A NEW TOPICAL TRANSDERMAL SILDENAFIL GEL FORMULATION TO IMPROVE SKIN FLAP VIABILITY IN A RABBIT MODEL

by

KHO SWEE LIANG

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LIST OF ABBREVIATIONS

AECUSM	Animal ethics committee, Universiti Sains Malaysia
ANOVA	Analysis of variance
ARASC-USM	Animal research and service center, Universiti Sains Malaysia
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNG	Cyclic nucleotide-gated channels
CO	Carbon monoxide
DAD	Diode array detection
ED	Erectile dysfunction
FDA	Food and Drug Administration, USA
FGF	Fibroblast growth factor
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectrometer
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPLC	High performance liquid chromatography
HPMC	Hydromellose
HQC	High concentration quality control
HUSM	Hospital Universiti Sains Malaysia
I.S.	Internal standard
KCl	Potassium chloride
LC-MS	Liquid chromatography – mass spectrometer

LOD	Limit of detection
LOQ	Limit of quantification
LQC	Low concenration quality control
mPa.s	Millipascal second (centipoise)
MQC	Medium concentration quality control
MS	Mass spectrometer
N/A	Not applicable
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NO	Nitric oxide
PBS	Phosphate buffered saline
PBS-Azide	Phosphate buffered saline with 0.02% sodium azide
PDE	Phosphodiesterase
PDE-5	Phosphodiesterase 5
PEG	Polyethylene glycol
PEG 300	Polyethylene glycol 300
PEG 400	Polyethylene glycol 400
PFU	Perfusion unit
QC	Quality control
QS	*Quantum sufficit*
RECON-USM	Department of reconstructive surgery, Universiti Sains Malaysia
RSD	Relative standard deviation

RT	Retention time
SD	Standard deviation
SDS	Sodium dodecyl sulfate
USA	United States of America
USD	U.S. Dollar
USM	Universiti Sains Malaysia
UV	Ultra violet
v/v	Volumer per voume
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
VEGF-B	Vascular endothelial growth factor B
VEGF-C	Vascular endothelial growth factor C
w/v	Weight per volume

RUMUSAN TRANSDERMAL TOPIKAL GEL SILDENAFIL YANG BARU

GEL SILDENAFIL TOPIKAL BARU UNTUK PENINGKATAN KEBOLEHHIDUPAN FLAP KULIT DALAM MODEL ARNAB

ABSTRAK

Kebolehhidupan flap kulit merupakan salah satu hasil yang paling diutamakan dalam pembedahan rekonstruktif. Pelbagai aplikasi agen farmakologi dengan rejimen dos yang berbeza telah menunjukkan kebolehan meningkatkan kebolehhidupan flap dengan darjah peningkatan yang berbeza-beza. Kajian ini bertujuan untuk menghasilkan satu perumusan gel topikal sildenafil sitrat yang berkesan dalam menambahbaik kebolehhidupan flap kulit. Pencirian ciri-ciri fizikokimia dan ujian kestabilan asas telah dijalankan pada perumusan gel tersebut. Pembedahan flap kulit (14 cm x 5 cm) berdasarkan arteri epigastrik atas kaudal telah dijalankan pada sembilan ekor arnab *New Zealand White* (julat berat antara 2.5-3.2 kg). Satu aliquot 1 ml gel yang mengandungi sildenafil sitrat (3 mg) disapukan serta-merta selepas pembedahan pada separuh hujung bahagian flap dalam kumpulan rawatan (n = 3), manakala 1 ml gel tanpa sildenafil dijadikan kumpulan kawalan kenderaan (n = 3), dan tiada sebarang ubatan digunakan untuk kumpulan kawalan (n = 3). Perfusi darah pada bahagian hujung flap dan indeks nekrotik flap kulit diukur secara berterusan selama 10 hari selepas pembedahan flap dengan

menggunakan meter pengaliran darah berasaskan Doppler laser (LDF) dan planimetri satah berdua. Kumpulan rawatan telah menunjukkan pengurangan signifikan pada indeks nekrotik (min ± sisihan piawai) berbanding dengan kumpulan kawalan dan kawalan kenderaan (kumpulan rawatan, 0.25 ± 0.43% *vs*. kawalan kenderaan 5.41 ± 3.50% *vs*. kawalan 5.41 ± 1.53%; p = 0.017). Peningkatan awal yang signifikan pada perfusi darah di bahagian hujung flap berlaku dalam 24 jam pertama selepas pembedahan ($p < 0.001$). Keputusan uji kaji *ex vivo* kadar penelapan dadah sildenafil merentasi kulit arnab (n = 3) menunjukkan bahawa kadar penelapan kandungan dadah melebihi separuh berlaku selepas enam jam (58.03%) dan penelapan dadah sebanyak 95.84% berlaku dalam tempoh 24 jam pertama. Pelepasan dadah perumusan gel sildenafil didapati menepati kinetik tertib pertama untuk tempoh 24 jam pertama dan pemalar eksponen pelepasan, n, didapati melebihi 1.0 dengan analisa persamaan *Korsmeyer-Peppas*. Hasil kedua-dua analisis kinetik ini menunjukkan bahawa perumusan gel sildenafil tersebut mempunyai ciri-ciri pelepasan dadah yang terkawal dengan pelepasan dadah berpanjangan. Secara kesimpulannya, kajian yang menggunakan model arnab ini memperlihatkan bahawa aplikasi tunggal perumusan gel sildenafil (3 mg/ml) berupaya meningkatkan perfusi darah peredaran mikro pada bahagian hunjung flap dan menambahbaik kebolehhidupan flap kulit.

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ABSTRACT

Skin flap survival is one of the most important outcomes in reconstructive surgery. Various applications of pharmacological agents with different dosing regimens have been shown to improve flap survival experimentally with varying degrees. This study aimed to produce an effective topical gel formulation of sildenafil citrate for the improvement of skin flap survival. Characterization of physicochemical properties and basic stability tests were carried out on the gel formulation. Skin flaps (14 cm x 5 cm) based on superficial inferior epigastric vessels was designed and raised on nine *New Zealand White* rabbits (weight range 2.5-3.2 kg). An aliquot of 1 ml gel consisted of sildenafil citrate (3 mg) was applied immediately post-surgery to the distal half of the flap in treatment group (n = 3) while 1 ml gel without sildenafil was applied to vehicle control group (n = 3), and none was applied to controls (n = 3). Blood perfusion at the flap distal end and mean necrotic index of the skin flaps were measured continually for 10 days after the flap surgery using laser Doppler flowmetry (LDF) and 2-plane planimetry, respectively. Necrotic index (mean ± standard deviation) in the treatment group was significantly lower compared to control and vehicle control group (Treatment group, 0.25 ±

0.43% *vs*. vehicle control 5.41 ± 3.50% *vs.* control 5.41 ± 1.53%; $p = 0.017$). Early blood perfusion improvement was observed in the treatment group with significant increase seen at flap distal end on the first 24 hours post-surgery ($p < 0.001$). *Ex vivo* drug release data of the sildenafil gel across rabbit skin (n = 3) showed that more than half of the drug content were released after six hours (58.03%) and 95.84% of the drug were released after 24 hours. The drug release in the first 24 hours was found to follow the first-order kinetic model, and the release exponent, n, was found to be greater than 1.0 using the Korsmeyer-Peppas equation. Both kinetic analyses showed that the current sildenafil gel formulation demonstrated controlled release properties for extended drug release. In conclusion, this study demonstrated that single application of the current gel formulation of sildenafil (3 mg/ml) significantly increased microcirculatory blood perfusion at the flap distal end and improved skin flap survival in rabbits.

CHAPTER 1

INTRODUCTION

1.1 An overview of skin flap surgery

1.1.1 Definition of flap

Flap surgery is a common procedure in plastic and reconstructive surgery. It is a technique performed to cover up an open wound where the edge of the wound cannot be closed primarily. The procedure is particularly useful in the reconstruction of bodily defects left behind by traumatic events, or after the removal of tumour growths such as cancer excisions from the head and neck region or mastectomy (Bagdas *et al.*, 2014; Khouri *et al.*, 1998).

The procedure in flap surgery involves the lifting of any type of tissue from the donor site, and moved to the recipient site, usually a wound with significant tissue loss. The piece of tissue transferred in this procedure is called a flap. In a broad sense, a flap is similar to a graft. However, a flap is a piece of living tissue raised from the donor site with its underlying vasculature intact, while a graft is a tissue transplanted without intact blood vessels (Gurtner and Neligan, 2017). This allows the flap to have a better survival rate compared to a graft as a graft's survival relies solely upon growth of blood vessels after being transplanted to the recipient site (Semer and Adler-Lavan, 2001).

Depending on the reconstructive requirements, a flap can take the form of many shapes with different tissue compositions. It can be as simple as a skin tissue advancement, or it can be a composite of different tissue types that includes muscle, fat, bone and fascia. Usually, the choice of flap tissue is made according to like for like basis, meaning that the donor site with similar tissue structure to the recipient site is preferred (Minas, 2017).

1.1.2 History of flap surgery

As early as 600 B.C., records of upper extremity flap reconstructions have been documented by Susruta of India in *Samahita*. According to the record, nasal reconstruction using a regional pedicled flap from the cheek have been described. During the Roman era, local tissue rearrangements of the head and neck region have been documented by Celsus (25 A.D.) and Oribasius (325 – 403 A.D.) using random circulation pedicled flaps. However, further documentation of flap surgery or any record of technical advancement is scarce for nearly a millennium, until the Renaissance era in Europe (Tschoi *et al.*, 2009).

During the early 15th century, Gustavo Branca of Italy and his son Antonio began experimenting and described the use of local forehead flap for the nose, which they gained insights from the texts of Susruta (Tenenhaus *et al.*, 2008). It was during this time that the first nasal reconstruction using a distant flap harvested from the arm was documented (McDowell *et al.*, 1952).

With advances in the accuracy of anatomic depictions from the Renaissance era, through the Industrial Revolution, leading towards the period of the world wars, coupled with vast improvements in surgical techniques became the basis for the improvement of flap surgery techniques. Flap surgery enters a phase of significant improvement in the period of the two world wars. During that period, it was realised that reconstruction of the extremities, particularly the hand was very important for maintaining the function of the upper limb. This led to the transfer of flap surgery techniques used in head and neck reconstruction onto the reconstruction of upper extremities. At the same time, improvements in distant flap reconstruction techniques allows for the advent of hand surgery, committed towards addressing major upper extremity trauma during World War II (Chambers *et al.*, 2010; Chambers and Ray, 2009; Fang and Chung, 2014).

With the arrival of microvascular free tissue transfer in the 20th century, flap surgery techniques took another leap forward. Before this, flap surgery was mostly localised pedicled flaps. The introduction of modified operating microscope by Julius Jacobson and Ernesto Suarez in the 1960s made free tissue transfer possible, thus bringing forth the era of free flaps which opened up endless possibilities and applications of flap tissue transfer in plastic and reconstructive surgery (Fang and Chung, 2014).

In the following decades, with the availability of information, surgical literature documenting each innovative technique could be widely disseminated, allowing continued development of flap surgery up to this date (Lamberty *et al.*, 1990).

1.1.3 Classification of skin flap

Skin flaps are a type of flap tissue that consist of skin and subcutaneous tissue lifted from the donor site together with its underlying vasculature. The flap may or may not come together with the fascia depending on its intended use. According to literature, skin flap comes in a multitude of shapes and forms, causing the classifications and naming of skin flap to be varied. The principle of classifying the skin flaps is intended to aid in the communication among surgeon, thus similar flaps can have different names on different occasions, depending on the purpose of communication. Nonetheless, the two most common principles of classifying skin flaps are based on the type of blood supply, or according to whether the flap is a pedicled or free flap (Hallock, 2004).

Based on the type of blood supply, skin flaps can be categorized into two major types, i.e. axial pattern flaps and random flaps. Axial pattern flaps are supplied by a specific, identifiable artery running along the longitudinal portion of the flap. The identified blood vessel (pedicle) will serve as the main blood supply for the flap, allowing the surrounding tissue to be detached from the donor site while maintaining the viability of the flap tissue. Axial pattern flaps have high success rates as a result of good circulation of blood supply from the pedicle. However, careful planning in locating the blood vessels that will be used as the pedicle is crucial and injury towards the pedicle will often result in failure of axial pattern flaps (Semer and Adler-Lavan, 2001).

On the other hand, random flaps are supplied through non-specified blood vessels, or in a more accurate manner, there is an absence of a main artery running across the random flap. Hence, the circulation of the random flap is dependent on the number of microvascular connections that the pedicle has. The more profuse the vascular connection, the greater the chance of survival of the random flap. Although the blood supply in a random flap may not be as reliable in an axially supplied flap, random flaps have its advantages in ease of application, allowing it to be created and used on almost any part of the human body (McGregor and Morgan, 1973).

Apart from classifying skin flaps according to the type of blood supply, it can be classified with reference to the pedicle of flap. According to this classification, there are two types of skin flaps, namely pedicled flaps and free flaps (Geiger *et al.*, 2016). Pedicled flaps are created with its original blood supply intact, thus the donor site is usually not too distant from the recipient site. This type of flap does not require microsurgical anastomosis of the pedicle to re-establish the blood flow in the tissue transferred. As such, the time required for the procedure is shorter compared to free skin flaps, and the fact that the blood supply in the flap tissue remained intact throughout the surgical process ensure a high chance of flap survival. Hence, the pedicled flap is preferred in cases where the patient could not undergo long hours of surgery (McCrory and Magnuson, 2002). Nonetheless, there is limited options for pedicled flaps as its shape, mobility and position of the flap are greatly hampered by the pedicle. The possibility of pedicle entanglement during the transposition of the flap tissue also poses risk of causing inadequate blood supply in the flap, increasing the chances of flap necrosis and failure (O'Neill *et al.*, 2010).

With the advancement of microsurgery and free tissue transfer, free flaps became a new option distinctive from the pedicled flap. Free flaps are created by total detachment from its donor site and transferred to the recipient site. Blood flow in the flap will then be re-established by surgical anastomosis of the arterial pedicle, and in some cases, anastomosis of the vein to increase venous output are also done. As it is not limited by the position of the pedicle, free flaps have a higher degree of freedom in terms of its location of harvest as well as position of the recipient site (Kruse *et al.*, 2010; Wu *et al.*, 2014). However, the use of free skin flaps is also met by many challenges. Since the flap tissue is totally detached from the donor site, there will be a significant period of ischemia experienced by the flap tissue, causing unwanted stress and injury to the tissue structure. Thrombotic occlusion and the subsequent ischemic reperfusion injury after the flap is being anastomosed will also cause unwanted flap necrosis, as well as flap failure in severe cases (Kerrigan and Stotland, 1993; Pattani *et al.*, 2010; Siemionow and Arslan, 2004).

1.2 Skin flap survival: Burden and challenges

Recent advances in surgical equipment and technique oversaw marked improvements in skin flap success rates. Yet, complications of flap surgery, including partial and total skin flap failure remained a challenge for practitioners across the globe. Management of these complications are often expensive, and the morbidity and psychological stress that follows can be a burden to both patients and practitioners. Although there is lack of comprehensive review showing global or regional statistics of flap failure, there are abundant literature of individual report by practitioners and institutions documenting failure rates of flap transfer ranging from 5% to 10% (Phillips *et al.*, 2012; Wang *et al.*, 2012b; Wu *et al.*, 2014).

In a paper published in 2011, it was reported that an estimated number of 19000 free tissue transfers were being performed in the United States alone in 2008 for a variety of reconstructive purposes. A total of 264 free flaps have been studied in a retrospective review in the Division of Plastic Surgery at Brigham and Women's Hospital over a period of five years revealed that thrombosis related flap complications was about 4.9% with 1.9% total failures. The average cost disparity between flap complication and case-controlled uncomplicated flaps was about USD$ 23246 which is a burden especially in developing countries (Bowman and Carty, 2011).

Another study conducted from January 2004 to April 2010 at Middlemore Hospital, New Zealand reported a success rate of 96% (Gao and Loo, 2011). However, a breakdown of the statistics reported shows that out of the 100 cases of free flap transfer, 21 cases presented with complications. Out of these, 14 cases were returned to the surgical table for salvage procedures. Among these, eight cases require a redo of the anastomoses. A total of four cases of total flap failure and five cases of partial flap failure were recorded.

1.3 Pathophysiology of skin flap failure

Ischemia of the flap tissue is the main culprit in most instances that lead up to flap failure. Ischemia is defined as a condition of inadequate blood supply to a certain area of tissue. Inadequate blood supply will lead to insufficient oxygen and nutrient intake by the cells in the area, forcing the tissue cells to adopt anaerobic metabolic pathways. Prolonged period of ischemia will lead to derangement of tissue health,

and will trigger a cascade of biochemical pathways that are detrimental to the survival of the tissue (Siemionow & Arslan, 2004).

In the case of free skin flaps, the tissue lifted from the donor site experience a period of ischemia as soon as it is detached from the donor site. Depending on the surgical conditions, this period have an average range of about 1.5 hour to 3 hours. During this period, the flap is being transferred to the recipient site and vascular anastomosis is being performed. Although it may not be evident at this point, technical and physiologic factors occurring during this time could cause unwanted flap loss (Siemionow and Arslan, 2004).

There is no definitive answer to how long the primary ischemic period will be tolerated, or when the injury from this period will become irreversible. The consensus is that increased ischemic period will lead to a decreased rate of flap survival, as well as a lower percentage of survival area. After the re-establishment of blood flow in the flap tissue, injury sustained during the ischemic period will most likely be reversed and the flap would survive. However, if an unwanted episode of secondary ischemia occurs, the injury induced will be even more lethal as a secondary ischemia is less tolerated by the tissue (Dorweiler *et al.*, 2007).

Flap loss is commonly categorised into partial and total flap failure. Partial flap loss is often associated with ischemia at the distal portion of the flap as a result of failure to maintain critical capillary pressure to perfuse the region. On the other hand, total flap failure is usually a consequence of secondary obstruction to the main supply vessel (pedicle), causing no-reflow phenomenon (Pang, 1990). Events of prolonged

ischemic period or occurrence of a secondary ischemia can be caused by a number of factors. These factors can be categorised into technical and physiological factors.

1.3.1 Technical factors related to skin flap failure

Technical factors are extrinsic factors that arise from problematic surgical procedures. While it may be unintentional, it could be reduced through experience and careful pre-operation planning by the surgeons.

Anastomoses of the arterial inlet or venous output that are not properly done is a common cause of prolonged ischemia in flap surgery. Injury to the arterial inlet or a faulty suturing while reconnecting the pedicle would lead to blood loss at the pedicle. Loss of hemodynamic pressure at the pedicle of flap will greatly hamper the ability of the pedicle to properly supply the flap tissue, which would then lead to ischemia of the flap, especially at the region distant to the pedicle (Granzow *et al.*, 2015).

In some cases, especially among pedicled flaps, folding and entanglement of the pedicle causing inhibition of blood flow at the pedicle can also be a factor of prolonged ischemia. This can happen during the process of transposing the flap to the recipient site, where the flipping and rotational movement of the flap tissue resulting in the folding of the pedicle (Vaienti *et al.*, 2013).

The size of the flap is also a factor causing prolonged ischemia. As the flap tissue is being supplied by a sole pedicle, the decrease in hemodynamic pressure as blood travels farther away from the pedicle means that the farther the tissue from the pedicle, the harder it is to be perfused by the pedicle. As a result, the distal region

relative to the position of the pedicle, are at risk of prolonged ischemia, especially in oversized flaps (Shasti *et al.*, 2017).

1.3.2 Physiological factors related to skin flap failure

Physiological factors of flap ischemia are intrinsic factors pertained to physiological changes that occur as a result of the flap surgery. These physiological factors include vasoconstriction of blood vessels in the flap tissue as well as occurrence of thrombosis in the flap.

Vasoconstriction of the blood vessels, either at the pedicle or at the peripheral branches of arteriole, will inhibit blood flow towards an area of flap tissue. This will result in partial or total ischemia of in that area. As soon as the flap is being detached from the donor site, vasoconstriction occurs as a result of diminished blood flow. Vasoconstriction of the vessels may also induce other physiological changes such as release of thromboxane A2 and other toxic metabolites that will promote free radical induced damage to the tissue (Dorweiler *et al.*, 2007).

Thrombotic events during and after the flap surgery will lead to occlusion of blood vessels, causing prolonged ischemia. If the thrombotic event occurs after the re-establishment of blood flow, a secondary ischemic insult will again cause more damage to the tissue. According to a study reported by Makiguchi and colleagues in 2012, thrombotic events occurred at a rate of 7.5% in total of 200 cases of flap surgery in the head and neck region (Makiguchi *et al.*, 2012). In a separate study by Bowman and Carty (2011), flap complications caused by thrombotic events were recorded at 4.9%.

1.4 Reperfusion injury

Reperfusion injury is another aspect of concern in the pathophysiology of flap tissue failure. Although it is important to ensure that the flap is properly perfused after the surgery, the establishment of blood flow after the ischemic period will bring about physiological changes in the blood vessels that may eventually lead to additional tissue injury. This type of injury is termed ischemic reperfusion injury in free tissue transfer. While the susceptibility of a flap towards reperfusion injury differs in different type of flap tissue, it is well noted that the extent of reperfusion injury is proportionate to the length of ischemic period before reperfusion occurs (Wang *et al.*, 2011; Widgerow, 2014).

During the reperfusion of flap tissue, inflammatory substrates that enters the tissue together with the restored blood flow, brings about free radical mediated damage to the tissue. These free radicals such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH), could cause structural changes to important biochemical molecules like amino acids, membrane surface proteins, cytochrome enzymes and nucleic acids (Dorweiler *et al.*, 2007; Eisenhardt *et al.*, 2012; Manson *et al.*, 1983; Siemionow and Arslan, 2004).

1.5 Methods in improving skin flap survival

Despite the high success rate of flap surgery, flap necrosis and failure still occur. This may result in a number of unwanted complications such as infections, loss of tissue function as well as cosmetic loss. Hence, many methods have been tried and studied over the decades to improve flap survival and to prevent post-operative

complications. These methods can be categorized into technical and pharmacologic interventions.

1.5.1 Technical intervention in improving skin flap survival

One of the common methods of improving the survival rate of flap tissue is by ischemic preconditioning, also known as a delayed flap. This is done by subjecting the donor tissue to short periods of ischemia prior to the surgery. The procedure is usually performed one to two weeks prior to the tissue transfer. In this way, the injury caused by the actual ischemic period during the flap surgery will be better tolerated by the flap tissue, resulting in higher rate of success (Ghali *et al.*, 2007).

Early effects of the delay procedure have been attributed to alteration of sympathetic tone, causing vasodilation by means of depleting norepinephrine in the sympathetic nerves. Other early effects include dilatation of choke vessels, as well as changes in tissue metabolism resulting in reduced metabolic requirements by tissue, less radical formations, and reduced infiltration of neutrophils and cell apoptosis allowing greater tolerance to ischemic insult (Maitz *et al.*, 1994). Late effects of delay procedure oversee a series of anatomical changes in the flap tissue prior to the flap surgery. These changes include increase in vascular density by means of angiogenesis and vasculogenesis through the increased production and release of cytokines and angiogenic factors such as vascular endothelial growth factor (VEGF) (Holzbach *et al.*, 2009).

Although the delay procedure is an effective technique in increasing the success rate of flap surgery, especially in reducing distal flap necrosis and partial flap failure, the technique requires a second surgical procedure and anesthesia which often leads to additional costs for both the healthcare system and patients (Ghali *et al.*, 2007; Harder *et al.*, 2008; Küntscher *et al.*, 2005).

1.5.2 Pharmacologic agents in improving flap survival

Although surgical refinement has greatly increased the survival rate of skin flaps, skin flap failures were still being reported at about 2 - 10% of the cases, and 5 - 25% of free flaps require re-exploration due to compromised circulatory functions (Karsenti *et al.*, 2010). Hence, in addition to improved surgical equipment, technique and procedural planning, plastic and reconstructive surgeons have experimented on various pharmacological agents to improve skin flap survival. These pharmacological agents often aimed to increase skin flap survival by improving blood perfusion in the transferred tissue or alleviating the effects of ischemic reperfusion injury.

As tissue injury of skin flaps follows a complex series of pathophysiologic pathways, where processes such as thrombosis, ischemia reperfusion injury or the no-reflow phenomenon, and vasospasm may combine to play a role in causing skin flap failure. Hence, different pharmacological agents with different mechanisms may account for the improvement of skin flap survival, which may include prevention of microcirculatory failure, the inhibition of inflammation, and the induction of ischaemic tolerance (Harder *et al.*, 2008). Thus, pharmacological treatments for complications in skin flap surgery may focus on thrombosis (anticoagulants),

ischemic reperfusion injury or no-reflow phenomenon (ischemic tolerance modulators such as antioxidants, free radical scavengers and growth factors), as well as vasospasm (vasodilators) (Hyza *et al.*, 2014).

Since the complication and failure of skin flap surgeries can manifest through a plethora of pathological processes, and in each case, a certain process predominates over the others, management of post-operative care in skin flap surgery depends greatly on the experience and individual preference of the surgeon in specific therapy selection (Hyza *et al.*, 2014). Throughout the years, various pharmacologic agents have been proposed for improving skin flap survival, yet, there is no general consensus on treatment guidelines (Pršić *et al.*, 2015). Although there were numerous animal studies on the different pharmacologic agents proposed along with few prospective clinical trials that dotted the literature, the lack of unified science behind the use of pharmacotherapy in the treatment of skin flaps resulted in anecdotal practice by individual surgeons with marked differences in medications, dosing and timing (Conrad and Adams, 2001). Owing to the extensive propositions of different pharmacologic agents in literature, many medications have been shown, to a certain extent, to be successful in improving skin flap survival. Thus, there are currently many pharmacologic agents that can be used in treating skin flaps, despite the absence of standard algorithm in their application. As such, drug choices may vary according to institutions, subjecting to the preferences of the practitioners as well as the availability of the individual medication (Ricci *et al.*, 2016). A few of the commonly employed pharmacologic agents are further discussed in the entailing sections.

1.5.2 (a) Anti-thrombotic agents

One of the major causes of flap failure is due to thrombosis of blood vessels in the flap tissue, resulting in inadequate blood flow. Hence, anti-coagulants and anti-platelet medications have been used to prevent thrombosis in the flap post-operatively. It is reported that about 96% of surgeons have used some form of antithrombotics post-operatively to improve flap survival (Glicksman *et al.*, 1997; Spiegel and Polat, 2007). Anti-thrombotics used include Aspirin, low molecular weight dextran, heparin, prostaglandin E1 and tissue coagulative factor inhibitors. Although individual experiments have reported some degree of improvement in flap survival by applying antithrombotics post-operatively, broad scale reviews and meta-analysis have shown little or no significant protective effect being conferred by anti-coagulant therapy (Askari *et al.*, 2006; Pršić *et al.*, 2015). A systematic review with multicentre, individual patient data meta-analysis by Schwartz and colleagues in 2015 have reported that antithrombotics did not significantly improve flap survival rate. They have also noted an increased in flap complications when heparin is administered in patients (Swartz *et al.*, 2015).

In another separate study, different antithrombotics were used on skin flaps in small animal models to elucidate the effects of these agents in improving the viability of skin flaps. However, the study shows that even though antithrombotics such as clopidogrel and hirudin improves the viability of skin flaps, these pharmacologic agents puts the subject at a higher risk of developing post-operative complications (Fichter *et al.*, 2016).

1.5.2 (b) Vascular endothelial growth factor

Growth factors, particularly vascular endothelial growth factor (VEGF), have the ability to induce vasodilation and angiogenesis. Thus, VEGF have long been suggested as a form of pharmacologic treatment to improve flap survival (Zhang *et al.*, 2004).

Vascular endothelial growth factor has been experimented on various animal models and have shown great promise in improving flap survival. Administration of VEGF can be done through intra-muscular, subcutaneous, subdermal, intra-arterial injections, systemic intake as well as topical applications. The treatment can be applied during the flap surgery (acute administration), or before the flap tissue is being raised for transfer (pre-operative treatment) (Kryger *et al.*, 2000).

The effects of VEGF include acute vasodilation and increased capillary permeation in flap tissue. It also stimulates migration and proliferation of endothelial cells, leading to formation of new microvessels. Formation of new microvessels eventually lead to increased vascular connections between the flap tissue and its underlying vascular bed (recipient site), promoting a variety of wound healing effects resulting in higher tissue survival rate (Fang *et al.*, 2014; Michlits *et al.*, 2007)

Although various studies on VEGF treatment on flap surgery have shown promising results in increasing flap survival, the relatively short half-life of VEGF causes the availability of VEGF in the flap tissue to be poorly sustained (Taub *et al.*, 1998). Attempts of increasing the availability of VEGF in situ by means of gene therapy have been experimented (Taub *et al.*, 1998; Yu *et al.*, 2003; Zheng *et al.*, 2008).

However, these studies are limited to animal trials and its application remain prospective at best at the moment. Furthermore, despite improvements in skin flap survival were demonstrated in a number of studies using VEGF, the overall effectiveness of VEGF in treating flaps were not consistent (Fang *et al.*, 2014). In some studies, the treated flaps shows survival rate as high as 97.2% (Yi *et al.*, 2005), while in some studies, the survival rate of treated skin flaps was only 64.3% (Liu *et al.*, 2005).

1.5.2 (c) Free radical scavengers and antioxidants

Release of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl, and the depletion of glutathione (an antioxidant) are responsible for causing cellular damage and tissue death in ischemic reperfusion injury (Dorweiler *et al.*, 2007). A number of pharmacologic agents have been studied to address the detrimental effects of reactive oxygen species generated after the reperfusion of skin flap (Campos and Yoshida, 2004). These agents include free radical scavengers and antioxidants.

Allopurinol is an inhibitor of xanthine oxidase, which reduces formation of reactive oxygen species. The effect of allopurinol on the survival of skin flaps have been studied for many years, yet the results remained controversial (Milcheski *et al.*, 2013; Moura *et al.*, 2009; Picard-Ami *et al.*, 1992; Rees *et al.*, 1994). A recent study reported in 2017 showed that systemic administration of allopurinol for one week prior to flap surgery showed protective effect on skin flaps (Ardakani *et al.*, 2017). However, there is still no uniform and practical way of applying allopurinol in flap surgery and thus its practicality as a choice treatment remain inconclusive.

Another recent study by Fukunaga and colleagues studied the efficacy of a novel radical scavenger nitrosonifedipine in reducing ischemic necrosis of skin flap in mouse models. Daily dose of nitrosonifedipine (30 mg/kg) suspended in normal saline containing 1% carboxymethycellulose as vehicle was injected subcutaneously at the centre of the flap for up to a week. The study demonstrates that nitrosonifedipine reduces the percentage area of skin flap necrosis (approximately 20% increase in viable area), and reduces cellular and tissue damage caused by oxidative stress (Fukunaga *et al.*, 2017).

Other antioxidants that have been tried to ameliorate skin flap survival includes vitamin C (ascorbic acid), melatonin, N-acetylcysteine, and superoxide dismutase. Although these agents have been shown to improve skin flap survival, neither of them has been tested and applied in standard clinical settings (Kerem *et al.*, 2014; Tunç *et al.*, 2016; Zaccaria *et al.*, 1994).

1.5.2 (d) Vasodilators

Another major type of pharmacologic agents that have been identified as potential choice of treatment of skin flaps are vasodilators. Reducing the incidence of vasospasm and vasoconstriction after the flap surgery greatly reduces the ischemic period of skin flaps, especially at the distal part of the flap. In addition, it also reduces the chances of a secondary ischemia caused by vasoconstriction. Vasodilators can be broadly categorized into five types, namely, calcium channel blockers, local anaesthetics, alpha antagonists, direct vasodilators and phosphodiesterase inhibitors (Vargas *et al.*, 2015).

Calcium channel blockers reduces intracellular calcium concentration in vascular smooth muscle cells by antagonising the voltage gated calcium channels. Decreased calcium concentration reduces rate of actin-myosin interaction, hence, relaxation of the vascular smooth muscle (Roland *et al.*, 1998). Popular calcium channel blockers include nicardipine, verapamil and nifedipine.

Local anaesthetics have spasmolytic effects on smooth muscles. Apart from that, it also prevents depolarisation and activation of calcium channel. These effects will allow the blood vessels to dilate, increasing blood flow into the flap tissue. Lidocaine is a type of local anaesthetic that have been widely tested for its effects in treating skin flaps (Hou *et al.*, 1987). It has been shown that 12% lidocaine is effective in resolving microvascular constrictions on many occasions (Beekman *et al.*, 1990; Beekman *et al.*, 1988) , while in some other studies, 20% lidocaine have been reported to be effective (Johnstone *et al.*, 1995; Puckett *et al.*, 1985). However, these studies are limited to animal studies. Since the safety of lidocaine dosage in excess of 2% have not been established for human use, the suitability of lidocaine for human flap surgery is very limited at this moment (Vargas *et al.*, 2015).

Activation of alpha adrenergic receptors stimulate release of calcium ions from the sarcoplasmic reticulum, which leads to contraction of vascular smooth muscles. Inhibition of these receptors allows the smooth muscles to relax, thus causing the blood vessels to dilate. Alpha adrenergic antagonists include phentolamine and chlorpromazine. Direct vasodilators such as sodium nitroprusside produces nitric oxide, which increases intracellular cyclic guanosine monophosphate, inhibition of myosin light chain kinase, and vasodilation. The efficacy of sodium nitroprusside as

a treatment for skin flap remain inconclusive as not all experimental results shown significant improvement after surgery. Furthermore, sodium nitroprusside produces harmful by-products such as methaemoglobin and cyanide in the body, increasing the metabolic burden on the patient (Huang and Li, 1991; Vargas *et al.*, 2015; W. Notodihardjo *et al.*, 1998).

Phosphodiesterase inhibitors causes vasodilation by inhibiting phosphodiesterase and inactivates cyclic guanosine monophosphate. This leads to a secondary inhibitory effect on myosin light chain kinase, causing relaxation of smooth muscle cells in the blood vessels. A few phosphodiesterase inhibitors such as papaverine, pentoxifylline and amrinone have been studied and reviewed on its efficacy in reducing vasospasm in flap surgery. Papaverine and pentoxifylline have been shown to be effective in animal studies, though its usability in a human clinical setting have not been studied (Evans *et al.*, 1997; Monteiro *et al.*, 1986; Vargas *et al.*, 2015). Amrinone, on the other hand, have been tried on human skin flap surgery. However, its adverse effects may hamper its application in clinical use (Ichioka *et al.*, 2001).

In recent years, a specific type of phosphodiesterase inhibitors, namely phosphodiesterase type 5 (PDE-5) inhibitors, have been the target of many studies on its effect on improving skin flap survival (Bandera *et al.*, 2010; Brewer *et al.*, 2012; Kayiran *et al.*, 2013). The notable examples of PDE-5 inhibitors are sildenafil, vardenafil and tadalafil. This type of drugs prevents the degradation of 3',5'-guanosine monophosphate (cGMP), which are responsible for vasodilation and relaxation of cavernosal smooth muscles. Currently, PDE-5 inhibitors are mainly indicated for the treatment of erectile dysfunction (Gresser and Gleiter, 2002;

Jackson *et al.*, 2005) and treatment of pulmonary hypertension in infants (Iacovidou *et al.*, 2012; Wang *et al.*, 2014). In a study reported by Kaya and colleagues in 2015, oral administration of sildenafil, vardenafil and tadalafil significantly improves the survival rate of skin flaps in rats (Kaya *et al.*, 2015).

1.6 Sildenafil as a potential treatment to improve skin flap survival rate

1.6.1 Brief history of sildenafil

Sildenafil is a type of PDE-5 inhibitor discovered by scientists in Pfizer's European Research HQ in Sandwich, UK. Figure 1.1 shows the chemical structure of sildenafil citrate. It was originally developed to treat angina by augmenting the nitric oxide mediated cyclic guanosine monophosphate synthesis pathway (cGMP) (Jackson *et al.*, 2005). Cyclic guanosine monophosphate causes arteries to dilate by initiating the relaxation of vascular smooth muscles, thus increasing blood flow in the arteries. Sildenafil does not directly increase nitric oxide levels like other commonly used nitrate drugs during the 1980s and 1990s. Rather, it inhibits the PDE-5 mediated hydrolysis of cGMP, resulting in increased cGMP levels. Hence, it was hypothesised that sildenafil can be used as a vasodilator without the adverse effects of nitric oxide (Connelly, 2017). Figure 1.2 shows a schematic diagram of how PDE-5 inhibitors such as sildenafil can augment the vasodilatory effect of the nitric oxide/cGMP pathway.

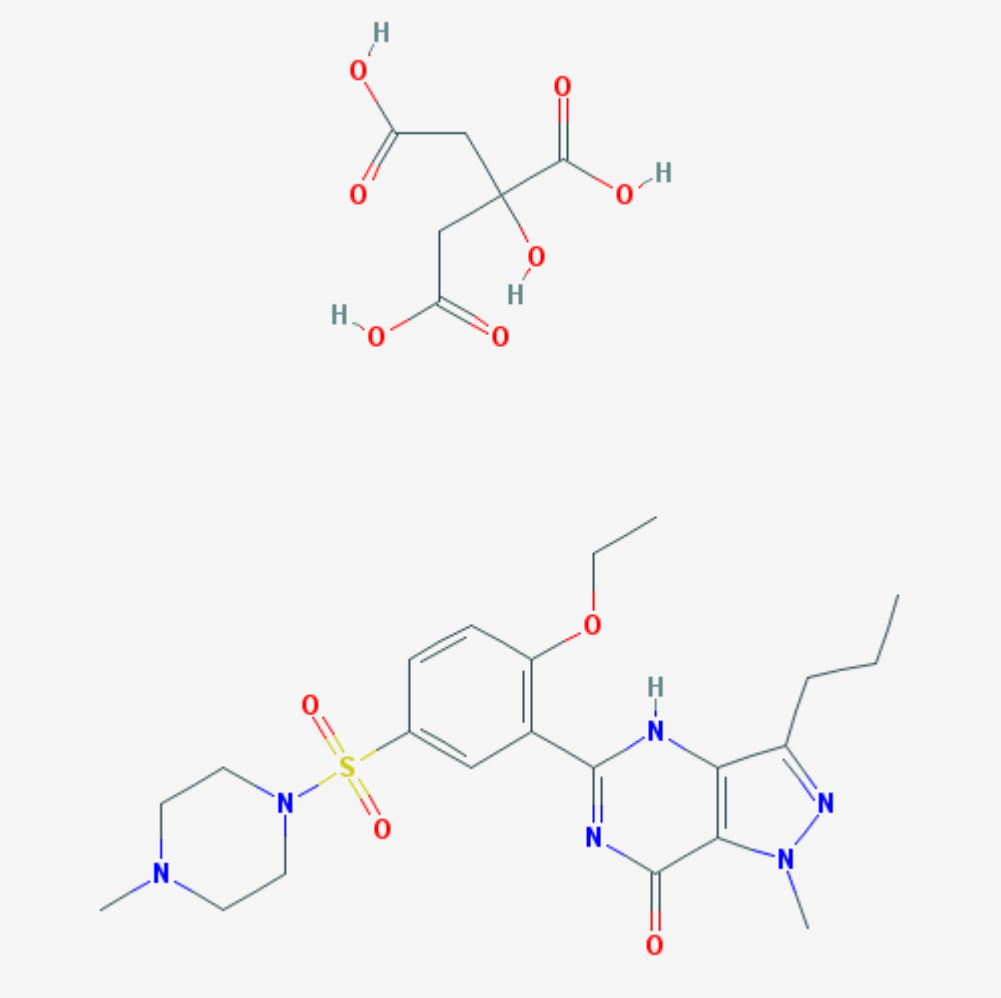

Figure 1.1 Chemical structure of sildenafil citrate. Source: PubChem, Compound Summary for CID 62853. Image retrieved on June 2nd, 2018. Image source: https://pubchem.ncbi.nlm.nih.gov/compound/Sildenafil_citrate#section=Top

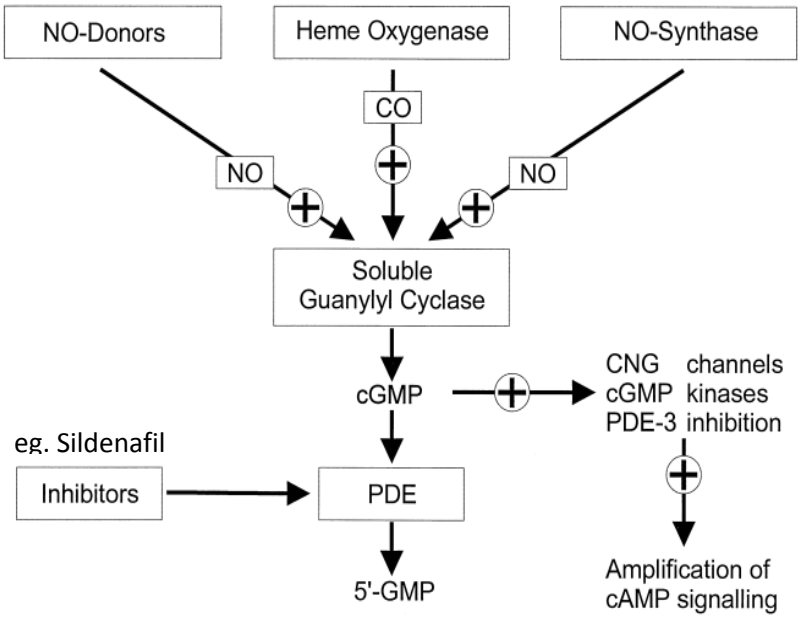

Figure 1.2 Molecular events in a soluble guanylyl cyclase signaling pathway. The soluble guanylyl cyclase can be activated pharmacologically and physiologically by the gaseous messengers (indicated by the (+) sign). Inhibitors of the cGMP PDE will amplify (+) responses downstream (e.g., on CNG channels, kinases) and, depending on the simultaneous presence of PDE-3, even amplify cAMP signaling.

Image source: Glossmann, H., Petrischor, G., & Bartsch, G. (1999). Molecular mechanisms of the effects of sildenafil (VIAGRA®). Experimental gerontology, 34(3), 305-318.

Abbreviations:

NO : Nitric oxide
CO : Carbon monoxide
cGMP : Cyclic guanosine monophosphate
CNG : Cyclic nucleotide-gated channels
PDE : Phosphodiesterase
cAMP : Cyclic adenosine monophosphate

Its relatively short half-life of four hours, and the fact that its haemodynamic properties have not been shown to be significantly superior over the nitrate drugs, there was limited usage of sildenafil to treat angina (Nichols *et al.*, 2002). Despite that, it was noticed that among the few of the noted side effects of sildenafil such as flushing, headache, dizziness, nasal congestion, heartburn, dyspepsia and visual disturbance (Dündar *et al.*, 2001; Moreira Jr *et al.*, 2000; Vobig *et al.*, 1999), penile erection stood out among them. At the time, there is no oral medication for the treatment of erectile dysfunction. Thus, the idea of utilizing sildenafil as a potential oral medication for erectile dysfunction (ED) prompted the scientists at Pfizer to switch focus and by March 27, 1998, the FDA of the United States of America approved the use of sildenafil as an oral treatment for erectile dysfunction (Jackson *et al.*, 2005).

Although sildenafil is currently only approved for the treatment of ED, ongoing investigations are being carried out to study its potential application in other clinical areas. These include studies on its potential in treating premature ejaculation (McMahon *et al.*, 2003; Salonia *et al.*, 2002), female sexual dysfunction (Laan *et al.*, 2002), as well as cardiovascular diseases (Lundberg *et al.*, 2015; Wang *et al.*, 2012a). The results of these studies are mixed and many of them are still in preclinical or early clinical studies (Jackson *et al.*, 2005).

1.6.2 Effect of Sildenafil on Skin Flap Survival

Vasodilators have been implicated to be useful in treating skin flaps to reduce necrosis area and to improve skin flap viability. Among the numerous vasodilators reported to have a beneficial effect on skin flap survival, sildenafil appears to be a