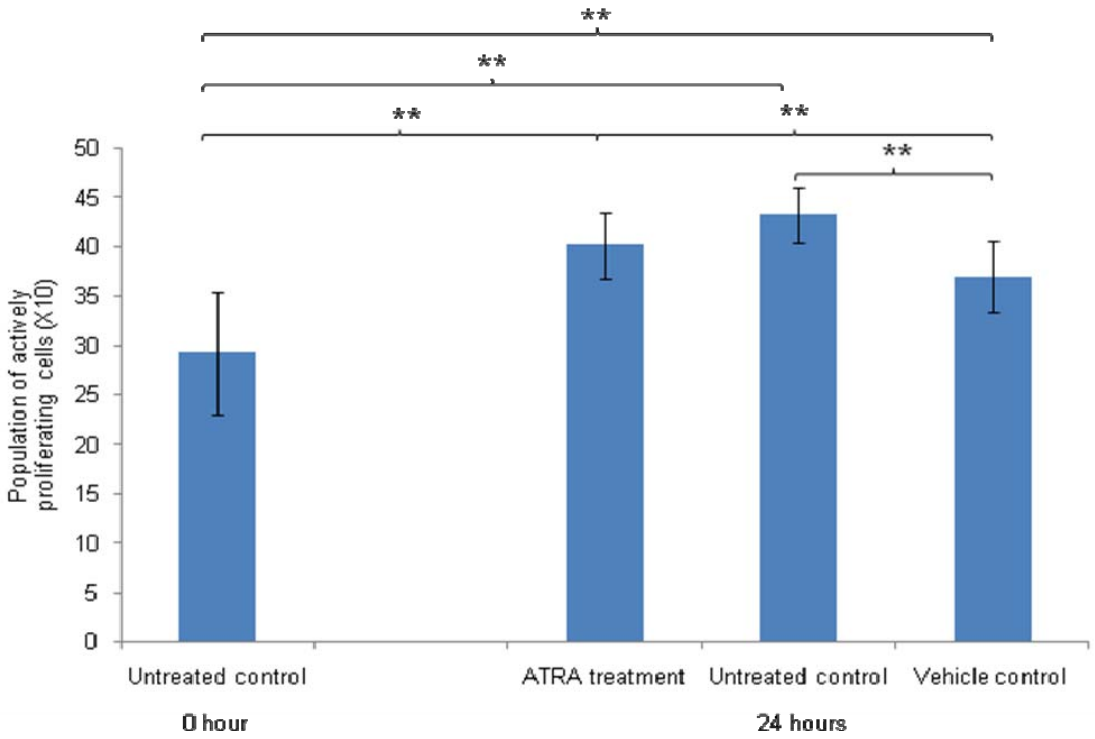



Figure 23: A graphical illustration of the number of actively proliferating cells in the co-cultures of HaCaT and HDF cells under different treatment regimes at 0 hour and 24 hours. The number of actively proliferating cells was significantly increased from 0 hour to 24 hours. The number of actively proliferating cells was significantly different when the ATRA treatment and the vehicle control or the untreated control and the vehicle control were compared at 24 hours after the start of the experiment (n=4, **p<0.01).

### 3.5.3 Comparison of numbers of actively proliferating cells in the HaCaT cultures with the co-cultures

There was no significant difference in the number of actively proliferating cells when the untreated HaCaT cultures at 0 hour were compared to the untreated co-cultures also at 0 hour (p=0.3075) (Fig. 24).

At 24 hours (Table 1 and Fig. 24), the number of actively proliferating cells in the HaCaT cultures with ATRA treatment was significantly different when compared to the co-cultures treated in a similar manner (p=0.0028). Similarly in the untreated cultures, there was a highly significant difference in the number of actively proliferating cells in the HaCaT cultures when compared to the co-cultures (p= 2.75E-06). However, there was no significant difference in the number of actively proliferating cells when the HaCaT cultures grown in vehicle control medium was compared to the co-cultures grown in a similar medium (p=0.2961) (Appendix X).

Table 1: Number of actively proliferating cells in the HaCaT cultures at 0 hour and 24 hours compared with the co-cultures of HaCaT and HDF cells at the same time period

| Time period | 0 hour | | 24 hours | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Untreated control | | ATRA treatment | | Untreated control | | Vehicle control | |
| | HaCaT | Co-culture | HaCaT | Co-culture | HaCaT | Co-culture | HaCaT | Co-culture |
| Number of actively proliferating cells per culture | 309.63 | 292.13 | 354.88 | 400.75 | 340.00 | 408.75 | 358.00 | 344.50 |
| Standard deviation of number of actively proliferating cells per culture (X10) | 5.94 | 6.24 | 3.04 | 3.27 | 2.06 | 2.82 | 1.65 | 3.58 |
| p-value | 0.3075 | | 0.0028 | | 2.75E-06 | | 0.2961 | |

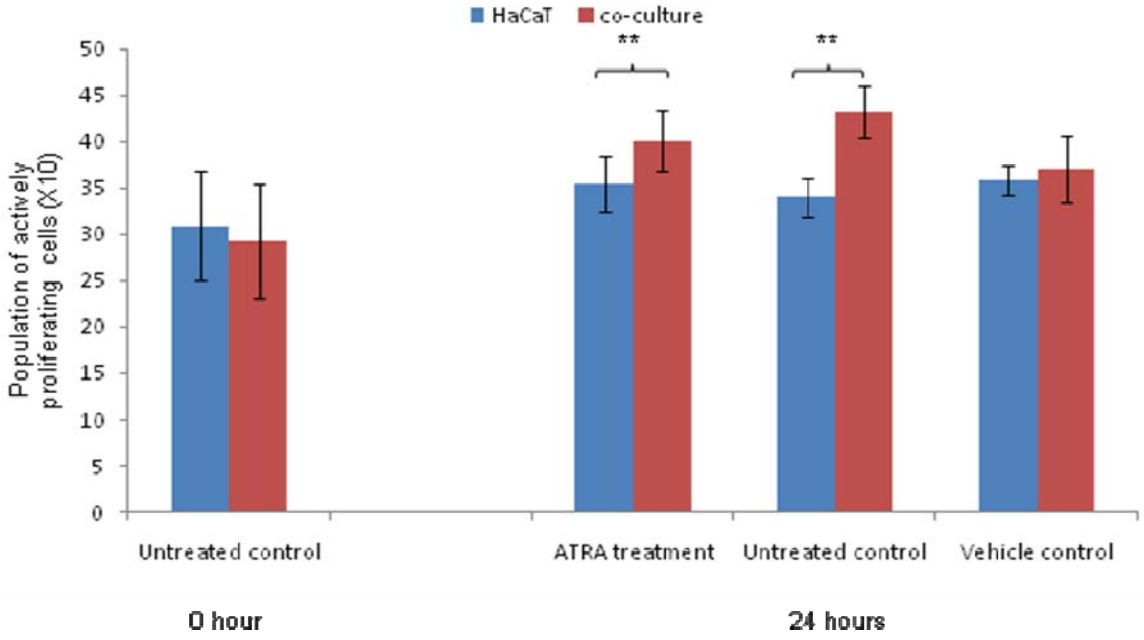



Figure 24: A graphical illustration of the comparison of the number of cells positive for Ki-67 immunolabelling in the HaCaT cultures and in the co-cultures of HaCaT and HDF cells at 0 hour and at 24 hours under different treatment regimes. The number of actively proliferating cells was significantly different when the HaCaT cultures and the co-cultures were compared for the ATRA treatment and the untreated control respectively at 24 hours (n=4, **p<0.01).

# CHAPTER FOUR

# DISCUSSION

The results of the present study showed that treatment of HaCaT cultures with 1μM ATRA or with the vehicle control (0.0015% DMSO in DMEM) did not have an effect on cell proliferation compared to untreated cultures (DMEM alone) as deduced by the colorimetric cell proliferation assay.

The non-proliferative effect of ATRA on the HaCaT cultures is in accordance with the report by Weninger *et al.* (1998) which also demostrated that ATRA at concentrations of 1μM or less, had no effect on proliferative activity in primary human keratinocyte cells. Weninger *et al.* (1998) further demonstrated using manual cell counts and quantification of total cellular protein, that treatment of epidermoid carcinoma and HaCaT cell lines with ATRA did not affect cell proliferative activity. In addition, as in the present study, Weninger *et al.* (1998) also reported that 0.01% DMSO (used as the vehicle control) did not have an effect on cell proliferation in cultures of primary human keratinocytes, HaCaT and epidermoid carcinoma.

In the HDF cell cultures, there was a noticeable, but not significant, inhibition in cell proliferation with the treatment of ATRA compared to the untreated control cultures. In addition, there was a significant decrease in cell proliferation in the cultures grown in vehicle control medium when compared to either the cultures treated with ATRA or

grown in DMEM alone. The non-proliferative effect of ATRA on the HDF cells in the present study is in contrast to reports (Nelson and Balian, 1984; Priestley, 1987) that demonstrated inhibited proliferation following treatment with 1μM ATRA of neonatal human dermal fibroblasts (Nelson and Balian, 1984), and fibroblasts cell cultures derived from normal human skin as well as from lesional and non-lesional skin of patients with psoriasis disease (Priestley, 1987).

It appears as though different concentrations of ATRA may have different effects. Harper and Burgoon (1982) investigated the effects of ATRA on the proliferation of fibroblast-like cells derived from rabbit, swine and human skins *in vitro*. Their results on fibroblast-like human skin demonstrated that proliferation was increased at a concentration of 0.33μM and 3.3μM ATRA but was inhibited at 13.2μM. In the same study, it was reported that proliferation of fibroblast-like rabbit skin was inhibited at concentrations of 0.05μM and 0.5μM (Harper and Burgoon, 1982). In addition, it was found that the concentrations of ATRA which inhibited proliferation of rabbit fibroblast-like cells had no effect on swine fibroblast-like cells, but promoted proliferation of fibroblast-like human skin (Harper and Burgoon, 1982).

The results of the Ki-67 immunolabelling analysis showed that the number of actively proliferating cells in the HaCaT cultures and in the co-cultures treated with ATRA were not significantly different when compared to the respective cultures grown in untreated control medium. This is in accordance with the results of the colorimetric cell proliferative activity for the HaCaT and the HDF cell lines in the present study in which ATRA was found to have no effect on cell proliferation. Interestingly, Lee *et al.*

(2009) while studying the transcriptional changes in normal human keratinocytes indicated that 1μM ATRA significantly induced, and at the same time, suppressed genes which are associated with the regulation of cell proliferation. Lee *et al.* (2009) proceeded to verify the cell proliferative effect of ATRA by determining the number of cells in ATRA-treated cultures at 24 and 48 hours following treatment using a colorimetric cell proliferation assay. The results however, showed moderate cell proliferation at both 24 and 48 hours, but cell proliferation was significantly increased in the cultures treated with ATRA compared to the untreated cultures. Lee *et al.* (2009) concluded that ATRA promotes cell proliferation in the keratinocytes despite observing a simultaneous expression and suppression of genes associated with cell proliferation. However, no effect of ATRA on the HaCaT, the HDF cell lines or the co-culture was found in the present study.

The number of actively proliferating cells in the co-cultures grown in the vehicle control medium was statistically significantly decreased when compared to either the co-cultures treated with ATRA or grown in untreated control medium. However, no effect of the vehicle control was found in the HaCaT cultures when compared to either HaCaT cultures treated with ATRA or untreated control medium. The inhibitory effect of DMSO on the co-culture and its non-significance in the HaCaT culture as deduced by the Ki-67 immunolabelling assays are in accordance with the results of the colorimetric cell proliferation assay in which the DMSO was inhibitory to cell proliferation in the HDF cells while it had no effect on proliferation in the HaCaT cells. The reason for the difference in the proliferative effect of DMSO in the HDF cells, HaCaT cells and in the co-culture cannot be ascertained in the present study.

In light of the inhibitory effect of DMSO on cell proliferation of the HDF cell line and the co-culture, but no effect on the HaCaT cultures, it thus appeared that DMSO was only inhibitory to the HDF cells. However, as ATRA was found to have no effect on cell proliferation in the HaCaT cells, HDF cells or co-culture as demonstrated in the present study, it thus appears that DMSO may have compromised the effect of ATRA. Unfortunately, suitable reports in the literature for comparison of the effect of DMSO on cell proliferation in HaCaT and HDF cell lines as well as in the co-cultures were not found. However, DMSO was implicated in compromising the positive effect of ATRA (in DMSO) on the number of insulin cells in cultures of embryonic chick pancreatic buds (Kramer and Penny, 2003). The present study thus suggests that DMSO had no effect on cell proliferation during 'wound' closure of the HaCaT culture, but that it appears to be inhibitory to cell proliferation during 'wound' closure in the co-cultures.

From the scratch 'wound' assays for both 'cell migration' and cell proliferation assays, it appeared that cells at the 'wound' edge of both the HaCaT cultures and the co-cultures proliferated and/or migrated to close the 'wound'. This is in accordance with the reports in the literature that cell proliferative and migratory phases during the process of wound healing overlap (Metcalfe and Ferguson, 2006; Sweitzer *et al.*, 2006; Dewor *et al.*, 2007). Buth *et al.* (2007) while studying the effect of cathepsin B (an extracellular degradation protease which is essential for cell migration) on regeneration of scratch 'wounds' in cultures of human epidermal cells reported that both cell proliferation and migration contributed to 'wound' closure. In another study using quantitative analysis, Dewor *et al.* (2007) indicated that proliferation contributed to about two-thirds and cell migration to about one-third of the actual 'wound' closure

of scratch-wounded primary human dermal fibroblasts. In light of the contribution of both cell proliferation and migration during the process of wound healing, it is thus impossible to rule out one of such process during the regeneration or closure of a wound (Matsuura *et al.*, 2007).

In the present study, 'cell migration' in the HaCaT cultures and in the co-cultures was not statistically significantly different when the cultures treated with ATRA and the untreated control cultures were compared. The non-significant effect of ATRA on cell migration in the HaCaT cultures and in the co-cultures in the present study is in accordance with the report by Lee *et al.* (2005). It was demonstrated that ATRA had a slight inhibitory effect on cell migration in primary human keratinocytes which had been pretreated with mitomycin C in order to arrest the cell cycle prior to scratching (Lee *et al.*, 2005). However, this inhibition of cell migration was not significant (Lee *et al.*, 2005). In another study, Lee *et al.* (2009) reported that ATRA at a concentration of 1μM induced (at 4 hours) and suppressed (at 24 and 72 hours) genes which are related to keratinocyte migration. It was suggested that the slight inhibition of cell migration as reported by Lee *et al.* (2005) could have been as a result of the down-regulation of genes associated with cell migration at 24 and 72 hours (Lee *et al.*, 2009).

There was a statistically significant difference in cell migration' in both the HaCaT cultures (at 10 and 24 hours) and the co-cultures (at 24 hours) when the ATRA treatment was compared to the cultures grown in vehicle control medium. As a result of a non-significant effect of ATRA on 'cell migration' or 'wound' closure in either the

HaCaT culture or the co-culture, the present study implicates DMSO (diluent for ATRA) as confounding the results. Unfortunately, there were no reports in the literature on the effect of DMSO on 'cell migration' or score closure with which to compare the results of the present study. The present study thus suggests that ATRA may not have a significant effect on cell migration during closure of scratch 'wounds' in the HaCaT cultures or the co-cultures.

It is interesting to find in the present study that ATRA had no significant effect on cell proliferation or cell migration. In a study of the molecular mechanisms through which ATRA regulates proliferation, it was reported that ATRA significantly stimulated the production of heparin-binding EGF-like growth factor (a stimulant of proliferation) in cultured normal human keratinocytes, organotypic culture of skin and in intact human skin (Stoll and Elder, 1998). In addition, it was found that ATRA promoted cellular interactions resulting in the release of stimulants for cell proliferation in cultures of skin cells (Stoll and Elder, 1998). The difference in the effect of ATRA on cell migration and proliferation in the present study compared to reports in the literature (Harper and Burgoon, 1982; Lee *et al.*, 2005; Lee *et al.*, 2009) could be attributed to the difference in the cell types. In addition, DMSO which is often used as a diluent of ATRA could also contribute to these contradictions as observed in the present study. On the other hand, it has been suggested that the contradictions in the reports of the effect of ATRA could be as a result of a complex sequence of actions which are not yet fully understood (Davies *et al.*, 1988; Watson *et al.*, 2004; Takami *et al.*, 2005).

In addition, the exact sequence or outcome of the bio-molecular effects of ATRA during wound healing could be difficult to establish in *in vitro* or in *in vivo* systems. It is important to know that the cellular activities and release of growth factors, cytokines, hormones and the expression of receptors for these molecules are regulated by gene transcription within the cells (Takami *et al.*, 2005; Arukwe and Nordbo, 2008) as well as a possible interaction from co-activators (Goyette *et al.*, 2000; Lee *et al.*, 2009). Under normal circumstances, it is expected that only the gene associated with a particular cellular activity in response to ATRA treatment should be expressed at any given time (Thanos and Maniatis, 1995; Lee *et al.* 2005; Metcalfe and Ferguson, 2006) but this is rarely the case. Instead, it is possible that a number of other factors which are either directly or indirectly linked to ATRA are simultaneously expressed with the desired gene (Thanos and Maniatis, 1995; Lee *et al.*, 2009). This thus leads to the expression of a multitude of genes associated with different cellular activities being expressed at the same time (Thanos and Maniatis, 1995; Lee *et al.*, 2005; Metcalfe and Ferguson, 2006; Lee *et al.*, 2009). Lee *et al.* (2009) while comparing genes which were regulated by ATRA at a concentration of 1μM with those induced by transforming growth factor-β (TGF-β) reported that several genes were regulated by TGF-β either in a similar manner or directly opposite to the effect of ATRA. It was stated that the co-activator (TGFβ) which was released by cells could augment or diminish the effect of ATRA (Lee *et al.*, 2009) which could be a contributing factor to the contradictions in the reports on the effect of ATRA. This simultaneous sequence of events in multiple expressions of genes or activity of co-activators thus makes tracing the path and understanding the role of ATRA in wound healing difficult (Lee *et al.*, 2005) either in an *in vivo* or *in vitro* system.

The present study found that cell migration was significantly increased in the HaCaT cultures grown in DMEM alone when compared to the co-cultures grown in the same medium. In the cultures treated with ATRA or cultures grown in vehicle control medium, cell migration was increased in the HaCaT cultures compared to the co-cultures. However, the increase in cell migration was not statistically significant. It thus appears that the co-culturing of the HaCaT cell line with the HDF cell line could have possibly decreased 'cell migration' or 'wound' closure in the co-cultures due to its bi-cellularity.

It is reasonable to state that the functionality of an *in vitro* skin system may not solely be dependent on the individual cell types in the cultures (Metcalfe and Ferguson, 2006). The complex structural organization of the *in vitro* system and the complex biological interactions which exist between the cells could also play an important role in the functionality of an *in vitro* skin model (Wakatsuki *et al.*, 2000; Grinnell, 2003). Apart from a possible influence of the bi-cellular nature of the co-cultures in the present study, it thus suggests that the decrease in migration in the co-cultures could be as a result of a complex interaction and/or the complexity arising from the *in vitro* condition.

Rorth (2002) in a review of initiation and guidance of migration by border cells during *Drosophilia* oogenesis emphasized that obvious differences exist between cells 'crawling in a dish' (*in vitro*) and cells migrating *in vivo*. These differences in the migration patterns could be attributed to difference in the resistance to movement within the two environments (Rorth, 2002). In his report, Rorth (2002) explained that

cells migrating in an *in vivo* environment encounter greater resistance caused by multifaceted cell-to-cell adhesions, complex biological interaction between cells, three-dimensional structural organization of the cells and multicellularity. It was emphasized that before cell migration can occur *in vivo*, these resistances have to be overcome, which thus requires “a strong protrusive force, traction and contraction” (Rorth, 2002). It is therefore the assumption of the present study that the layered nature and the bi-cellularity of the co-cultures mimicked certain characteristics of human skin. This was demonstrated by the decrease in cell migration in the co-cultures compared to the HaCaT cultures as illustrated by Rorth, 2002.

In addition, the number of actively proliferating cells in the co-cultures in the present study was greater than in the HaCaT cultures for the 24 hours treatment regime. It could be suggested that the increase in the number of actively proliferating cells in the co-culture may be due to the increased numbers of cells in the co-cultures (both HaCaT and HDF cells) compared to the HaCaT cultures. At the start of the experiment (0 hour) however, there was no statistically significant difference in the number of actively proliferating cells when the HaCaT cultures and the co-cultures were compared.

The number of actively proliferating cells in the co-cultures either treated with ATRA or grown in DMEM alone was significantly higher when compared to the HaCaT cultures cultured in a similar manner 24 hours after the start of the experiment. It thus appeared that cell proliferation around the ‘wound’ region was greater in the co-cultures when compared with the HaCaT cultures. Biological interaction between skin

cells have been reported to influence or promote the process of wound healing (Stoll and Elder, 1998; Metcalfe and Ferguson, 2006). Evidence has also shown that keratinocytes and fibroblasts secrete numerous growth factors (Boyce *et al.*, 1996) such as transforming growth factor-α and basic fibroblast growth factor which have been reported to facilitate the process of wound healing (Root and Shipley, 1991; Boyce *et al.*, 1996; Metcalfe and Ferguson, 2006). It thus suggests that cell proliferation could have been promoted in the co-cultures in the present study due to a possible complex cellular interaction between its two cell types compared to the HaCaT culture which contained only one cell type. The possibility of complex cellular interaction in the co-culture as deduced by comparison of the number of actively proliferating cells in the HaCaT culture with the co-culture further supports the assumption of the present study that the co-culture mimicked certain characteristic of human skin.

It is important to re-emphasize that the complete recreation of skin *in vitro* has not been achieved (Lim *et al.*, 2002; Boyce and Waden, 2002; Suhonen *et al.*, 2003; Maas-Szabowski *et al.*, 2004; Metcalfe and Ferguson, 2006) and neither has vascularization of the skin been achieved *in vitro* (Metcalfe and Ferguson, 2006). Although a porous acellular-glycoaminoglycan matrix (extracellular matrix) was not used in the co-culture in the present study, there are convincing reports in the literature that fibroblasts secrete extracellular matrix such as collagen type I, *in vitro* (Hata and Senoo, 1989; Ohgoda *et al.*, 1998; Moulin *et al.*, 2000; Maas-Szabowski *et al.,* 2004; Lee *et al.*, 2006). The ability of fibroblasts to secrete extracellular matrix was similarly reported by Lee *et al.* (2006) in which a confluent culture of human

dermal fibroblasts was used as a dermal equivalent in developing an organ culture of skin. In addition, it was reported that a HaCaT cell line used in a scratch-wound assay synthesized collagen type IV which is a constituent of dermal skin (Buth *et al.*, 2007). In light of a possible existence of a complex cellular interaction and the bi-cellular nature as well as complex adhesion in the co-cultures in the present study, it suggests that the co-culture mimics or simulates the complex biological interactions and the multicellular nature of intact human skin.

## CONCLUSION

The present study found that ATRA had no effect on cell proliferation in either of the individual cell lines used in this study or in the co-cultures. The DMSO (vehicle control) inhibited proliferation of the HDF cells as well as the co-cultures but did not appear to have an effect on the HaCaT cultures. It thus seems that the inhibitory effect of DMSO on proliferation appears to be directed towards the HDF cells as well as the co-cultures which contained HDF cells. ATRA also did not have an effect on 'migration' of cells in the HaCaT cultures or the co-cultures during 'wound' closure. In light of the failure of ATRA to promote 'cell migration' and proliferation during wound closure of the HaCaT culture and the co-cultures in the present study, it would seem that the activity of ATRA was compromised in the presence of DMSO. This may also substantiate why there is controversy in the literature regarding the effect of ATRA, as DMSO is often the diluent for ATRA. It is recommended that the exploration of the effect of ATRA at this particular concentration (1μM) without the use of the diluent (DMSO) would be beneficial in understanding the true effect of ATRA.

In future studies, it would be interesting to investigate the wound healing capabilities of ATRA on primary cultures derived from human skin to determine whether differences exist. In addition, an *in vivo* study on the effect of ATRA needs to be performed to ascertain its possible effect during wound healing, which would complement the findings of the present study. It would also be beneficial to investigate whether ATRA would express or suppress certain genes which are associated with cell proliferation and migration during wound healing in either an *in vitro* or *in vivo* situation.

As a result of a slowed ‘migration’ of cells in the co-culture compared to the HaCaT culture as demonstrated in the present study, it is believed that the co-culture more closely mimicked certain biological characteristics peculiar to intact skin. The present study thus recommends its use as a model of human skin for studies on scratch-wound assays.

## APPENDIX

## I

Preparation of 1µM ATRA

The preparation of serial dilutions of ATRA from the stock solution was performed as follows:

- 100mg of ATRA powder was dissolved in 5ml 100% DMSO

Molecular formula of ATRA= 300.44

$$\text{ATRA concentration} = \frac{\text{mass}}{\text{Volume X Molecular mass}}$$

$$= \frac{100\text{mg}}{5\text{ml X } 300.44}$$

$$= 0.0666\text{M or } 66600\mu\text{M}$$

Using dilution formula ($C_1V_1=C_2V_2$) the ATRA stock solution was diluted in the culture medium to 10000µM ATRA

Where $C_1$ = 66600µM; $V_1$ = unknown; $C_2$ = 10000µM and $V_2$ = 1000µL

$$\text{The volume of stock solution} = \frac{10000 \text{ X } 1000}{66600}$$

$$= 150.15\mu\text{L}$$

Therefore 150.15µL of ATRA stock solution was dissolved in 849.85µL of culture medium to prepare 10000µM ATRA.

Using a dilution factor of 10, the concentration of 10000μM ATRA was further diluted four times i.e. to 1000μM, 100μM, 10 μM and finally to 1μM (desired concentration).

In the same manner as the preparation of 1μM ATRA, serial dilutions of DMSO from stock solution (100% DMSO) were performed exactly in the same order as the preparation of 1μM ATRA.

Using dilution formula, $C_1V_1=C_2V_2$

Where $C_1$ = 100%; $V_1$ = 150.15μL; $C_2$ = unknown and $V_2$ = 1000μL

$$\text{Concentration of DMSO} = \frac{100 \text{ X } 150.15}{1000}$$

$$= 15.15\%$$

Using a dilution factor of 10, the concentration of DMSO was diluted four times (equivalent to the number of times ATRA was diluted in preparing 1μM ATRA) to a final concentration of 0.0015%.

**II**

Counting of cells using a haemocytometer chamber

From a confluent culture in a 25ml Nunc® culture flask,

- 1ml of trypsinized cells was suspended in 4ml culture medium
- 20µl of this cell suspension was mixed with an equal volume of 0.4% trypan blue (a dilution factor of 2) and then mixed thoroughly
- Using a pipette, 20µl of this suspension was carefully placed into the two compartments of a clean and dry hemocytometer chamber
- Only the viable cells (clear cells) within the five squares were counted
- The average of two counted cells was used

Using the formula below,

Cells per ml = average cell count per square X dilution factor X $10^4$

For example, if average of 58 cells were counted from 5 squares

Then, cells per ml = $\frac{44 \text{ X } 2 \text{ X } 10^4}{5}$

= $1.76 \text{ X } 10^5$ cells per ml

The desired cell concentration was obtained by diluting the cell suspension.

**III**

Preparation of calcium and magnesium-free phosphate buffered saline (CMF-PBS)

The following salts were into 1 litre de-ionized water:

| | |
|---|---|
| NaCl | 8.77g |
| $Na_2HPO_4$ | 1.15g |
| KCl | 0.20g |
| $KH_2PO_4$ | 0.18g |

The pH of the solution was adjusted to 7.4 by adding 1M NaOH. The solution was then sterilized and stored in 4ºC.

**IV**

Preparation of reagents for immunocytochemistry

i. Phosphate buffered saline (PBS)

The following salts were added into 1 litre de-ionized water

| | |
|---|---|
| NaCl | 8.00g |
| $Na_2HPO_4$ | 1.15g |
| KCl | 0.20g |
| $KH_2PO_4$ | 0.20g |

The pH was adjusted to 7.4 by adding 1M NaOH and stored in 4ºC.

ii. Tris-Buffered Saline (TBS)

The following salts were added into 1 litre de-ionized water

| | |
|---|---|
| TRIS-BASE | 6.055g |
| NaCl | 8.766g |

The pH was adjusted to 7.4 by adding 1M NaOH and stored in 4ºC.

iii. Diaminobenzidine tetrahydrochloride (DAB) reagent

7µl of 30% $H_2O_2$ was added into 10 ml water

1mg/ml DAB was added into 10ml PBS

The two solutions were prepared prior to use. The two solutions were then mixed together and filtered with a filter paper.

**V**

## Statistical tests for colorimetric cell proliferation assays

| Depend: Cell proliferation | Statistical test: HaCaT cell line proliferation assay<br>Independent (grouping) variable:<br>ATRA treatment; Untreated control; Vehicle control<br>Kruskal-Wallis test: (3, n= 6) p =0.2811 | | |
|---|---|---|---|
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.2980 | 0.1735 |
| Untreated control | 0.8939 | 0 | 1 |
| Vehicle control | 0.5205 | 0.8102 | 0 |

| Depend: Cell proliferation | Statistical test: HDF cell line proliferation assay<br>Independent (grouping) variable:<br>ATRA treatment; Untreated control; Vehicle control<br>Kruskal-Wallis test: (3, n= 6) p =0.002005 | | |
|---|---|---|---|
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA | 0 | 0.1495 | **0.0051** |
| Untreated control | 0.4486 | 0 | **0.0051** |
| Vehicle control | **0.0152** | 0.01522 | 0 |

**VI**

## Statistical tests for 'wound' closure analyses

| Depend: Wound closure | HaCaT cultures | | |
|---|---|---|---|
| | Independent (grouping) variable: ATRA treatment; Untreated control; Vehicle control<br>Kruskal-Wallis test: (3, n=12) | | |
| | 2 hour (p=0.4941) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.3708 | 0.2855 |
| Untreated control | 1 | 0 | 0.8852 |
| Vehicle control | 0.8564 | 0.0152 | 0 |
| | 4 hours (p=0.8554) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.8399 | 0.6650 |
| Untreated control | 1 | 0 | 0.6650 |
| Vehicle control | 1 | 1 | 0 |
| | 6 hours (p=0.4472) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.7075 | 0.2366 |
| Untreated control | 1 | 0 | 0.4025 |
| Vehicle control | 0.7098 | 1 | 0 |
| | 8 hours (p=0.2103) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.5067 | 0.0689 |
| Untreated control | 1 | 0 | 0.4025 |
| Vehicle control | 0.2069 | 1 | 0 |
| | 10 hours (p=0.0576) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.2145 | **0.0166** |
| Untreated control | 0.6435 | 0 | 0.3408 |
| Vehicle control | **0.0497** | 1 | 0 |
| | 24 hours (p=0.1134) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.9770 | **0.0404** |
| Untreated control | 1 | 0 | 0.141 |
| Vehicle control | 0.1212 | 0.4229 | 0 |

| Depend: Wound closure | Co-cultures | | |
|---|---|---|---|
| | Independent (grouping) variable: ATRA treatment; Untreated control; Vehicle control Kruskal-Wallis test: (3, n=12) | | |
| | 2 hour (p=0.1970) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.5834 | 0.0689 |
| Untreated control | 1 | 0 | 0.3123 |
| Vehicle control | 0.2069 | 0.9370 | 0 |
| | 4 hours (p=0.9340) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.7508 | 0.7950 |
| Untreated control | 1 | 0 | 0.9770 |
| Vehicle control | 1 | 1 | 0 |
| | 6 hours (p=0.3863) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.2602 | 0.2366 |
| Untreated control | 0.7807 | 0 | 0.8852 |
| Vehicle control | 0.7098 | 1 | 0 |
| | 8 hours (p=0.2895) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.2855 | 0.1260 |
| Untreated control | 0.8564 | 0 | 0.8399 |
| Vehicle control | 0.3781 | 1 | 0 |
| | 10 hours (p=0.3036) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.2145 | 0.1749 |
| Untreated control | 0.6435 | 0 | 0.9770 |
| Vehicle control | 0.5246 | 1 | 0 |
| | 24 hours (p=0.0425) | | |
| | ATRA treatment | Untreated control | Vehicle control |
| ATRA treatment | 0 | 0.0531 | **0.0226** |
| Untreated control | 0.1593 | 0 | 0.7075 |
| Vehicle control | 0.06773 | 1 | 0 |

**VII**

## Statistical test of the comparison of 'wound' closure between the HaCaT cultures and the co-cultures

| Depend: Wound closure | HaCaT cultures versus co-cultures | | | | | |
|---|---|---|---|---|---|---|
| | Independent (grouping) variable: HaCaT Vs. Co-culture<br>Mann-Whitney test: (2, n=12) | | | | | |
| | ATRA treatment | | | | | |
| Group | Sample | Mean rank (HaCaT) | Mean rank (co-culture) | p value | U | exact p value |
| 2 hours | 12 | 6.417 | 6.083 | 0.8399 | 68 | 0.8428 |
| 4 hours | 12 | 5.958 | 6.542 | 0.7075 | 65 | 0.7125 |
| 6 hours | 12 | 6.708 | 5.792 | 0.5444 | 61 | 0.5512 |
| 8 hours | 12 | 7.542 | 4.958 | 0.0782 | 41 | 0.0780 |
| 10 hours | 12 | 7.958 | 4.542 | 0.0194 | 31 | **0.0173** |
| 24 hours | 12 | 7.667 | 4.833 | 0.0531 | 38 | 0.0519 |
| | Untreated control | | | | | |
| Group | Sample | Mean rank (HaCaT) | Mean rank (co-culture) | p value | U | exact p value |
| 2 hours | 12 | 6.417 | 6.083 | 0.8399 | 68 | 0.8428 |
| 4 hours | 12 | 5.958 | 6.542 | 0.7075 | 65 | 0.7125 |
| 6 hours | 12 | 7.083 | 5.417 | 0.2602 | 52 | 0.2657 |
| 8 hours | 12 | 7.542 | 4.958 | 0.0783 | 41 | 0.0780 |
| 10 hours | 12 | 7.333 | 5.167 | 0.1410 | 46 | 0.1432 |
| 24 hours | 12 | 7.958 | 4.542 | 0.0194 | 31 | **0.0173** |
| | Vehicle control | | | | | |
| Group | Sample | Mean rank (HaCaT) | Mean rank (co-culture) | p value | U | exact p value |
| 2 hours | 12 | 6.042 | 6.458 | 0.7950 | 67 | 0.7987 |
| 4 hours | 12 | 5.833 | 6.667 | 0.5834 | 62 | 0.5899 |
| 6 hours | 12 | 6.208 | 6.292 | 0.9770 | 71 | 0.9774 |
| 8 hours | 12 | 7.167 | 5.333 | 0.2145 | 50 | 0.2189 |
| 10 hours | 12 | 7.083 | 5.417 | 0.2602 | 52 | 0.2657 |
| 24 hours | 12 | 7.417 | 5.083 | 0.1124 | 44 | 0.1135 |

**VIII**

Statistical test of the comparison between two independent counts of actively proliferating cells in the HaCaT cultures and the co-cultures

| Depend: Cell proliferation (Ki-67 immunolabelling) | HaCaT cultures | | | | | |
|---|---|---|---|---|---|---|
| | Independent (grouping) variable: Count 1 versus count 2<br>Mann-Whitney test (2, n=4) | | | | | |
| Group | Sample | Mean rank (count 1) | Mean rank (count 2) | p value | U | Exact p value |
| Untreated control (0 hour) | 40 | 20.40 | 20.10 | 0.9089 | 788.0 | 0.9117 |
| ATRA treatment (24 hours) | 40 | 19.79 | 20.71 | 0.7325 | 763.5 | 0.7284 |
| Untreated control (24 hours) | 40 | 19.79 | 20.71 | 0.7191 | 763.5 | 0.7286 |
| Vehicle control (24 hours) | 40 | 21.09 | 19.41 | 0.5169 | 733.0 | 0.5212 |

| Depend: Cell proliferation (Ki-67 immunolabelling) | Co-cultures | | | | | |
|---|---|---|---|---|---|---|
| | Independent (grouping) variable: Count 1 versus count 2<br>Mann-Whitney test (2, n=4) | | | | | |
| Group | Sample | Mean rank (count 1) | Mean rank (count 2) | p value | U | exact p value |
| Untreated control (0 hour) | 40 | 20.06 | 20.44 | 0.8897 | 785.0 | 0.8889 |
| ATRA treatment (24 hours) | 40 | 20.10 | 20.40 | 0.9109 | 788.0 | 0.9118 |
| Untreated control (24 hours) | 40 | 19.02 | 21.48 | 0.3440 | 701.5 | 0.3447 |
| Vehicle control (24 hours) | 40 | 19.72 | 20.78 | 0.6842 | 757.5 | 0.6856 |

**IX**

Statistical tests for the number of actively proliferating cells in the HaCaT cultures and the co-cultures

| Depend:<br>Cell proliferation (Ki-67 immunolabelling) | HaCaT cultures<br>Independent (grouping) variable:<br>Untreated control (0 hour); ATRA treatment; Untreated control; Vehicle control<br>Kruskal-Wallis test (4, n=4) | | | |
|---|---|---|---|---|
| | Untreated control (0 hour) | ATRA treatment (24 hours) | Untreated control (24 hours) | Vehicle control (24 hours) |
| Untreated control (0 hour) | 0 | **0.0026** | 0.0555 | **0.0018** |
| ATRA treatment (24 hours) | **0.0158** | 0 | 0.2423 | 0.8174 |
| Untreated control (24 hours) | 0.3330 | 1 | 0 | 0.1113 |
| Vehicle control (24 hours) | **0.0108** | 1 | 0.6676 | 0 |

| Depend:<br>Cell proliferation | Co-cultures<br>Independent (grouping) variable:<br>Untreated control (0 hour); ATRA treatment; Untreated control, Vehicle control<br>Kruskal-Wallis test (4, n=4) | | | |
|---|---|---|---|---|
| | Untreated control<br>0 hour | ATRA treatment<br>(24 hours) | Untreated control<br>(24 hours) | Vehicle control<br>(24 hours) |
| Untreated control<br>(0 hour) | 0 | **1.27E-07** | **3.67E-09** | **0.0036** |
| ATRA treatment<br>(24 hours) | **7.64E-07** | 0 | 0.6033 | **0.0020** |
| Untreated control<br>(24 hours) | **2.20E-08** | 1 | 0 | **2.11E-05** |
| Vehicle control<br>(24 hours) | **0.0216** | **0.0059** | **0.0001** | 0 |

**X**

Statistical test of the comparison of the number of actively proliferating cells between the HaCaT cultures and the co-cultures

| Depend: Cell proliferation | HaCaT cultures versus Co-cultures<br>Independent (grouping) variable: Hacat cultures; co-cultures<br>Mann-Whitney test (4, n=4) | | | | | |
|---|---|---|---|---|---|---|
| Group | Sample | Mean rank (HaCaT) | Mean rank (Co-culture) | p value | U | exact p value |
| Untreated control (0 hour) | 40 | 21.58 | 18.92 | 0.3051 | 693.5 | 0.3075 |
| ATRA treatment (24 hours) | 40 | 16.36 | 24.14 | 0.0024 | 489.0 | **0.0028** |
| Untreated control (24 hours) | 40 | 14.16 | 26.34 | 0.0001 | 312.5 | **2.75E-06** |
| Vehicle control (24 hours) | 40 | 21.61 | 18.89 | 0.2972 | 691.0 | 0.2961 |

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