In vitro and *in vivo* oxygen delivery applications and its limitations

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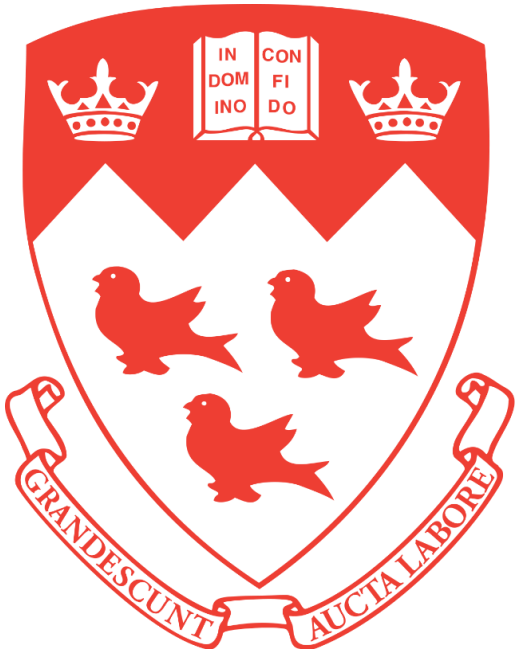

October 2019

A thesis submitted to McGill University in partial fulfillment of the requirements for the degree

of

Doctor of Philosophy

Dedication

This document is dedicated to my family and friends

"I believe that scientific knowledge has fractal properties, that no matter how much we learn, whatever is left, however small it may seem, is just as infinitely complex as the whole was to start with. That, I think, is the secret of the Universe."

— Isaac Asimov

Acknowledgement

I would like to thank Dr. Jake Barralet for your hard work, guidance, patience, and occasional push that I have sometimes needed. I have learned a lot during these past years, this would not have been possible without you.

I would like to express sincere appreciation to my committee members: Dr. Jake Barralet, Dr. Svetlana Komarova, Dr. Lucie Lessard and Dr. Simon Tran, for your valuable advices and guidances.

I would like to thank Dr. Mirko Gilardino and Dr Kevin Watters for your teachings and contributions to this work.

I would like to thank Dr. Huaifa Zhang and Dr. Hani Shash for your contribution to this work.

I would like to thank the staff in Animal Resource Division of the Research Institute of the McGill University Health Center for their help and support.

I would like to thank every lab member from Dr. Barralet's and Dr. Merle's lab: Dr. Baptiste Charbonnier, Zishuai Zhang, Francis McEachern, Aslan Baradaran, Andrew Gorgy, Marianne Comeau-Gauthier, Nazhat Yafi, Daniela Vieira, Mohamed Abdulla, Sophie Maillard, Dr. Omaer Syed for your friendship, help and support through the past years.

Author contribution and statement of originality

1. Bioinnorganic materials for wound healing

Benjamin Dalisson, Jake Barralet

- Benjamin Dalisson gathered the literature and wrote the manuscript
- Jake Barralet supervised the study, directed and revised the manuscript

Originality: For the first time we reviewed the effects of different inorganic molecules and ions on the different phases of wound healing and their possible application in the development of new wound dressings.

2. Anti-necrotic oxygen releasing wound dressings

Benjamin Dalisson, Huaifa Zhang, Hani Shash, Kwatters, Mirko Gilardino, Jake Barralet

- Benjamin Dalisson designed the experiments, prepared and tested the materials, performed the surgeries, measurements, analyzed the data wrote the manuscript
- Huaifa Zhang designed the material
- Hani Shash advised on and preformed pilot surgical procedures
- Kevin Watters trained and directed Benjamin Dalisson for histological section analysis
- Mirko Gilardino trained Benjamin Dalisson for the surgeries and cosupervised the study.
- Jake Barralet supervised the study and revised the manuscript

Originality: For the first time a topical oxygen releasing wound dressing was developed to prevent necrosis of the tissues and improve wound healing speed in an ischemic ear wound model.

3. Preservation of ischemic skin flaps using oxygen delivery implants

Benjamin Dalisson, Jake Barralet

- Benjamin Dalisson designed the material, the experiments, performed the surgeries, measurements, data acquisition and analysis and wrote the manuscript
- Jake Barralet supervised the study and revised the manuscript

Originality: An oxygen releasing implant was developed and implanted *in vivo* to evaluate the ability of subcutaneous oxygen delivery to prevent necrosis in rat full thickness ischemic skin flaps as well as its limitations. Unlike a previously reported material differences in necrosis were not short lived.

4. Ongoing work: Self-oxygenating bioinks

Benjamin Dalisson, Jake Barralet

- Benjamin Dalisson designed the materials, the experiments, performed the experiments, measurements, data acquisition and analysis and wrote the manuscript
- Jake Barralet supervised the study.

Originality: For the first time oxygen releasing microparticles were developed and incorporated in a gel with a high density of cells (10^8cell/mL) with the intent of creating a bioink able to prolong cell survival in tissue engineered constructs *in vitro*.

Abstract

Blood's major function aside from being a source of immune cells, is to supply oxygen, nutrients and to provide a sink for metabolic wastes. Tissues are reliant on a sufficient blood flow to maintain their viability, and if the blood flow is reduced or interrupted, tissues ultimately die at a point known as the ischemic limit. Oxygen is a major limiting factor of tissue survival during ischemia: it is an essential component of the oxidative phosphorylation that produces energy for cells. It is not uncommon to find clinical situations in which oxygen concentration fails to meet metabolic requirements, and even a mild ischemia e.g. due to diabetes can impair wound healing. A more severe and prolonged ischemia due to a trauma or a surgery may result in much more extensive damage.

Many different technologies have been reported to address clinical issues related to oxygen insufficiency such as hyperbaric oxygen therapy (HOBT), perfluorocarbon technologies, hemoglobin-based carriers, peroxides, etc. Amongst all of these technologies, peroxides, e.g. hydrogen peroxide, sodium percarbonate and calcium peroxide have the particular advantage of producing oxygen *in situ*. Indeed, while it would require several hundreds of grams of red blood cells, several kilograms of perfluorocarbons or plasma to deliver one liter of pure oxygen gas, less than gram of peroxides would be required. However, peroxides have an inherent toxicity that makes them of limited value for cell and tissue applications wherein direct of close or direct contact with tissues required.

This thesis investigates the potential benefits of a calcium peroxide-based biomaterial previously developed in our laboratory to deliver oxygen locally. The rapid decomposition of the compound, that forms both oxygen and hydrogen peroxide, is controlled using hydrophobic polymers. Inorganic catalysis transforms the toxic hydrogen peroxide byproduct into water and oxygen thus reducing the inherent toxicity of the compound. The material was processed into different forms and tested in topical, subcutaneous and as an *in vitro* cell support or self-sustaining scaffold. This is important because common issues resulting in insufficient or damaged vascularization such as ageing, diabetes, radiotherapy, trauma, etc., are not easily treatable and give rise to significant reduction in quality of life and increase healthcare costs. Indeed, tissue necrosis may occur at the center of large ischemic wounds and spread to the surrounding healthy tissue causing significant tissue loss. An oxygen releasing wound dressing was designed and used as a patch to prevent necrosis and promote healing in large ischemic wounds.

Similarly, skin flaps are an important approach to reconstructive wound repairs that when performed, damage a part of the skin's vascularization and can lead to ischemia if not sufficiently perfused. Outcomes of these procedures are worsened by common comorbidities like diabetes, radiotherapy, etc., and often result in necrosis. To date the *in vivo* delivery of oxygen to prevent ischemic damages has hardly been studied. A subcutaneous implant generating oxygen was developed to determine if the sub-dermal oxygen delivery could significantly prevent ischemic necrosis and to study the limitation of oxygen delivery in a rat ischemic skin flap model.

Absence of a blood flow can also be an issue in tissue engineered constructs, whether it is *in vitro* or upon implantation, as they do not possess intrinsic functioning vascularization. For this reason, the size of the constructs and their application are limited. Oxygen releasing microparticles were

formed so that they could be incorporated to engineered constructs and mimic blood's ability to deliver oxygen locally in order to support the size increase of tissue engineered constructs.

In this thesis we provide compelling proofs of concept that oxygen delivery with biomaterials can augment conventional treatments and improve tissues and cells survival. These materials are a first step towards building systems able to mimic blood functions.

Résumé

En plus d'être une source de cellules immunitaires, le sang a pour fonction d'apporter de l'oxygène, des nutriments et est un puits pour les déchets métaboliques. Les tissus dépendent d'un flux sanguin suffisant pour maintenir leur viabilité et, si le flux sanguin est réduit ou interrompu, les tissus meurent à un point appelé limite ischémique. L'oxygène est un facteur limitant majeur de la survie des tissus pendant l'ischémie : c'est un composant essentiel de la phosphorylation oxydative qui produit de l'énergie pour les cellules. Il n'est pas rare de trouver des situations cliniques dans lesquelles la concentration en oxygène ne répond pas aux besoins métaboliques, et même une légère ischémie, par exemple en raison du diabète, peut nuire à la guérison des plaies. Une ischémie plus grave et prolongée due à un traumatisme ou à une intervention chirurgicale peut entraîner des dommages beaucoup plus importants.

De nombreuses technologies différentes ont été rapportées pour traiter les problèmes cliniques liés à l'insuffisance en oxygène, tels que l'oxygénothérapie hyperbare (HOBT), les technologies perfluorocarbonées, les supports à base d'hémoglobine, les peroxydes, etc. Parmi toutes ces technologies, les peroxydes, comme le peroxyde d'hydrogène, le percarbonate de sodium et le peroxyde de calcium présentent l'avantage particulier de produire de l'oxygène *in situ*. En effet, alors qu'il faudrait plusieurs centaines de grammes de globules rouges, plusieurs kilogrammes de perfluorocarbones ou de plasma pour délivrer un litre d'oxygène pur, il faudrait moins d'un gramme de peroxydes. Cependant, les peroxydes ont une toxicité inhérente qui les rend d'une valeur limitée pour les applications de cellules et de tissus nécessitant un contact direct ou étroit avec les tissus.

Cette thèse étudie les avantages potentiels d'un biomatériau à base de peroxyde de calcium mis au point dans notre laboratoire pour délivrer de l'oxygène localement. La décomposition rapide du composé, qui forme à la fois de l'oxygène et du peroxyde d'hydrogène, est contrôlée à l'aide de polymères hydrophobes. La catalyse inorganique transforme le sous-produit toxique, le peroxyde d'hydrogène, en eau et en oxygène, réduisant ainsi la toxicité inhérente du composé. Le matériau a été traité sous différentes formes et testé dans des environnements topiques et sous-cutanés ainsi que sur un support cellulaire *in vitro* pour un échafaudage autonome. Ceci est important car les problèmes courants résultant d'une vascularisation insuffisante ou endommagée, tels que le vieillissement, le diabète, la radiothérapie, les traumatismes, etc., ne peuvent pas être traités facilement et entraînent une réduction significative de la qualité de vie et augmentent les coûts de soins de santé. En effet, une nécrose tissulaire peut se produire au centre de grandes plaies ischémiques et se propager aux tissus sains environnants, entraînant une perte importante de tissu. Un pansement libérant de l'oxygène a été conçu et utilisé comme patch pour prévenir la nécrose et favoriser la guérison des grosses plaies ischémiques.

De même, les lambeaux cutanés constituent une approche importante des réparations de plaies reconstructives qui, lorsqu'elles sont effectuées, endommagent une partie de la vascularisation de la peau et peuvent conduire à une ischémie s'ils ne sont pas suffisamment perfusés. Les conséquences de ces procédures sont aggravées par des comorbidités courantes telles que le diabète, la radiothérapie, etc., et aboutissent souvent à une nécrose. À ce jour, l'apport in vivo d'oxygène pour prévenir les dommages ischémiques n'a pratiquement pas été étudié. Un implant sous-cutané générant de l'oxygène a été développé pour déterminer si l'apport d'oxygène sous-cutané pourrait prévenir de manière significative la nécrose ischémique et étudier la limitation de l'apport d'oxygène dans un modèle de lambeau cutané ischémique de rat.

L'absence de circulation sanguine peut également être un problème dans les constructions d'ingénierie tissulaire, à la fois *in vitro* ou lors de l'implantation, car elles ne possèdent pas de vascularisation. Pour cette raison, la taille des constructions et leur application sont limitées. Des microparticules libérant de l'oxygène ont été fabriquées pour être incorporées dans des constructions d'ingénierie tissulaire et imiter la capacité du sang à libérer de l'oxygène localement afin de supporter leur augmentation de la taille.

Dans cette thèse, nous fournissons des preuves convaincantes que l'apport d'oxygène avec des biomatériaux peut augmenter les traitements conventionnels et améliorer la survie des tissus et des cellules. Ces matériaux constituent un premier pas vers la construction de systèmes capables d'imiter les fonctions sanguines.

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BG	Bioglass
Ca^{2+}	Calcium ions
CaO_2	Calcium peroxide
CHO	Chinese hamster ovary
Cl^-	Chlorine ions
CO^2	Carbon dioxide
CO_3^{2-}	Carbonates ions
CoQ10	Coenzyme Q10
DFO	Deferoxamine
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial NOS
Fe_3O_4	Iron oxide
FGF	Fibroblast growth factor
GF	Growth factors
H+	Proton
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2CO_3	Carbonic acid
HBO	Hyperbaric oxygen
HCO_3^-	Bicarbonate ions
HIF-1α	Hypoxia Inducible Factors alpha

HUVEC	Human umbilical vein endothelial cell
iNOS	Inducible NOS
K^+	Potassium
MDCK	Madin-Darby Canine Kidney
MMP	Matrix metalloprotease
mRNA	Ribonucleic messenger
Na^+	Sodium
NADH	Nicotinamide adenine dinucleotide
$NaHCO_3$	Sodium bicarbonate
NBCs	Sodium/bicarbonate cotransporters
nNOS	Neuronal NOS
NO	Nitric oxide
OμP	Oxygen releasing microparticles
PGDF	Platelet derived growth factor
Pi	Inorganic phosphate
PMNs	Polymorphonuclear neutrophils
RNA	Ribonucleic acid
ROS	Reactive oxygen species
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
Zn	Zinc

Contents

List of figures

List of tables

List of Equations

Chapter 1 - Introduction

1 Ischemia

Ischemia is a restriction of blood supply to tissues causing a shortage of oxygen and nutrients that are needed for cellular metabolism.[1] It generally is caused by problems with blood vessels, and results in damage or dysfunction of tissue. The reduced blood supply will fail to sustain delivery of oxygen and nutrients necessary to meet metabolic requirements, leading to an inadequate removal of metabolic wastes. Ischemia can be partial (reduced blood flow in the tissues) or total (no blood flow). Reduction of blood flow can have different causes such as atherosclerosis or vasoconstriction and will lead to partial ischemia, which in turn will results in chronic issues (chronic ischemia) like non-healing wounds, chronic pain, etc. On the other hand, the sudden total occlusion of a blood vessel due to thrombosis or embolism will result in acute problems (acute ischemia) such as stroke or necrosis of tissues. It is not uncommon that chronic ischemia due to a precondition like atherosclerosis results thrombosis and lead to acute ischemia. If impaired blood flow lasts long enough, it triggers the ischemic cascade that will eventually lead to tissue and organ malfunction or damage. This chapter will focus on the causes of ischemia, the normal the metabolic mechanisms that are be involved in ischemia and how these processes are impaired, leading eventually to tissue malfunction and ultimately tissue death.

1.1 Cause of ischemia

1.1.a Atherosclerosis

Atherosclerosis is a disease in which the buildup of plaque narrows the inside of an artery. Because it develops slowly over time, symptoms are rarely apparent. [2] Depending on which arteries and

organs are affected it can result in coronary artery disease, stroke, peripheral artery disease, or kidney problems. Plaque buildup is most often found where there is a decreased shear stress or turbulence (bifurcations, bends) .[2] Plaque accumulation results in a swelling of the inner wall of the artery, narrowing the blood flow. It is composed of mostly debris, lipids, calcium, variable amount of fibrous connective tissues and inflammatory cells [3] that have taken up oxidized low-density lipoprotein. Activated macrophages release chemoattractants which in turn recruit more macrophage and smooth muscle cells as well as lymphocytes and other inflammatory cells. This accumulation alters the endothelial wall and lead to platelet deposition and form a fibrous cap. Platelet-derived growth factor is released by the platelets, which participates to the recruitment of more plaque precursors. This cycle repeats and progressively leads to a large buildup of plaque and narrows blood vessels even further. Inflammation increases activity of matrix metalloproteinases and other enzymes weakening the fibrous cap, leading to plaque instability. Rupture may occur leading to plaque hemorrhage and thrombus formation. [2]

1.1.b Arterial thrombosis

Arterial thrombosis refers to a blood clot that develops in an artery and obstructs or stops the blood flow. It usually occurs after degradation or rupture of an atherosclerotic plaque, forming a platelet-mediated thrombus and can cause ischemic injuries. It can have devastating consequences when affecting major organs, such as cardiac ischemia and stroke, which are the most severe clinical manifestations of atherothrombosis. Ischemia can arise slowly from the progression of atherosclerotic disease or acutely in the case of vascular or intracardiac thromboembolization. [4]

1.1.c Arterial embolism

Arterial embolism is a sudden interruption of blood flow due to an embolus adhering to the wall of an artery blocking the blood flow. It shares many similarities with thrombosis but is a unique condition. Unlike thrombosis, that occurs when a thrombus develops and obstructs a blood vessel, embolism occurs when a piece of a blood clot, foreign object, or other substance obstructs a blood vessel.[5] Thromboembolism the major type of embolus being a blood clot and is the major cause of infarction.

1.1.d Vasculitis

Vasculitis is a group of inflammatory disorders that destroy blood vessels by inflammation. Both arteries and veins are affected. The vascular inflammation may cause the narrowing of the vessels or occlusion and leads to ischemia. It may also result in vessel rupture and hemorrhage.[6]

1.2 Impaired blood flow and organs

Organs and tissues oxygen consumption varies depending on their size, cell density, etc. In arterial blood oxygen concentration is usually 104 to 146 μmol/L (10 to 15% dissolved oxygen, 70 to a 100mmHg).[7] The equilibrium between the delivery and the consumption results in a wide variety of physoxic oxygen levels.[8] When the blood flow is disrupted and results in ischemia of tissues or a whole organ, and the rate of oxygen delivery is lower than that of consumption, the oxygen depletion results in hypoxia.

Sustained or chronic changes in the environment induce changes in the metabolic machinery. Chronic heart ischemia exhibit few or no symptoms. The heart adapts to reduced oxygen delivery to prevent reversible damage by a mechanism known as hibernating myocardium resulting in a

switch from fat to glucose metabolism and an augmented glycogen storage. In the case of peripheral arterial disease and chronic limb ischemia the symptoms varies, depending on the severity of the ischemia, from no symptoms or claudication to ischemic ulceration and necrosis. [9] However, tissues can adapt by slowly creating new vessels as hypoxic conditions are a stimulus for angiogenesis.[10]

In case of acute ischemia, myocardial blood flow is insufficient to match the metabolic activities of myocardial cells, the imbalance between myocardial oxygen supply and consumption results in a rapid hypoxia of myocardial cells and causes myocardial ischemia.[11] Chest pain is the most common symptom of acute myocardial infarction and is often described as a sensation of tightness, pressure, or squeezing, principally due to cardiomyocytes contracture caused by an overload of calcium in the cells, that will eventually result in necrosis.[12] The brain consumes a massive amount of oxygen and glucose, and an ischemic episode that would result in the reduction of about 50% of its blood flow could result in a loss of energy production, followed by a calcium and glutamate overload inside the cells and in the case and ultimately tissue necrosis.[13] Acute limb ischemia also follow a similar pathway of loss of energy production, cells overload, swelling and ultimately death. [14]

2 Cellular injury

The decrease of oxygen availability and metabolic substrates decreases the energy available to the cells and in turn leads to reversible or non-reversible cell injury. The extent of this injury is determined by the severity of ischemia, its duration, the metabolic and physical changes, etc.

2.1 Biological processes

2.1.a Energy production

In animal cells and non-photosynthetic cells, adenosine triphosphate (ATP) is generated mainly by aerobic oxidation, which consist in glucose and oxygen are metabolization to form carbon dioxide (CO_2) and water (H_2O). Glycolysis is the initial steps in the oxidation of glucose and occur in the cytosol in both eukaryotes and prokaryotes and does not require oxygen (***Equation 1-1***). It results in the production of ATP and pyruvic acid that in turns serve the production of acetyl CoA (**Equation 1-2**). Acetyl CoA is then used with oxygen in the Krebs cycle to further produce ATP (**Equation 1-3**). Products of Krebs cycle are finally used in the electron transport chain, the final steps, which also require oxygen and generate most of the ATP (**Equation 1-4**). In eukaryotes, this later stage of aerobic oxidation occurs in mitochondria (***Figure 1-1***).

Equation 1-1: *Glycolysis:* $2\ ADP + Glucose \rightarrow 2\ Pyruvic\ Acid + 4H^+ + 4\ ATP$

Equation 1-2: *Acetyl CoA formation:* $2\ \mathrm{Pyruvic\ Acid} + 2\ \mathrm{CoA} \rightarrow 2\ \mathrm{Acetyl\ CoA} + 2\ \mathrm{CO_2} + 2\ \mathrm{H^+}$

Equation 1-3: *Krebs Cycle:* $2\ \mathrm{PAcetyl\ CoA} + 3\ \mathrm{O_2} \rightarrow 2\ \mathrm{ATP} + 4\ \mathrm{CO_2} + 6\ \mathrm{H^+}$

Equation 1-4: *Electron Transport System:* $12\ \mathrm{H^+} + 3\ \mathrm{O_2} \rightarrow 32\ \mathrm{ATP} + 4\ \mathrm{H_2O}$

Equation 1-5: *Overall reaction:* $\mathrm{Glucose} + 6\ \mathrm{O_2} \rightarrow 6\ \mathrm{CO_2} + 6\ \mathrm{H_2O} + 36\ \mathrm{ATP}.$

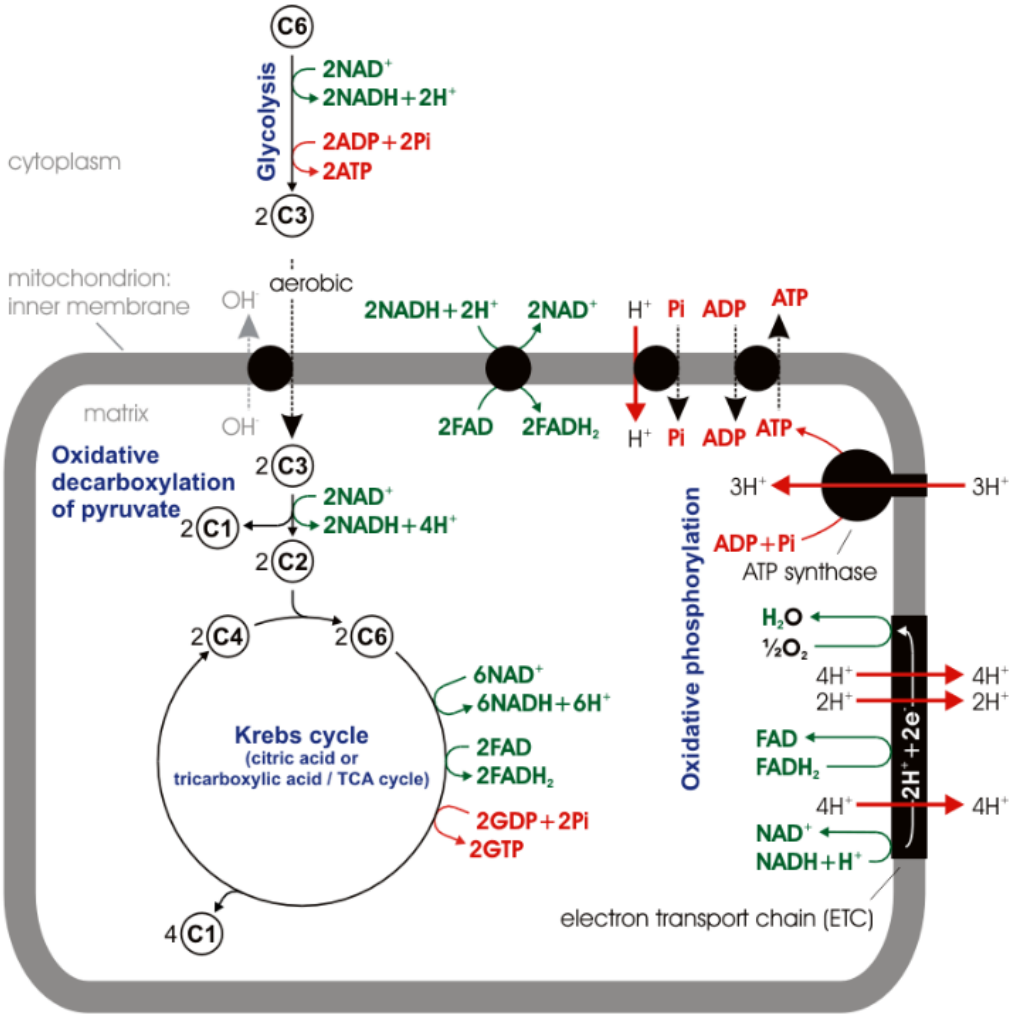

Figure 1-1: Stoichiometry of aerobic respiration in eukaryotic cell. Numbers in circles indicate counts of carbon atoms in molecules, C6 is glucose $C_6H_{12}O_6$, C1 is CO_2. Mitochondrial outer membrane is omitted. Reproduced and modified from open source image. [15]

2.1.b Ionic exchanges across cell membrane

Cell membranes are composed of a phospholipid bilayer with protein, glycoproteins, glycolipids, carbohydrates, etc., embedded in the membrane or tightly adsorbed to the surface of the membrane. The hydrophobic ends of the phospholipid molecules are directed toward the middle of the

membrane and the hydrophilic ends toward the outside of the membrane forming the inside and outside of the cells. [16] Diffusion is the process by which gases, liquids and dissolved molecules or ions tend to blend because of their spontaneous motion caused by thermal agitation. All diffusing substances tends to move from regions of higher concentration to regions of lower concentration until an uniform distribution appear at equilibrium. [17]

Cell membranes have a fixed negative charge on their outer and inner surfaces at physiological pH because of acidic nature of the phospholipids of the bilayer and the proteins it contains as their isoelectric point is acidic. The number of ionized charge groups is affected by pH and ionic strength, and a too low or high binding of proton (H^+) or cation on the outer surface can result in a depolarization of hyperpolarization of the membrane.

A selective and regulated migration of ions and molecules across the membrane is essential for cellular homeostasis. Maintenance of cell pH, volume, intake of nutrients and waste removal depends on membrane transport.

The hydrophobicity of the phospholipid bilayer turns the cell membrane into an impermeable barrier that excludes the passage of hydrophilic ions (anion, cation) or non-electrolyte molecules. Their selective passage across the barrier, is mediated through membrane transport proteins located in the bilayer. Transport proteins subdivided depending on their functions into channels, pumps and carriers depending on the different mechanisms by which they mediate ions and non-electrolytes transport [18]

Two types of transport mechanism can be distinguished: passive transport is defined as movement of a solute from a region of high electrochemical potential on one side of the cell membrane to a region of lower electrochemical potential on the opposite side. Active transport is defined as

movement of a solute from a region of low electrochemical potential or concentration on one side of the cell membrane to a region of higher electrochemical potential or concentration on the opposite side, thus transferring ions or non-electrolytes across the membrane in an opposite direction to the prevailing electrical gradient and/or chemical concentration gradients. To perform the work, active transport mechanisms require energy through ATP hydrolysis.

Amongst active transporter two class can be distinguished: primary active transport, like ion translocating ATPases or pumps, is directly coupled to ATP hydrolysis and use the energy to form an ion electrochemical potential difference. Secondary active transport mechanisms, like cotransporters and counter-transporters, are indirectly coupled to ATP hydrolysis and transfer the energy from one solute electrochemical potential difference to the formation of a second solute electrochemical potential difference.

In normal condition, the electrochemical gradient is orientated from the outside of a cell towards the inside as the extracellular space possesses a higher electrochemical potential than the inside (***Table 1-1***)

Table 1-1: Summary of the Ion Distributions in Most Types of Cells. Reproduced and modified from Sperelakis et al (2012) [16]

Ion	Extracellular Distribution (mM)	Intracellular Distribution (mM)
Na+	145	15
Cl^-	100	5[a]
K^+	4.5	150
Ca^{2+}	1.8	0.0001
H^+	0.0001	0.0002

a Assuming Cl^- is passively distributed and resting E_m is −80 mV. The extracellular H^+ concentration is given for pH 7; it would be 40 nM at pH 7.4.

There are three main types of ATP-driven ion pumps: The F-type, the V-type and the P-type.

F-type ion pumps are found in chloroplasts and bacteria, these proteins seem to function exclusively to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi).

V-type are structurally similar to F-type pumps and actively transport H^+ using ATP hydrolysis, thereby coupling H^+ transport to ATP hydrolysis or synthesis.

P-type ATPases are a superfamily widely distributed throughout the plant and animal kingdoms, and they catalyze the transport of a wide variety of cations, heavy metals, and phospholipids. They are critical to maintain cell viability. [19]

Proton exchanges

The distribution of H^+ cations between the extracellular and intracellular space is influenced by the negative membrane potential, which tends to cause intracellular H^+ accumulation and acidification of the cell due to a number of mechanisms. H^+ ions alone can only enter by protein-mediated membrane transport because the phospholipid membrane is totally impermeable to H^+. [20] Virtually all of the H^+ ions within a cell are buffered by reversible binding to weak acids and bases, resulting in a low free H^+ ion activity. [21]

If H^+ ions were passively equilibrated across the cell membrane, the intracellular pH would be more than one pH unit lower than the extracellular pH. However, the extracellular pH remains relatively constant near 7.4, and the intracellular pH in a resting cell is about 7.2. This difference between the cellular and extracellular space indicates that H^+ ions actively equilibrated across membranes. In intact cells, the cellular H^+ concentration remains low because H^+ is transported out against the inward-directed electrochemical gradient. Several membrane transport mechanisms and buffer systems contribute to the decrease in the intracellular H^+ concentration.

Amongst the many proteins at the surface membrane of cells, several are dedicated to the active transport of acids and bases across the membrane. Extremely important to cellular pH regulation, they have been extensively studied and have been divided into five groups: (1) direct H^+ exchange with another cation; (2) those that move bicarbonate ions (HCO_3^-) and carbonates ions (CO_3^{2-}); (3) H^+-ATPases; (4) anionic weak base and sodium (Na^+) cotransporters; (5) anionic weak bases and chlorine ions (Cl^-) exchangers.

The sodium-proton exchanger is the classical pH-regulating transport system present in most cell types. This exchanger responds to cellular acidification. The general scheme is that the set point for activation of the sodium-proton exchanger is close to normal internal pH of 7.2, implying that acidification of the cell activates H^+ extrusion in exchange for the influx of one Na^+. This activation ensures a normalization of internal pH.

Amongst the other pH-regulating transport systems, the sodium/bicarbonate cotransporters (NBCs) are the most important. The entry of bicarbonate shifts the CO_2/bicarbonate equilibrium decreases H^+ concentration. H^+ efflux from the cell could also be mediated by lactate/H^+ cotransporters (monocarboxylate transporter isoforms).

Some organellar compartments maintain their functions by having a different pH than the pHi. For intracellular organelles like endosomes, lysosomes and storage granules, maintaining an internal pH of 5–6 is essential for this function.

ATP production is the main role of mitochondria. It requires a H^+ gradient across the inner mitochondrial membrane: the electron transport chain allows to bring H^+ from mitochondria to the cytoplasm across the inner mitochondrial membrane. This H^+ extrusion establishes a H^+ gradient

that renders mitochondria alkaline when compared to the cytoplasm (Intramitochondrial pH varies between 7.5 and 8.0). Then a passive H^+ influx from the cytoplasm into mitochondria occurs through a membrane-bound ATPase that produces ATP. [20] To achieve its purpose, mitochondria, are using different H^+ pump (***Figure 1-2***):

Complex I (Ubiquinone oxidoreductase or NADH dehydrogenase), in eukaryotes, is located in the inner mitochondrial membrane and it is the entry point of most electrons involved in the respiratory chain. It is a H^+ pump driven by electron transport. It oxidizes NADH generated by the Krebs cycle and catalyzes the transfer of electrons to coenzyme Q10 (CoQ10). As this reaction and the electron transfer occur, the resulting electron current powers an active extrusion of H^+ from the mitochondria. [22, 23]

Complex III (Cytochrome bc1 or Coenzyme Q) is the second enzyme in the mitochondrial respiratory chain that couples the electron transfer from the coenzyme Q10 from Complex I to cytochrome C. The electron transfer from this reaction drives H^+ extrusion from the mitochondria. [23]

Complex IV (Cytochrome C oxidase), is the terminal enzyme of the respiratory chain reduces O_2 to H_2O using the electron transport chain. In the process, it translocates H^+ across the membrane. [23]

ATP synthase is an enzyme that couples the electrochemical gradient created by the difference H^+ between the inner membrane space of the mitochondria and the intermembrane space of a cell to drive synthesis ATP from ADP and Pi.

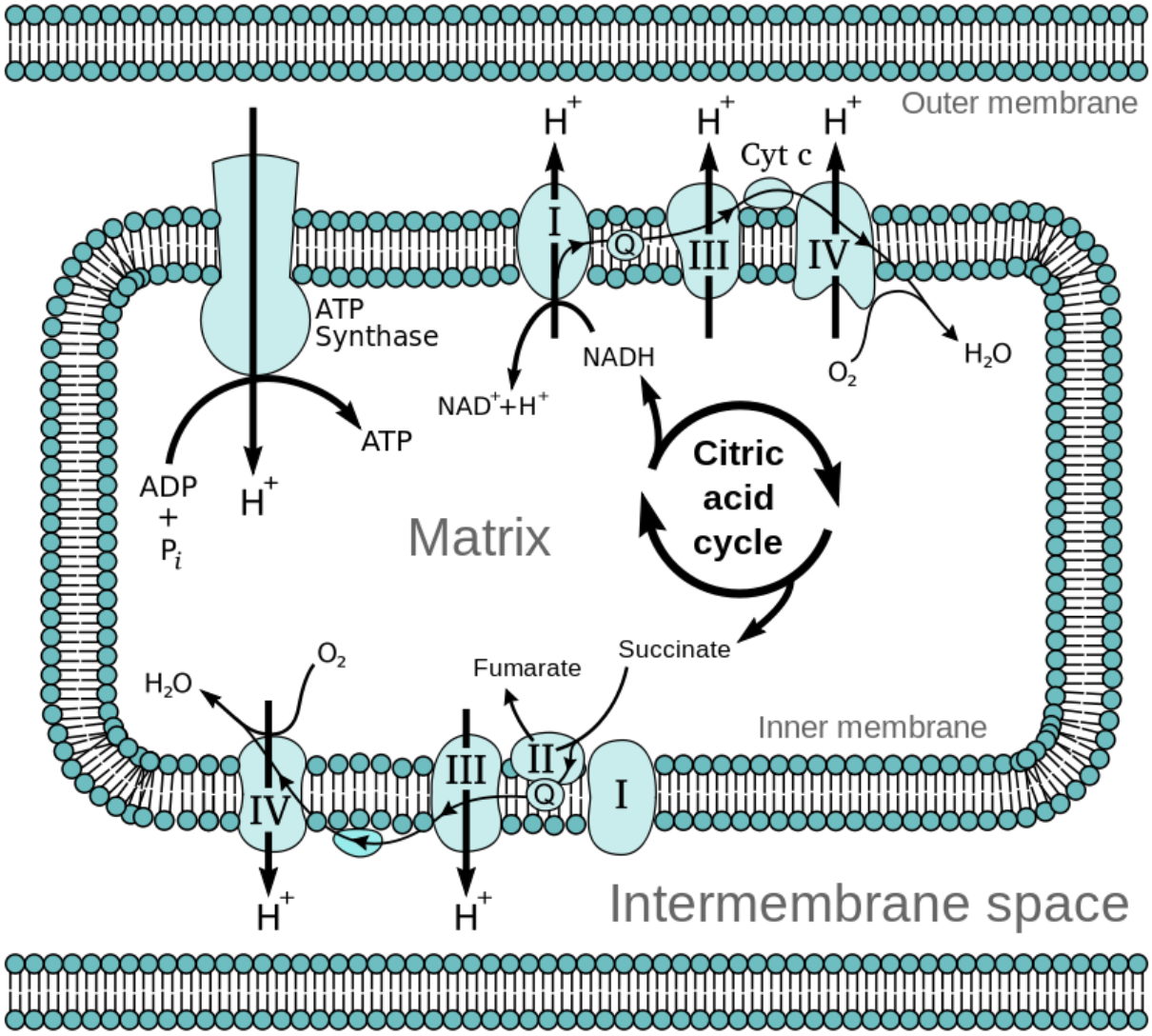

Figure 1-2: ATP Synthesis as seen from the perspective of the matrix. Conditions produced by the relationships between the catabolic pathways (Citric Acid Cycle and Oxidative Phosphorylation) and structural makeup (Lipid Bilayer and electron transport chain) of matrix facilitate ATP synthesis. Reproduced and modified from open source image.[24]

Sodium exchanges

Sodium channels are highly selective transporters of sodium ions across cell membranes, working by encapsulation of the sodium ion in a large molecule's cavity.[25] They play a major role in in many different physiological processes ranging from locomotion to cognition.

The sodium–proton (Na^+/H^+) exchanger (or antiporter) is a membrane protein that transports Na^+ into the cell, and H^+ out of the cell or organelles.[26] They are pH dependent and help to regulate intracellular pH, sodium levels, and cell volume.[27] Their dysfunction is associated with a number of different diseases, and they are well-established drug targets.[28, 29] Na^+/H^+ exchanger will bind Na^+ in the extracellular space and exchange it against a H^+ from the intracellular space through a series of conformational changes for which the mechanistic steps have not reached a consensus but would consist in a conformational change induced by alkaline pH, exposing the Na^+ binding site. The ion binding leads to further conformational changes resulting in the exchange of Na^+ and H^+.[27, 30]

Sodium-potassium (Na^+/K^+) exchanger is found in the plasma membrane of all animal cells and is responsible for the active transport of Na^+ and potassium (K^+) across the membrane. It is responsible for both the extrusion of Na^+ outside the cells against its concentration gradient, and for the intake of K^+ also against its concentration gradient using ATP. (***Table 1-1***).[31] The pump mechanism is decomposed into a set of six elementary and reversible reactions consisting in the binding of ATP Na^+ ions by the enzyme, ATP hydrolysis inside the cell resulting in the enzyme phosphorylation, followed by a conformational change and the extrusion of the Na^+ ions and their dissociation. The subsequent binding of K^+ ions outside the cells induce a dephosphorylation resulting in a conformational change and the binding of ATP and the intake of the K^+ ions.[31, 32]

Calcium exchanges

Calcium exchanges through the cellular membrane are governed by calcium channels, calcium exchangers and calcium pumps.[33]

Calcium channels, as described previously, are pores that allow calcium ions (Ca^{2+}) to flow through cells membrane following their concentration gradient. Their opening/closing is tightly regulated by either the membrane potential (voltage-gated) or by chemicals (ligand-gated). There is a wide variety of Ca^{2+} channels distributed on cells membrane and organelles and serving different functional and regulatory purposes from cell contraction, secretion, synaptic transmission, enzyme regulation, protein phosphorylation/dephosphorylation, and gene transcription.[33, 34]

Sodium-calcium exchanger (Na^{+}/Ca^{2+}) is an antiporter channel that removes calcium from cells. It uses the energy stored as the electrochemical gradient of Na^{+} by allowing Na^{+} to flow down its gradient across the plasma membrane in exchange of Ca^{2+}. Na^{+}/Ca^{2+} exchanger is one of the most important cellular mechanisms for removing Ca^{2+}, it has a low affinity for Ca^{2+} thus allowing a rapid ion transport but requires large concentrations of Ca^{2+} to be effective. Its ions movements are reversible and determined by electrochemical forces, and the direction of the exchange determined by intracellular concentration of Na^{+} and Ca^{2+}.

Calcium pumps are responsible for the active transport of calcium out of the cell for the maintenance of the Ca^{2+} electrochemical gradient across the cell membrane and plays a crucial role in cell signaling.[35] Three types of Ca^{2+} ATPase have been described in animal cells located in either the cell membrane or different organelles.[36, 37]

A simplified reaction scheme, valid for all three Ca^{2+} pumps, consist in two basic conformational states of the pumps, one that have a high Ca^{2+} affinity in the cytosol, and one with a low affinity that releases the ion in the extracellular space. The reversible transition between the two states and the migration of Ca^{2+} through the membrane is based upon phosphorylation of the enzyme by reaction with ATP Ca^{2+} binding in the first state, and a release of inorganic phosphate after the release of Ca^{2+} in the second state.

2.1.c Ischemic Cascade

The ischemic cascade is the series of overlapping cellular events occurring when the blood supply is disrupted. Although the different types of cells react differently, there is an overall common process leading to cell death. In blood, the glucose concentration is regulated between 1.4 mmol/L and 6.2 mmol/L [38], and oxygen concentration 104 to 146 µmol/L.[7] According to the overall reaction of ATP production, during ischemia the first limiting factor is oxygen. Without oxygen the ATP production cycle is limited to a yield of 2 ATP for 1 glucose. In absence of a functioning Krebs cycles, glucose is turned into lactates and the intracellular pH increases. Without ATP the Na^+/K^+ ATPase stop its functions leading to a Na^+ buildup in the cells, followed by a water influx inside the cells causing a cell swelling (***Figure 1-3***). The resulting depolarization of the cell membrane cause the Na^+/Ca^{2+} transporter to malfunction and a Ca^{2+} accumulation inside the cells. The high Ca^{2+} content excites proteases [39] and triggering catabolic processes mediated by lipases and nucleases that will degrades the cell membrane and increase its permeability, leading to further depolarization. At the same time Ca^{2+} will cause an increase in reactive oxygen species (ROS) production that will also damage the cells.[40] The swelling of the cells will eventually lead to necrosis. In addition, in this harmful environment, mitochondria's membrane is disrupted and open its permeability transition pore which further decrease the ATP production and the releases of

apoptotic factors and start the apoptotic cascade.[1, 41] In neurons, the high intracellular Ca^{2+} content causes glutamate release which in turn causes an increased calcium intake. These alterations and thus the degree of tissue injury vary with the magnitude of the decrease in the blood supply and with the duration of the ischemic period.

2.1.d Reperfusion injury

Reestablishing blood supply in ischemic tissues will restore oxygen and nutrient necessary to the production of ATP and normalization of the extracellular pH. However sudden reperfusion can have devastating consequences for cells as it triggers a cascade of events that exacerbate tissue injury. The mechanisms underlying reperfusion injury are complex. First the reintroduced oxygen first feeds the generation of ROS, then the intracellular pH normalization increases Na^+ intake through the Na^+/H^+ exchanger, and by extension Ca^{2+} through the Ca^{2+}/Na^+ exchanger, thus aggravating the calcium overload and permeability transition pore opening. Following these direct effects, reperfusion has been shown to result in endothelial dysfunction as well as a pronounced inflammatory response, further aggravating tissue damage (***Figure 1-3***).[1]

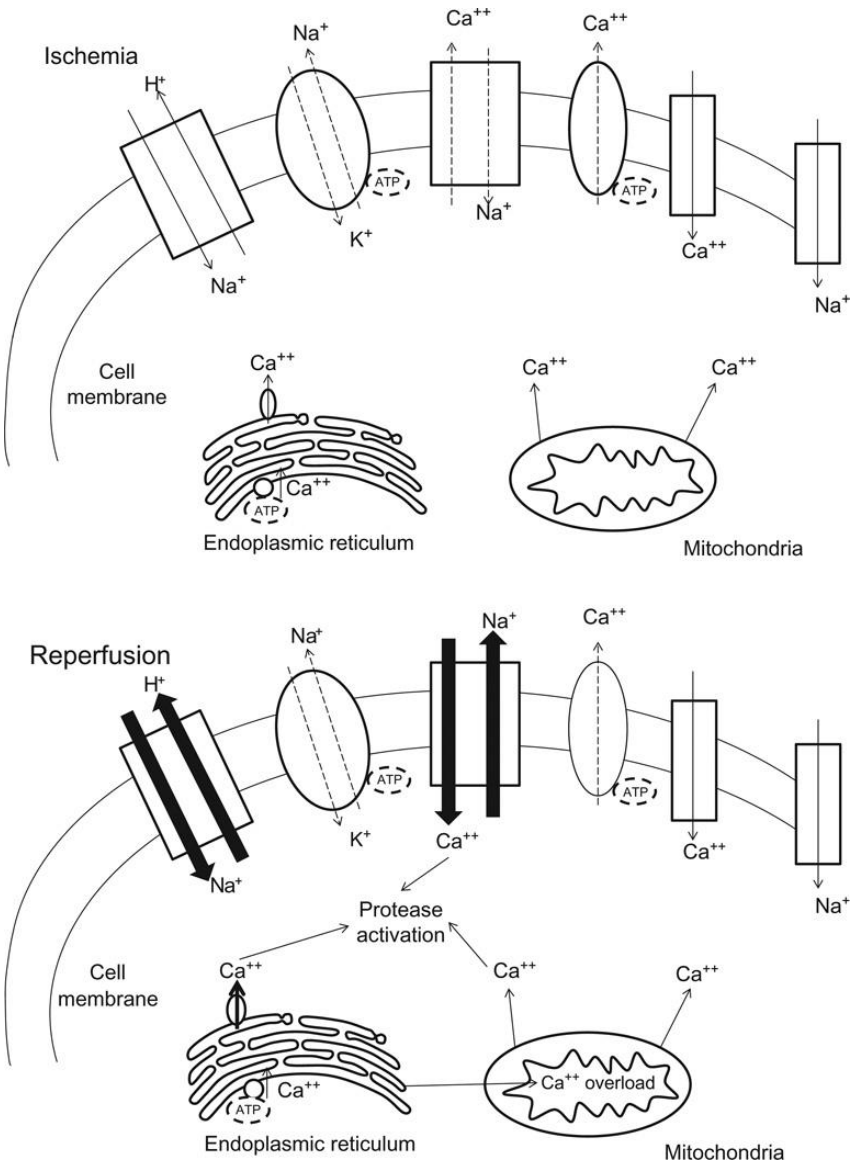

Figure 1-3: Major pathologic events contributing to ischemic (Upper Panel) and reperfusion (Middle Panel) components of tissue injury. Reproduced with permission from Kalogeris et al (2012).[1]

2.2 Cell death

Cellular death can occur following different types processes. Necrosis will occur after extrinsic factors (loss of energy, toxic molecules, etc.). Apoptosis and autophagy are programmed cell death that will be initiated by cellular signaling mechanisms (***Figure 1-4***).

2.2.a Apoptosis

Apoptosis is the process of programmed cell death. It occurs during development and aging, as a mechanism to maintain cell populations in tissues, a proper development and functioning of the immune system, embryonic development, chemical-induced cell death, etc. A dysfunction of apoptosis is common in different conditions like neurodegenerative diseases, ischemic damage, autoimmune disorders or cancer.[42] Apoptotic mechanisms can be divided into extrinsic pathways (upon ligation of death receptor like tumor necrosis factor (TNF) receptors) and intrinsic pathways (mitochondrial damage by ischemia, radiations, etc.).[43] Apoptosis will result in different simultaneous processes of cell degradation (deoxyribonucleic acid (DNA), protein, membranes), and will form apoptotic bodies and express of ligands for phagocytic cell receptors. During ischemia, cells undergo different damage capable of initiating apoptosis. If ischemia is prolonged, then necrosis occurs as the apoptotic processes are energy dependent. However, if energy production is restored, the apoptotic cascade that was initiated may proceed.

2.2.b Autophagy

Autophagy is an important process that balances energy sources in cells during periods of nutrient stress by self-degradation. It also serves to remove misfolded or aggregated proteins, damaged organelles such as mitochondria, endoplasmic reticulum, etc.; or eliminates intracellular pathogens. It is induced by starvation and is a key component of the adaptive response of cells to nutrient

deprivation that promotes survival until nutrients become available again.[44] It will consist in the sequestration of the target and the transport to lysosomes followed by their degradation and utilization of end products.[45]

2.2.c Necrosis

Necrosis is an irreversible cell injury resulting in the premature death of cells in living tissue by autolysis. It is caused by external factors, such as infection, trauma, ischemia, etc.; which result the swelling of cells and their organelles, mitochondrial dysfunction, lack of nuclear fragmentation, plasma membrane rupture, and release of their cellular content into the extracellular space.[46]

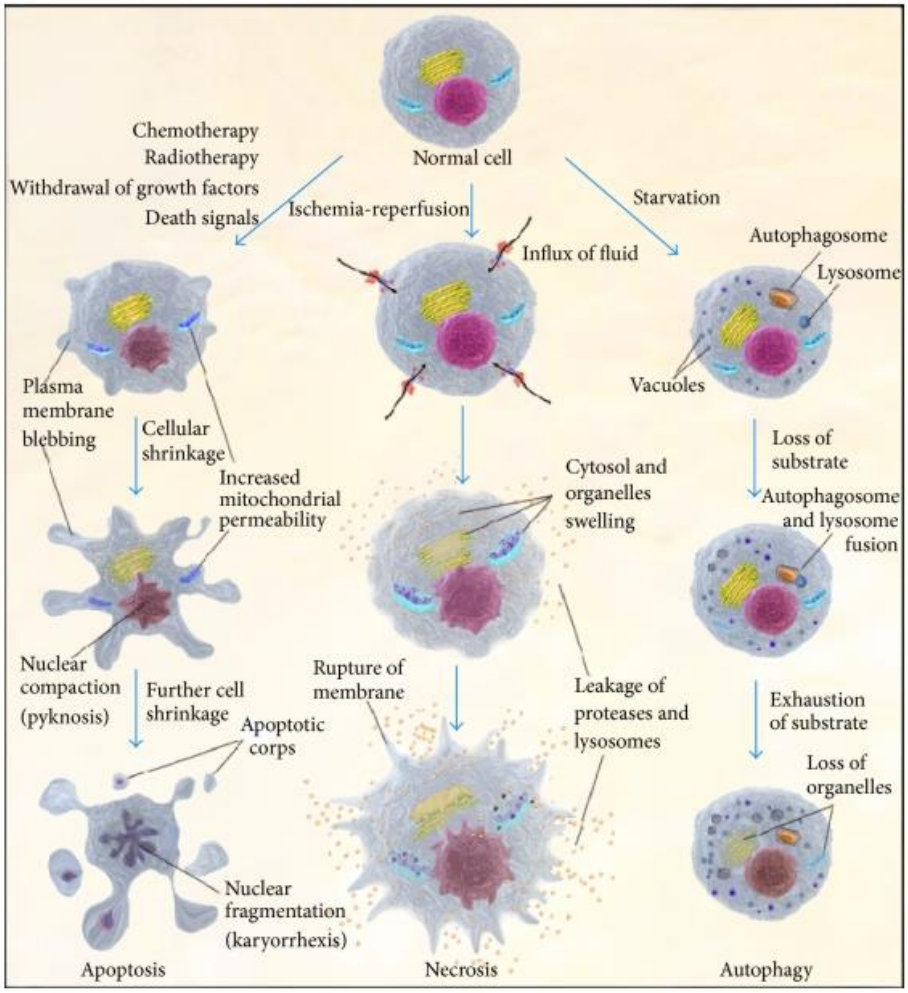

Figure 1-4: The three major pathways of cell death. Cells can be directed to different programmed cell death mechanisms depending on several factors. In the left, the apoptosis pathway is represented with the characteristic cellular shrinkage and formation of the apoptotic bodies without leakage of contents. In the middle, the necrotic pathway shows the cytosol and organelle swelling and rupture of plasma membrane with subsequent leakage of cellular contents. In the right, autophagy is illustrated with the appearance of vacuoles, the autophagosome, and its fusion with the lysosome, which ends in organelle digestion. Reproduced from Nunes et al.[47]

3 Rationale

The overall aim of this work is to use oxygen delivery biomaterials in different formats to address *in vivo* and *in vitro* ischemia and avascular wounds and to evaluate their limitations.

Oxygen is crucial for survival and function of many cells. To prevent ischemia-induced necrosis different clinical methods have been used: pneumatic compression, HBOT, angioplasty, bypasses, drug treatments, exercises programs, etc.[9, 48]

However, during mild ischemia, with or without treatment, even if the blood flow is sufficient to maintain cell viability it is often insufficient to sustain some basic functions like healing. Oxygen delivery for ischemic wounds has been widely described in the literature [49] and carried out through various methods. Hyperbaric oxygen therapy (HBOT) is a medical treatment that aim to expose a patient for several hours at a time to an oxygenated atmosphere in a chamber at hyperbaric pressures to increase blood oxygen content by a factor of 3.[50] First hyperbaric chambers were conceived in 1662 and used to treat decompression sickness successfully in 1937 [51, 52], but the use of hyperbaric oxygen only appeared in 1955. Today it is often used to help improving chronic wound healing or free flap take after surgical reconstruction, yet to this day, to the exception of decompression sickness, there is a lack of evidence supporting its medical use. It is a systemic and intermittent delivery approach that relies on a functioning vasculature, and its effect on different types of wounds have not found to be significant and are still debated [50, 51, 53-56], as well as for skin grafting.[57]

3.1 Topical oxygen delivery

Topical oxygen delivery consists in increasing the amount of oxygen directly above the wound bed and has been shown to stimulate significantly chronic wound healing in both rats and humans.[56, 58, 59] Different technologies have been developed and are commercialized: portable oxygen chambers (O2Boot®, O2Sacral®, TWO2®) that are relatively intrusive, oxygen concentrators (EPIFLO®, NATROX®, TransCuO2®) that are more convenient but require the wearing of an oxygen generating source, have been shown to promote wound closure of non-healing ulcers in patients.[60, 61] Newer oxygen pre-loaded wound dressings like Oxyband™ has been reported to improve healing in human burns [62], and OxygenSys™ prevented partially skin flap necrosis in pigs. [63]. Mednoxa delivers oxygen through catalysis of hydrogen peroxide but has not yet been shown to be effective in preclinical and clinical settings as it is an early stage start-up. An experimental sodium percarbonate/calcium peroxide based oxygen releasing wound dressing [64] has also been shown to promote full thickness surgical wound healing in pigs by 10%.

However, topical oxygen delivery to chronic wounds in clinical settings is not always sufficient to prevent necrosis.[65, 66] Most prior studies focus on promote healing in patients with impaired healing and none have intended to salvage tissues that will otherwise undergo necrosis. In theory local and sustained supply of oxygen, one of several essential nutrients for most cell types, could prolong ischemic survival and thereby prevent to onset of tissue loss. In chapter 3 we modified the method of Ahn & Mustoe [67] to create necrotic ischemic wounds and used a previously developed and proprietary calcium peroxide formulation developed in our laboratory as a dressing patch changed every three days to determine if the patch could mitigate necrosis. To our knowledge no biomaterial is capable of preventing necrosis in such wounds.

3.2 *In vivo* oxygen delivery

Oxygen can penetrate no more than 0.4mm through intact skin [68] and so topical delivery is likey effective on in open wounds. Systemic oxygen delivery methods such as HBOT and normobaric oxygen delivery rely on a functioning vasculature with which to deliver the super-physiologically oxygenated red blood cells.[57] Only a few studies have investigated implantable oxygen delivery systems. Harrison et al (2007) [69] reported the use of sodium percarbonate embedded in a Poly(D,L-lactide–co–glycolide) able to release oxygen over three days and implanted as a film under skin flaps to temporarily delay the onset of necrosis up to three days in full thickness skin flaps. Ward et al (2013) [70] also reported that reported that injections of 0.08mg of sodium percarbonate releasing oxygen over a period of 24h in ischemic limbs allowed partial preservation of muscle contractility during acute ischemia. No studies developing these concepts has been published to date.

In chapter 4 we investigate the fabrication and use of an oxygen delivery implant able to sustain the release of oxygen over 14 days and assess its efficacy in preventing necrosis in a full thickness skin flap model and examine the limitations of the technique.

3.3 High density cell cultures

The interest in increasing cell density from normal culture (5×10^6cells/mL) to high density cell culture (10^7 to 5×10^8cells/mL) [71] lies in the construction of bioreactors with increased biomolecules, protein or vaccine production [72], as well as the creation of tissue engineered near physiological cell density constructs (typically ~$1\text{-}5\times10^8$cells/mL).[73]

A wide variety of bioreactors for cell cultures in suspension is available [72] and allow the homogeneous optimization (medium input, oxygenation, waste removal, etc.) by combination of different methods (stirring, oxygen bubbling, tangential flow, etc.) [74] and allow culture of high

cell densities (2.14×10^8cells/mL).[75, 76] Bioreactors for engineering tissues are more limited dues to low diffusion through cells themselves and their ECM. Tissue engineered constructs do not possess functioning vasculature, and the movement of medium through the structure is limited to diffusion or flow through channels and pores that can become occluded as the tissue grows [77] *in vivo*, cells are usually found no more than 100µm from capillaries [78] and at physiological densities, oxygen diffusion will be limited to a maximum of 200µm.[79, 80] This limits both the size of the scaffolds that can be build as well as their cellular density depending on the tissues and their metabolism (at physiological densities, 5 to 8mm for cartilage, 100-200µm for cardiac tissues).[77]

3D-Bioprinting has the potential to create constructs from a mixture of cells, biomaterials and bioactive agents, also known as bioinks, allowing spatial control of cell position to facilitate the creation of tissue engineered structure, but do not yet match the required resolution to build complex organs with vascular networks.[81] Even if some bioprinting methods allow the creation of organs and tissue mimics with physiologically relevant densities (Laser assisted bioprinting, 10^8cell/mL) [82], The resulting constructs however still suffers from the limitation of tissue engineered constructs mentioned previously. Techniques like PFC, HOBT, haemoglobin-based carriers have allowed improvement of oxygen delivery to increase size viability, protein expression and production of said constructs.[83] However, these means of delivery are external to the construct, therefore also require conduits and are subject to diffusion limitation. *In situ* oxygen generation may have the potential to mimic capillaries in tissues and organs and deliver oxygen in a homogeneous manner throughout the constructs. Different techniques like solid peroxides, photosynthetic biomaterials, myoglobin-polymer-surfactant, polymer-encapsulated oxygen microbubbles, etc. have been reported to have potential applications *in vitro* to maintain cell

survival during hypoxic or anoxic events [83], yet no use of *in situ* oxygen releasing materials in constructs at physiological density has been reported.

In chapter 5 we attempt to maintain cell viability at 1×10^8cells/mL in alginate beads (ø 1.7mm) by using calcium peroxide microparticles (ø 10-200µm). We hypothesise that the new bioink that can sustain cells could ultimately allow building large engineered constructs at physiological densities without a functioning vasculature.

3.4 Peroxide based oxygen delivery

Oxygen generating biomaterials were based on the use of calcium peroxide (CaO_2) and its reaction with water to form oxygen and hydrogen peroxide, that will further decompose into oxygen, following **Equation 1-6** to **Equation 1-8**:

Equation 1-6: $\mathrm{CaO_2 + H_2O = Ca(OH)_2 + \frac{1}{2}O_2}$

Equation 1-7: $\mathrm{CaO_2 + 2\ H_2O = Ca(OH)_2 + H_2O_2}$

Equation 1-8: $\mathrm{2\ H_2O = O_2 + 2\ H_2O}$

Reaction of peroxide with water is fast and can release oxygen and also hydrogen peroxide (H_2O_2) in amounts which can be potentially cytotoxic. CaO_2 was embedded in a hydrophobic polymer (polycaprolactone, PCL) to reduce and control the reaction rate. To further reduce H_2O_2 potential cytotoxicity, iron oxide (Fe_3O_4), a biocompatible inorganic catalyst [84], was used to catalyze the decomposition of H_2O_2 [85, 86] into oxygen according to a Fenton-Haber-Weiss catalytic reaction (***Figure 1-5***).

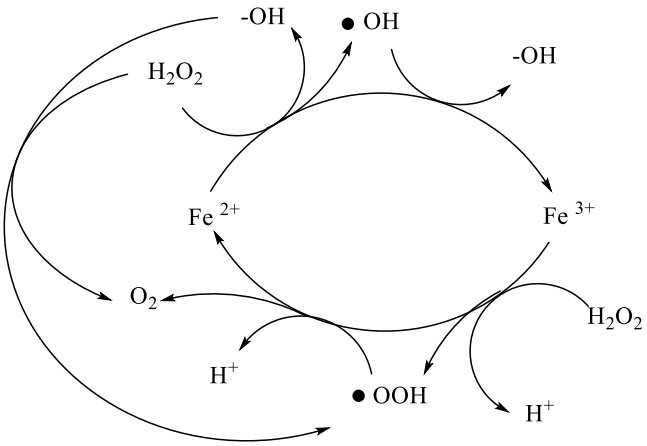

Figure 1-5. Fenton-Haber-Weiss catalytic reaction.[87, 88]

Chapter 2 - Bioinorganic and wound healing

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Abstract

Wound dressings and the healing enhancement (increasing healing speed and quality) are two components of wound care that lead to a proper healing. Wound care today consists mostly of providing an optimal environment by removing waste and necrotic tissues from a wound, preventing infections, and keeping the wounds adequately moist. This is however often not enough to re-establish the healing process in chronic wounds: with the local disruption of vascularization, the local environment is lacking oxygen, nutrients, and has a modified ionic and molecular concentration which limits the healing process. This disruption may affect cellular ionic pumps, energy production, chemotaxis, etc., and will affect the healing process. Biomaterials for wound healing range from simple absorbents to sophisticated bioactive delivery vehicles. Often placing a material in or on a wound can change multiple parameters such as pH, ionic concentration, osmolarity, and it can be challenging to pinpoint key mechanism of action. This article will review the literature of several inorganic ions and molecules and their potential effects on the different wound healing phases and their use in new wound dressings.

Key words: Wound healing, chronic wound, wounds dressing, bioinorganic.

Introduction

Before considering the role that inorganic ions and molecules might play in improving and accelerating healing, it is necessary to consider current materials used for dressings and what physical functions they play that also greatly affect healing outcomes. The clinical variability of wounds and their underlying cause combined with the lack of a control wound, compared to the reproducibility and ease of studying bilateral injuries in preclinical animal models, also means that trials can be hard to statistically power. Consensus as to effectiveness is then sometimes lacking, for example honey has been used for millennia to treat wounds but some wounds are not healed faster by its use. [89-91]

The wound healing process is divided into orderly and overlapping phases: haemostasis, inflammation, proliferation and maturation. They are tightly regulated by a series of external and internal stimuli such as growth factors (GF) and cytokines, resulting in regeneration of the damaged skin. [92, 93] Acute wounds are injury to the skin resulting from abrasion, avulsion, incision, laceration, and puncture that heals according to the normal wound healing process. Burns are acute wounds that differs from other acute wound by its systemic impact. [94, 95]

Disruption of the normal healing process can result in non-healing wounds (chronic wounds). This impaired healing is often a consequence of an underlying condition (e.g.: diabetes, age, ischemia) or of external factors (e.g.: infections). [96] These systemic preconditions often result in a partial or total restriction of the blood flow to a wound that in turns disrupt the healing process by limiting the amount of nutrients, oxygen, waste removal and homeostasis. In the U.S. alone, 6.5 million suffer from chronic skin ulcers [92], making restoration or enhancement of wound healing one of the major challenges in healthcare.

Wound care today consists of wound cleansing, disinfection, closure if needed and dressing. Wound dressings should provide optimum parameters for the healing process: prevention of infections, removal of debris and wastes, proper oxygenation, and a moist environment that allow migration of cells and wound closure. It also needs to account for type of wounds and the patient precondition. [95-97] Traditional dressings (gauze, lint, plasters, bandages, cotton wool, etc.) are mostly used to protect the wound from contamination but they also fail to provide a moist environment. Modern dressings have been designed to account for the cause and type of wound. They allow modification of the physical parameters of the wound environment [98] (***Table 2-1***), or are bioactive by either playing an important role in healing process (biomaterials derived generally from natural tissues or artificial sources to provide a extracellular support to cells) or delivering bioactive molecules that enhance migration and proliferation of cells, etc. Tissue engineered skin substitutes are modern dressings that are cell-containing matrices or acellular matrices that release bioactive molecules to accelerate epithelialization. Negative pressure wound dressings apply a vacuum to the wounds in order to drain exudate. [96] Its efficiency is widely discussed [99-101], and appears to maintain a moist environment, optimize blood flow, remove exudates, and applies pressure to promote wound closure.

Table 2-1: Representative list of the different types of modern dressings, their advantages and disadvantages

Dressings types	Advantages	Drawbacks	Examples of Commercial brands	Ref
Semi-permeable film	O_2 and CO_2 permeant, impermeable, and	Low exudate absorption	Opsite™, Tegaderm™, Biooclusive ™	[102]

	can adapt to the shape			
Semi-permeable foam	Allows gaseous exchange, and absorbs exudates	Not suitable for low exudating wounds	Lyofoam™, Allevyn™, Tielle™	[103, 104]
Hydrogels	Suitable for all stages of wound healing	Low mechanical strength and exudate accumulation	Intrasite™, Nu-gel™, Aquaform™	[105]
Hydrocolloid	Permeable to water vapor but impermeable to bacteria, debridement and wound exudate absorption	Not indicated for neuropathic ulcers or highly exudating wounds	Granuflex™, Comfeel™, Tegasorb™	[106, 107]
Alginate	Limits wound exudates and minimizes bacterial contamination, accelerate healing	Inhibits keratinocyte migration, not suggested for dry wound, third degree burn wound and severe wounds with exposed bone	Sorbsan™, Kaltostat™, Algisite™	[108]
Bioactive (collagen, hyaluronic acid, chitosan, alginate, elastin)	Facilitate cell migration and ECM production	Low mechanical strength and exudate accumulation		[109-116]
Tissue engineered skin substitutes	Facilitate and stimulate cell migration, proliferation and ECM production	Low exudate absorption, risk of infection, antigenicity	Alloderm™, Integra™, Laserskin™, Biobrane ™, Bioseed™, Hyalograft3-D™	[117]
Medicated Dressings	Can be tuned to exhibit specific properties (Antibacterial, proliferation promotion, wound debridement, etc.)		Cutisorb™, Debridace™	

Bioglass	Can stimulate cells function using by releasing ions	Toxic potential of ions in contact with physiological tissues	DermaFuse™ Mirragen™	[118]

Providing an optimal environment for the wounds to heal is often not enough to re-establish the healing process in chronic wounds. When a patient suffers from a blood flow limiting precondition not only is exchange of nutrients and wastes limited, but there is also a localized imbalance of ions and molecules affecting cell homeostasis. Bioinorganics is a field that manipulates inorganic ions to lead to a variety of therapies. [119-121] Systemic inorganic ion deficiency and overload effects on health are well documented. For example iron overload and deficiency lead to thrombosis suggesting an involvement of iron in the coagulation cascade. [122] Most of this knowledge is based on systemic delivery and little is known about localized delivery for localized effect. This article will review the literature of different organic ions and their effects on the different wound healing phases and their use in new types of wound dressings.

Protons, calcium ions, carbonate ions, etc. are also deeply involved in cell biology through ionic pumps and channels. They participate in ionic exchanges between the cell and the extracellular environment and drive energy production, cells migration, etc. [123]

Inorganic ions or molecules, and more particularly metal ions, are essential catalytic and structural elements of some proteins, enzymes, transcription factors, and can inhibit or increase their expression level and activity sometime through conformational changes.

Compared with biologic/organic agents there have been relatively few studies focusing on the influence of inorganic ions on wound healing even though some have demonstrated a potent influence. This review focuses on the influence of a selection of inorganic ions or molecules on the different phases of wound healing, their use to promote wound healing *in vivo* and their potential use in the development of new dressings.

Wound Healing Process

Haemostasis

In the normal wound healing process, haemostasis is the first stage of wound healing occurring within the first minutes after wounding. This process involves (i) vasoconstriction that reduce the blood flow; (ii) the formation of a platelet plug: platelets express receptors and (iii) the formation of a fibrin clot around the platelet plug (***Figure 2-1***). [124]

Inflammation

Within an hour after clotting, polymorphonuclear neutrophils (PMNs), become the predominant immune cells in the wound bed up to two days after the injury [125] and phagocytize debris and kill bacteria by releasing free radicals (respiratory burst) and break down damaged tissues through proteases. PMNs either undergo apoptosis after two days or are degraded by macrophages. After monocytes then enter the wound bed they mature into macrophages that participate in phagocytosis, secreting several growth factors and cytokines. promoting angiogenesis and indirectly stimulate granulation tissue formation, and re-epithelialization.

Proliferation

Before the inflammatory phase has ended, factors released by macrophages activate fibroblasts, endothelial cells and keratinocytes from surrounding tissues that start migrating and proliferating, laying down a new extracellular matrix and forming granulation tissue, gradually forming a barrier between the wound and the environment and closing the wound.

Maturation

Maturation of the wound can start as early as three days after wounding. During this phase, the previously lay down collagen matrix is slowly remodeled.

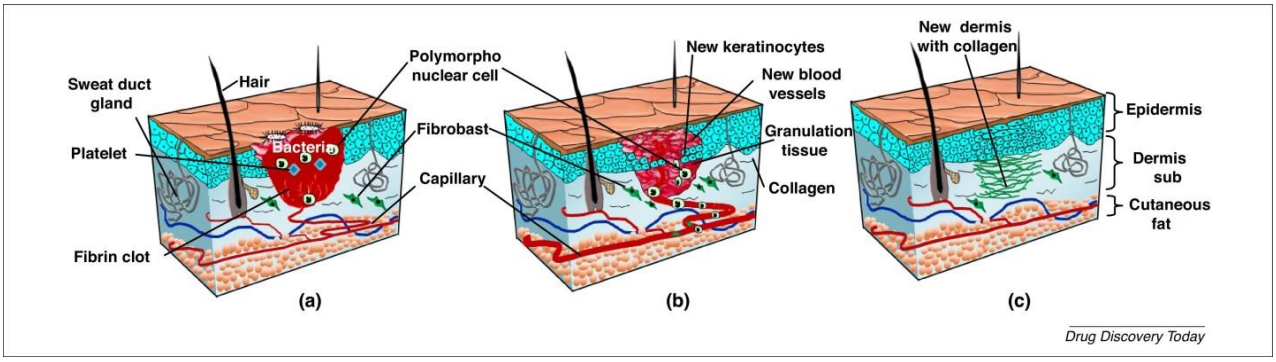

Figure 2-1: Schematic representation of different phases of wound healing. a) Haemostasis and Inflammation: Following injury the wounded area is filled with blood clots which seal the wound and creates haemostasis. Followed by the hypoxic inflammation phase, where the bacteria, neutrophils and platelets are abundant in the wound bed. b) Proliferation: Endothelial cells migrate into the clot where they proliferate and form new blood vessels. Following migration and proliferation of fibroblasts cells they deposit extracellular matrix and form granulation tissue. At the wound edge, keratinocytes proliferate and migrate along the injured dermis and above the provisional matrix. c) Remodeling phase: includes wound contraction and collagen deposition by fibroblasts. Finally, neo-epidermis completely covers the wound. Reproduced with permission from Mohanty et al. [126]

Calcium

Calcium can form stable coordination complexes with organic molecules (enzymes, proteins, etc.). It acts as an electrolyte and is vital to the health of the muscular, circulatory, and digestive systems;

is indispensable to the building of bone; and supports synthesis and function of blood cells. It is involved in energy production/consumption in eukaryotic cells through calcium channels and pumps and is required for the cells to assume their roles, and an alteration of calcium level will have an impact on the wound healing process summarized in ***Figure 2-2***.

Haemostasis

Assembly of procoagulant complexes and generation of thrombin requires calcium. It mediates the interaction and activation of different factors of the coagulation cascade. Increased calcium concentration can increase clot formation speed by increasing the rate of fibrin-monomer polymerization. [127-129] Accumulation of calcium in vascular smooth muscle cells results in their contraction and leads to vasoconstriction, an important phase for the haemostatic process. [130]

Inflammation

A correlation has been shown to exist between calcium concentration and the inflammatory and proliferative activity in the different layers of the skin. Exchanges of calcium ions between the extra/intracellular spaces of neutrophil modulate their respiratory burst and mobility in the early stage of inflammation. [131, 132] Calcium entry in the neutrophils is necessary for the generation of superoxide, an increase or decrease in extracellular calcium concentration leads to a similar variation in superoxide production. At a later stage of the inflammatory response, calcium has a chemokine effect regulating macrophages and monocytes. [133] Monocytes migrate toward Ca^{2+} in a dose-dependent manner, which will in turn play a role in the formation of granulation tissue and angiogenesis process by secretion of various factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). They will also participate to the removal of neutrophils. [134, 135]

Proliferation

A decrease in calcium concentration diminishes fibroblast adhesion and proliferation. [136] On the contrary, a high calcium content can increase keratinocyte differentiation, thus inhibiting their proliferation. [137]

Extracellular calcium can upregulate expression of different angiogenesis-related genes involved in the production of cytokines and growth factors important for endothelial cells proliferation and migration. [138-140] For instance, it has been claimed that calcium phosphate coatings increase angiogenesis. [141] Controversially, the use of calcium channel blockers has been demonstrated to have a positive impact on wound healing [142-145] exhibiting faster wound closure, with better fibroblast and endothelial cell proliferation and an improved collagen deposition. These effects have been demonstrated in both acute and diabetic wound models and appear to be mostly due to an enhancement of nitric oxide production in endothelial cells. [146-149]

Calcium-based materials

Because calcium is the main component of mineral in bones and teeth, calcium-based biomaterials (calcium carbonate, sulphate and phosphates) have been used for orthopaedic and dental applications [150] Those that are poorly soluble have a property known as osteoconduction whereby bone grows along the material's surface. While widely exploited for osteointegratration through osteoconduction, most of what is known about osteoconduction was empirically determined in preclinical models and the biological mechanism by which these materials support bone growth is not known. Only a few studies have been performed on the utilization of calcium-based biomaterials for acute/chronic wounds and indeed superficially it is counter intuitive to introduce high modulus ceramics to a soft and painful wound bed. Calcium crosslinked alginate

has been widely investigated mostly because of its ability to keep a wound moist and absorb exudates while delivering active agents. [151]

Calcium chloride or calcium carbonate nanoparticles [152] (topical application and intravenous injection), calcium phosphate particles (local injection [153], and as cream for topical application [154]) have demonstrated the potential of calcium delivery to improve wound healing. Calcium-containing bioactive-glasses have been studied for different kinds of applications (drug delivery agent, bone tissue engineering, antibacterial agent, etc. [155]). In wound healing, utilization of calcium-containing bioglasses have exhibited stimulatory effects on endothelial cells [156] and fibroblast [157] proliferation, migration, and expression of proteins and growth factors *in vivo*. Bioglasses are composed of several different ions and the so the effect of calcium within this material is yet to be understood, as are also the specific mechanism of action of bioglasses on wound healing. Ethylenediaminetetraacetic acid (EDTA) is a molecule that can chelate metals like calcium, iron, magnesium, etc., have been used in rat brass comb burn model and resulted reduced damage from reactive species. [158] Furthermore EDTA has also exhibited antibacterial properties. [159]

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is naturally produced and degraded by enzymes inside and outside cells where it serve as a signaling molecule in a variety of biological processes from cell death and cell proliferation [160-163] to protein secretion [164] and gene expression. [165, 166] It has the property to dismutate to generate oxygen or radicals and can potentially be cytotoxic due to its high oxidizing potential. [167, 168] ***Figure 2-2*** summarizes some of the literature indicating which phase of wound healing may be affected by altering hydrogen peroxide levels.

Haemostasis

Hydrogen peroxide has demonstrated potential as a haemostatic agent [169, 170]: it triggers the activation of platelets via the cyclooxygenase pathway and also appears to stimulates platelet derived growth factor (PGDF) activation and aggregation. [170]

Inflammation

Hydrogen peroxide level increase significantly during the inflammatory phase [171] and have a variety of effects: [172] briefly, hydrogen peroxide induces leukocytes recruitment by macrophages through mRNA expression and increases their adhesion to endothelial cells. It participates to the creation of molecule with high oxidative potential that act as bactericide or are turn into free radicals through Fenton reaction. It is also able to enhance expression of inflammatory genes and pro-inflammatory cytokines. A defective production of hydrogen peroxide can cause a persistence of the inflammation phase and impair wounds.

Proliferation

A sub-lethal concentration of hydrogen peroxide (up to 500µM) has been shown to increase keratinocyte proliferation and migration in scratch wound models. [160, 173, 174] Moreover it has been shown to increase VEGF expression in keratinocytes [175, 176], macrophages [177] and vascular smooth muscle cells [178] through ribonucleic messenger (mRNA) expression. Increased VEGF level are also observed *in vivo* in full-thickness wound model in mice. [171] Taken together these results are implying that hydrogen peroxide within a certain range (up to 500µM) can enhance vascularization in wound healing. Furthermore, endothelial cells chemotaxis has been reported to be mediated through hydrogen peroxide and cyclooxygenase-2 interactions. [179]

Clinical and Preclinical Studies

Hydrogen peroxide has long been clinically used in wound management for its bactericidal, fungicidal and sporicidal properties [180] and as an haemostatic agent. [169] It was shown to facilitate healing in full-thickness wound model in mice [171, 181] when used at low concentration (10 to 50 mM) concentrations, it was not found beneficial when used at a commercial concentration (166 to 975mM) [182] and could even slow down wound healing.

Clinical studies on hydrogen peroxide [183] and drugs containing hydrogen peroxide [184] have been developed to treat cutaneous infections and were found beneficial to wound healing and skin graft intake in cases of burns and ulcers.

As described previously, honey has been used as a dressing in wound management. Amongst the previously described properties, it also contains glucose oxidase that allows the release of low hydrogen peroxide concentration from the conversion of glucose to gluconic acid [185, 186] that would be beneficial to wound healing.

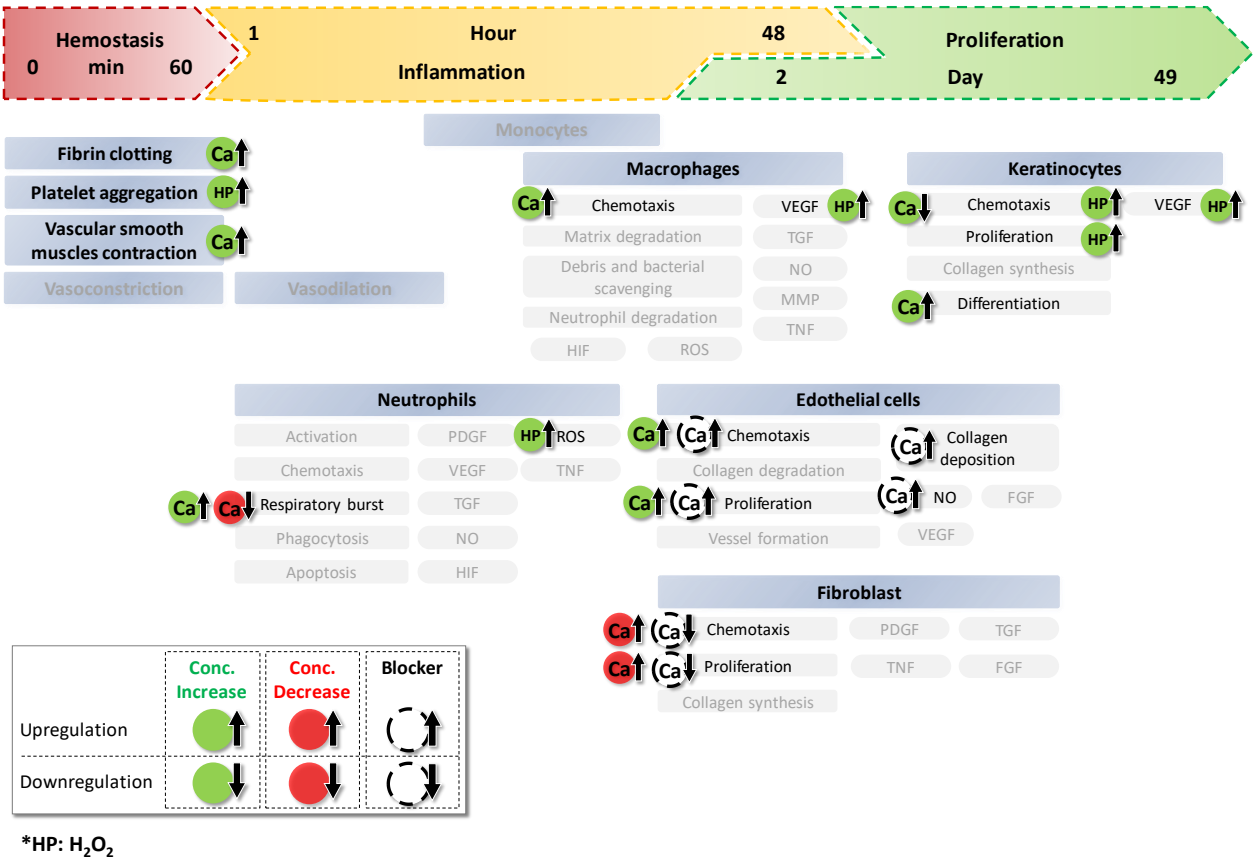

Figure 2-2: Summary reported effects of calcium and hydrogen peroxide level changes on cells involved in stages of wound healing. With the exception of fibroblasts, elevated calcium levels generally have pro-chemotactic effects. When administered at non-toxic concentrations, hydrogen peroxide can promote keratinocyte proliferation and migration.

Molecular Oxygen

Oxygen is a highly reactive gas, an oxidizing agent that readily forms oxides with most elements as well as with other compounds. By mass, oxygen is the third-most abundant element. At standard temperature and pressure, it is usually found as dioxygen (O_2, molecular oxygen). In biology oxygen is at the center of many biological systems. Without it many cells cannot, it allows aerobic

respiration and efficient ATP production in cells. A lack of oxygen reduces energy production in cells and will eventually lead to cell death through the ischemic cascade In wound healing, after injury, oxygen availability decreases in the wound due to of the absence of functioning vasculature resulting in a hypoxic environment.[187] Thus, alteration of oxygen level will influence the different wound healing phases (***Figure 2-3***).

Inflammation

During inflammation, most of the oxygen is consumed by inflammatory cells (mainly neutrophils) to produce reactive oxygen species during the respiratory burst. [188] However, when approaching the proliferation phase, oxygen overload leads to the generation of reactive oxygen species and to the persistence of the inflammation. It has been demonstrated that Intermittent hypoxic environment initiate release of growth factors (transforming growth factor (TGF), TNF and VEGF) from platelets and monocytes [189], and a continuous hypoxia inhibits VEGF release. [190]

Proliferation

Elevation of oxygen levels in the skin through the utilization of hyperbaric oxygen, (HBO), has been shown to stimulate the reconstruction of an epidermis by enhancing fibroblast proliferation (*in vitro* and *in vivo*) [191, 192] as well as proliferation, migration, and differentiation keratinocytes. [191, 193] Interestingly hypoxia has been shown to also increase keratinocyte motility *in vitro*. [173]

Oxygen is needed for collagen synthesis and release (production of collagen is proportional to the oxygen tension). [190, 194-196] It has been demonstrated that an optimum oxygen concentration exists for collagen production in fibroblasts. [197]

Exposure of endothelial cells to low oxygen tension results in a inhibition of Nitric oxide production (NO) as NO synthase requires arginine, NADPH and oxygen [198], which in turns participates to in a variety of physiological processes and is beneficial to wound healing. [199] Topical oxygen treatment was associated with higher VEGF expression in the wound edge tissue. [56]

Oxygen and wound healing

Oxygen delivery for wound healing has been widely described in the literature [49] and carried out through various methods, ranging from systemic approaches like HBO [50, 53-55] which has been shown to be effective for some cases of delayed wound healing [53-55] but is not always found to bring statistically significant improvements to healing [56], it also presents several disadvantages like the need for pressure chamber (***Figure 2-4***), risks of cataract maturation, barotrauma, pneumothorax, oxygen toxicity seizures, etc., [200] with only a temporary effect as oxygen levels are systemically elevated by HBO for the 1-2 hour treatment time, but return to baseline within 3 minutes up leaving the chamber [50] ; to local delivery using perfluorocarbons or Inorganic peroxides and percarbonates that either allowed to temporarily delay necrosis appearance in ischemic skin flap models [69] although the influence of the produced peroxides have not been discussed, or increased wound closure, re-epithelialization, epidermal thickness, collagen content of dermis and neovascularization. [201] Recently polymer/perfluorocarbon-based oxygen loaded particles have been prepared as a mean of oxygen delivery [202], but only allowed and increased oxygen level in blood during few minutes.

Boron

Boron is found in nature as various oxides of B(III), often associated with other elements. Boron is similar to carbon in its capability to form a stable covalent bond. It has long been used with its derivative in chemistry as a catalyst (Suzuki reaction) as it is able to act as a Lewis acid and can form adducts with Lewis bases (e.g.: amines, alcohol, carboxylic acids). Thus, like iron, it can bind to proteins and nucleotides and modify their stereochemistry or stabilize some structures. Boron bio-absorption is mainly through inorganic and organic forms that are easily transformed in boric acid in acidic conditions. [203] It is considered as a probably essential element and has roles in steroid hormone metabolism, bone development, and cell membrane maintenance. It also affects various mechanisms in animals including carbohydrate, mineral metabolism, energy consumption, the regulation of several enzyme activities and embryonic development.[204] Boron deficiency effects are still being researched, it has however been found to affect calcium and bone metabolism, steroid hormones and brain function. [205] In wound healing, boric acid and borax solutions have historically been used as antiseptics in wound treatment but are no longer recommended because of their potential toxicity. [203] Sodium tetra-borate decahydrate (Borax) has extensively been used to form hydrogels as an ionic crosslinker for various applications including wound healing (***Figure 2-3***). [206, 207]

<u>Haemostasis</u>

There is no direct evidence of boron being involved in haemostasis. However increased boron concentration in the blood appear to exacerbate the effects of low vitamin K level which can result in bleeding and death. [208]

Inflammation

Boron is reported to be involved in stabilization of the antigen-receptor complex on lymphocyte surface, as well stabilization their proliferation. It is also hypothesized that similar effects could be observed in inducing the synthesis and secretion of pro-inflammatory cytokines by macrophages. It is a regulator of the immune and inflammatory reactions and macrophage polarization. [209]

Proliferation

Low concentrations of boron (up to 0.5% w/v) in medium have been reported to be nontoxic to fibroblast cultures with no effect on their proliferation. It appears to inhibit the proteoglycan chain and collagen synthesis, but increase the release of these molecules which increases the turnover of the extracellular matrix [210-212]; these effects, added to boron's ability to enhance protease, collagenase and cathepsin D activities in fibroblasts [213], make boron an active effector of the extracellular matrix turnover in wound healing. Boron also enhances TNFα synthesis in fibroblasts [210] (a pro-inflammatory cytokine important for lymphocyte proliferation), the recruitment of monocytes, and the promotion of angiogenesis.

Boron does not appear to enhance keratinocyte proliferation but stimulates their migration and extracellular matrix production by modulating matrix metalloproteases. [214, 215]

It is observed *in vitro* for boron and its derivatives an inhibition of endothelial cells tube-like structure formation of and micro-vessel sprouting of endothelial cells in a dose-dependent manner. [216] However, an *in vitro* dynamic study of borate-based bioactive glass fibers demonstrated that boron could promote angiogenesis via stimulation of fibroblast VEGF secretion. [217]

Boron and wound healing

Boric acid and its derivative's ability to bring improvements to wound healing when used as an ointment is unclear and appear to not entirely related to pH, but also to their ability to regulate the production of extracellular matrix, protein and collagen. [211, 218] Boron can be used to fabricate variety of dressings: boron based gels [216, 219] have been found to significantly increase the proliferation, migration of dermal cells as well as growth factor and gene expression levels. The use of a combination of boron and Pluronics has been shown to improve wound healing full thickness excisional wounds [220, 221] by enhancing fibroblast migration, superoxide dismutase activity, fibroblast growth factors and TNF-β level, VEGF and TNF-α. Boron based bioglass are ceramic similar but more soluble than silica based bioglass, this results in a higher ions release (boron and calcium) dissolution where it stimulates angiogenesis and improve collagen deposition thus improving wound healing in full thickness wounds and diabetic wound models. [222-225] Doping of such material with copper ions has demonstrated increased properties [224], and a doping with different ions (zinc, iron, strontium) are considered. [226]

Zinc

Zinc is a trace element and is the most abundant intracellular metal and the second most abundant in the body after iron. Zinc is ubiquitously found in the body, with 85% stored in muscle and bone, 11% in the skin and liver, and the rest in other tissues. Zinc is located intracellularly and in extracellular matrix in epidermal and dermal tissues in the form of protein complexes where zinc acts as a stabilizer of cell membranes and an essential cofactor. It also plays a central role in mitosis, migration, and maturation. [227]

More than 3000 unique human zinc proteins have been identified, suggesting that more than 10% of the human genome encodes zinc proteins essential in enzymatic and structural roles, transport

and storage, DNA repair, replication, and translation. [228, 229] Zinc can relocate calcium by modifying calcium dependent processes [230-232] and participate to cells membrane stability. Zinc is crucial in countless physiologic processes; it is essential in growth, immune function, tissue maintenance, and wound healing. There is a marked increase in zinc during inflammatory phase of wounds, zinc levels in the wound des can increased by 15–20% within 24 hours and up to 30% with granulation tissue formation and proliferation. [227] ***Figure 2-3*** summarizes some of the literature indicating which phase of wound healing may be affected by altering zinc levels.

Haemostasis

One of the symptoms of zinc deficiency is tendency to bleed and impaired platelet aggregation [233, 234] which can be reversed by zinc supplementation. An important enzyme that takes place in platelet activation is the calcium-dependent protein kinase. Zinc deficiency decreases platelet calcium capture, reducing their activity; this is possibly linked to defective calcium channels due to a lack of zinc that usually protect them by chelation. [235]

Thrombocytes accumulate zinc ions, platelet stimulation during blood clotting involves a local increase of the zinc concentration at the site of blood-vessel injury [236, 237] which affects haemostasis through plasma clotting factors, platelet aggregation, and platelet interactions between themselves and with endothelial cells. [235] Adding calcium ions to a fibrin oligomer solution causes its polymerization and that this phenomenon is intensified several times after addition of zinc ions at physiological levels. [238]

Inflammation

The immune system is highly proliferative, and thus particularly susceptible to Zn deficiency. [239] Zinc ions function as chemo-attractants for some immune cells: a super-physiological Zn

concentration induces PMN chemotaxis *in vitro* and a deficiency leads to reduced PMN chemotaxis, phagocytosis and NAPDH production in association with reduced production of ROS. Zinc deprivation reduced monocyte adhesion and maturation of macrophages therefore the production of cytokines was also modified. [240]

Proliferation

Zinc-dependent matrix metalloproteases, capable of degrading essentially all components of the ECM, are synthesized by various cell types in the wound, notably keratinocytes, macrophages, fibroblasts, and endothelial cells. [241] Like copper and manganese, zinc enhance keratinocyte migration: supplementary zinc promotes induction of some integrin subunits that influence keratinocyte motility in the healing phase. [242, 243]

Zinc in wound healing

Zinc supplementation (topical and systemic) in case of zinc-deficient rats has allowed to re-establish proper healing in full thickness wounds, [244] however a systemic supplementation to non-deficient rats did not improve wound healing. [227] Local application of a zinc oxide cream on a full thickness wound was found to increase wound contraction [245] and wound debridement in rats full-thickness scald burns; [246] it was also reported to advance epithelialization in surgical wounds in rat. [247] Interestingly in partial thickness wounds in pigs, zinc oxide was found beneficial to wound healing, however zinc sulphate [248] or zinc gluconate [249] were found to have no or even deleterious effects on healing. Zinc oxide was found to have no influence on granulation tissue formation [250] and a local application of zinc oxide on granulating wounds in rats was shown to have no effect on the healing rate [251] which correlates results obtained with zinc oxide added to hydrocolloids dressing on full thickness wounds in pig. [252] However locally

applied zinc oxide has been reported to enhance the repair of ulcerated skin in patients [253] and clinical study of gauze impregnated with zinc oxide applied on ulcer promoted the removal of necrotic tissues [254] and improved wound closure. [255-257] Similar results were obtained in a clinical study of Zinc tape that also led to reduction of wound debris and necrotic material in burns. [258]

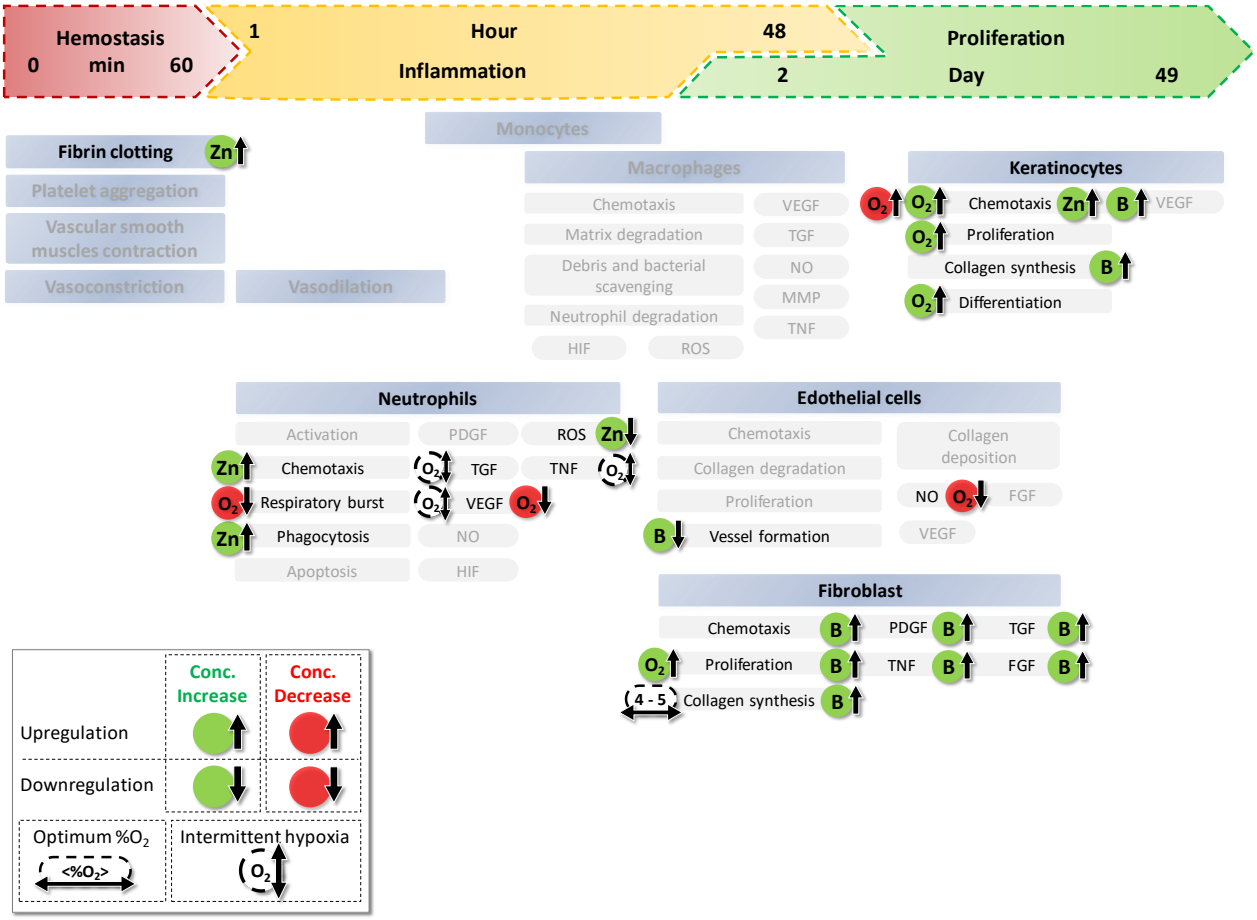

Figure 2-3: Summary of re reported effects of oxygen, boron and zinc level changes on wound healing. Zinc is mostly reported to have an influence on neutrophils, and boron to affect mostly the proliferation phase. All phases of wound healing are reported to be modified by altering oxygen levels.

Figure 2-4: Multiplace hyperbaric oxygen treatment chamber. Reproduced with permission from Mortensen. [259]

Iron

Iron is an important metal for many different biological processes. It can be found as a stable free ion (Fe^{3+}) in plasma (50 to 250 µmol/dL) usually bound to proteins or enzymes, mostly heme where which serves to transport oxygen. Like most ions, iron concentrations are tightly regulated [260] and a deficiency or an overload can have severe consequences. [261] In its free ionic form iron may form reactive oxygen species via Fenton reactions [262, 263] and may impair some cells functions during wound healing (***Figure 2-6***)

Haemostasis

Iron deficiency can lead to reactive thrombocytosis, which in turns can lead to thrombosis. On the other hand, similar effects can be observed with iron overload that causes an increased production of hydroxyl radical that accelerate thrombus formation. [122] Thrombin conversion to fibrin is

sensitive to iron concentration; after wounding, during the early stage of healing, it bounds to the protein and has a stabilizing effect, preventing the formation of a fibrin clot. [264]

Inflammation

Iron deficiency has been also shown to delay and decrease neutrophil activation. [265] Under its free ionic form, iron is involved in the regulation of reactive oxygen species (via Fenton reactions) and can be a nutritional for bacteria. Macrophages play major roles in iron recycling during the inflammation phase. [266, 267] In the case of skin injury, sequestration of iron allows a decrease in radical production and inhibits bacteria proliferation, whereas an overload of iron can cause a persistence of inflammation and damage to tissues. [268] Sequestering of iron can serve to promote the degradation of foreign bodies after the phagocytosis by generation of radicals. [267]

As iron is a cofactor of Hypoxia Inducible Factors alpha (HIF-1α) hydroxylation [269, 270], its chelation or deficiency can induce HIF-1a expression leading to an accumulation of the factor by its stabilization thus simulating hypoxic conditions and the appropriate response from hypoxia responsive elements (erythroprotein, VEGF, etc.) and facilitate the adaptation to hypoxic conditions. [271, 272] However, H1F-1a increases macrophage recruitment and mobility allow expression of pro-inflammatory cytokines as well as neutrophil survival resulting in sustained inflammation. [269, 273] Cell permeable iron inhibits VEGF receptors and consequently inhibits endothelial cells proliferation, migration and viability. [274] In addition, the increased level of VEGF and HIF-1a due to iron chelation promotes angiogenesis [270, 274, 275] which is beneficial to wound healing.

Iron chelation in wound healing

Several proteins and enzymes can bind with iron, either for transport and regulation or to assume their function. Amongst them, lactoferrin is involved at many different levels of the healing process. [276] This protein has a strong affinity for iron ($K \sim 10^{22} M$) and is completely bound to iron (holo-lactoferrin) up to pH 4, and releases it completely at pH2.5 to form apo-lactoferrin. [277] It can be synthesized by glandular epithelial cells and polymorphonuclear neutrophils and is attributed several host defenses activities namely: antibacterial, antiviral, and antifungal. [277] Briefly, in addition to its iron chelating properties, lactoferrin is first involved during the early inflammatory phase where it promotes the production of proinflammatory cytokines, then during the proliferation-remodeling phase by promoting fibroblast migration and proliferation as well as extracellular matrix synthesis (collagen and hyaluronan production).[276]

Iron chelation has been used to modify wound healing. Deferoxamine (DFO) is one of the most widely studied iron chelators and is clinically used to treat iron overload. In the case of topical wound healing, topical DFO has been shown to improve wound closure in diabetic wound model and diabetic pressure ulcers [278-280] by modulating the expression of cytokines and growth factors and to facilitate angiogenesis. Topical application of deferoxamine on ischemic skin flaps has resulted in a reduction of necrosis by two fold. [281] Similarly, in diabetic wound models other iron chelators such as deferiprone, kojic acid and ciclopiroxolamine resulted in a faster wound closure. [282-284] In parallel, topical application of a metal chelator lotion has been demonstrated to protect burn wound progression by reducing oxidative stress. [158] Combination of deferoxamine with a polymer carrier has been recently carried out and could potentially improve iron chelation treatments by reducing its toxicity [285] and facilitate the incorporation in dressings. [286]

Iron Based Biomaterials

Iron is a degradable metal and has been used for bioabsorbable metallic stents and plate systems. [287] Direct topical utilization of iron in wound healing has been mainly limited to the magnetite (Fe_3O_4) phase. Because it is insoluble and biocompatible, it is easy to functionalize and has often been used in medical imagery or as a magnetic drug delivery agent. In the case of incisional wounds, a treatment with magnetite bound thrombin was exhibiting a better healing than thrombin treatment alone, potentially due to the stabilization of thrombin against its natural inhibitors. [288] The use of magnetite as a component in a hydrocolloid dressing also exhibited a faster healing than an undressed wound [289], this could however be due to the effect of the dressing alone.

Copper

Copper is a metal that possesses two common ionic forms (Cu(I) and Cu(II)). Like iron and boron, it forms coordination complexes with ligands and can bind organic molecules to influence their activity and stability. Copper proteins have diverse roles in biological electron transport and oxygen transportation, processes that exploit the easy interconversion between Cu(I) and Cu(II). Copper appears to facilitate iron intake [290], and a deficiency can produce symptoms similar to anaemia, neutropenia, bone abnormalities, hypopigmentation, osteoporosis, hyperthyroidism, abnormalities in glucose and cholesterol metabolism, etc. It also can impair wound healing. [291] An accumulation of copper in tissues caused by Wilson's disease causes can result in liver disease, neuropsychiatric symptoms, cardiomyopathy, etc. ***Figure 2-6*** summarizes some of the literature indicating which phase of wound healing may be affected by altering copper levels.

Proliferation

Copper has been demonstrated as a potent angiogenic factor [292] and is used to stimulate vessels formation in different biomaterials [293] like stainless steel stents, ceramics and hydrogels [224, 294-296] and even as guide to direct vascular growth. [297] When added as a bolus to culture medium (up to 0.5μM) has been shown to increase endothelial cell proliferation, but not fibroblast *in vitro*. [298] It also increases keratinocytes migration through integrin expression. [242, 299] Activation of platelet-derived growth factor (PDGF) signaling is copper dependent [300] and a decrease in copper levels or inactivation of high affinity copper uptake protein 1 can cause significant inhibition of angiogenesis. [301] Indeed, copper chelation has been trialed clinically as an anti-angiogenic therapy for tumor control. [302] Copper also has a clear effect on VEGF production from keratinocytes where an increase in copper concentration results in a significant increase in production of the factor [303] mediated by HIF-α [304]. Copper has a synergistic effect with VEGF or FGF-2 in enhancing angiogenesis *in vitro* [305] and promotes tube-like formation as well as VEGF and FGF-1 expression from endothelial cells. *In vivo* implantation of copper sulphate doped ceramics demonstrated the possibility to enhance and guide micro-vessels formation in ceramic implants. [297, 306] Matrix metalloproteases (MMP) involved in wound healing are also affected by copper: low copper concentration (0.3–3μM) stimulate their activity, and high concentration (1–100μM) stimulates their expression in fibroblasts [307] which stimulates cell proliferation, for wound re-epithelization, and extracellular matrix (ECM) remodeling. [308] It has also been demonstrated that even low concentrations of copper ions can crosslink type I collagen fibrils when under mechanical stimuli, resulting in enhanced mechanical properties of the collagen construct and with increased resistance to proteolytic enzymes.[308]

Copper in wound healing

Topical copper sulphate treatment on rats has demonstrated to significantly accelerated wound closure un murine full thickness excisional wound model. [303] Copper-doped borate bioactive glass microfibers stimulated the proliferation, migration and tube formation of human umbilical vein endothelial cell (HUVEC), and the expression levels of angiogenic-related genes of fibroblasts *in vitro* and accelerated the healing of the full-thickness skin wounds in rats (***Figure 2-5***). [224]

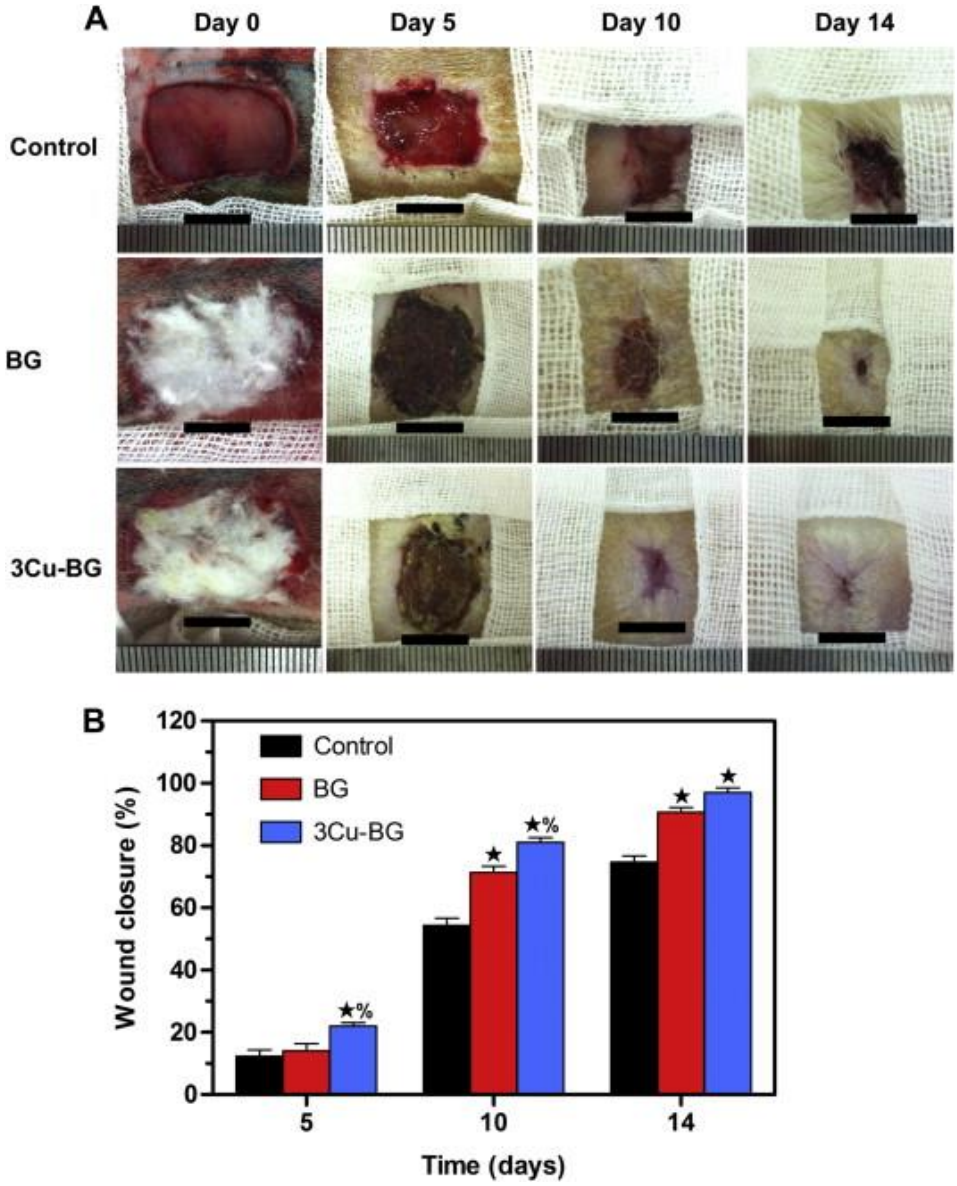

Figure 2-5: (A) Representative images of full-thickness skin defects in rodents, left untreated (control) or treated with the BG or 3Cu-BG microfibers, at 0, 5, 10 and 14 days post-surgery. (Scale bar = 10 mm). (B) Percent wound closure for the untreated defects (control) and the defects treated with the bioglass (BG) or 3Cu-BG microfibers at 5, 10 and 14 days post-surgery. Mean ± SD; n = 6. ★$p < 0.05$ compared to control; %$p < 0.05$ compared to BG. Reproduced with permission from Zhao et al. [224]

Copper GHK complex

GHK is a tripeptide with the amino acid sequence glycyl-histidyl-lysine that functions as a complex with Cu(II) and naturally occurs in humans. The GHK-Cu complex induce various well documented effects in tissues remodeling [309]: it has an anti-inflammatory action, can act as a chemoattractant of endothelial cells and macrophages, activate the synthesis and production of the extracellular matrix and induce angiogenesis, it is involved in numerous gene up- and down regulation and has been widely studied for its role in skin regeneration. [310]

In wound healing, the topical application of the complex as a cream accelerated the wound closure of ischemic wounds in rats [311] when compared to application of the peptide alone. Similar results were obtained for full thickness wounds in rabbits [245] when compared to commonly used zinc oxide ointment, and also for full-thickness pad wounds in dogs [312, 313], pig punch biopsy wounds where it was found to increase granulation tissue formation by increasing blood vessel formation and the level of antioxidant enzymes [245, 314] a stimulating collagen production in fibroblasts. [315] In diabetic wounds it was found to also increase the rate of wound contraction. [316] Interestingly systemic injections of the complex produced similar results for mice, rats and pigs in full thickness wound model. [317] Surprisingly, clinical studies failed to reach clinical significance [309] even though the complex did improve healing of diabetic ulcers clinically. [318]

Magnesium

Magnesium is an alkaline earth metal in the same group as calcium that possesses only one ionic form (Mg(II)) and like other metals is able to coordinate molecules: it is a cofactor for hundreds of enzymes, involved in the transfer, storage, and utilization of energy. [319] It coordinates DNA duplication, influence ribonucleic acid (RNA) translation, ribosome assembly and the opening and closure of ions channels and its influence in the different wound healing phases is summarized in

Figure 2-6. A magnesium deficiency can contribute to coronary atherosclerosis or thrombosis [320], hypermagnesemia can lead to similar issues, but is very rare.

Haemostasis

Systemic injections of magnesium sulphate have been shown to have an anti-thrombotic effect by inhibiting platelet-dependent thrombosis thus increasing the time needed for coagulation [321, 322], despite the fact that magnesium accelerates factor-VII calcium-mediated induced coagulation. [323] It also naturally compete with calcium by antagonizing calcium channels, potentially modulating the vascular smooth muscle contractibility leading to vasoconstriction and vasodilatation. [324]

Inflammation

Acute magnesium deficiency induces an inflammatory response suggesting that a reduced extracellular magnesium might be responsible for the activated state of immune cells. [325] It was suggested that anti-inflammatory effects of magnesium are mainly related to its ability to antagonize calcium channels [326] and that high magnesium levels can impair the immune function. Magnesium deficiency in rats can also cause early activation of neutrophils resulting in a higher production of radicals during the respiratory burst than non-deficient rats [327], an increase in extracellular magnesium concentration is able to attenuate the neutrophil respiratory burst. [328]

Proliferation

When added as a bolus to culture medium, magnesium ions can not only stimulate the proliferation of endothelial cells but also increase their chemotaxis [329-332] and a lack of it has a reversible

inhibitory effect [333]. Supraphysiological magnesium levels in combination with low calcium levels promotes keratinocytes and fibroblast migration. [334, 335]

Magnesium in wound healing

Magnesium inhibits calcium ions entering cells by blocking N-methyl-D-aspartate receptors, which causes an antinociceptive effect. [287] Although magnesium has been studied to develop biodegradable implants for bone repair or vascular stents [287, 336], there are only very few studies on its applications for wound healing. There are case reports of magnesium sulphate being used on patients with infected war wounds [337] as well as in ulcers [338] resulted in healing. Magnesium dietary supplementation was recently found to be beneficial to diabetic foot ulcer healing in a clinical study. [339]

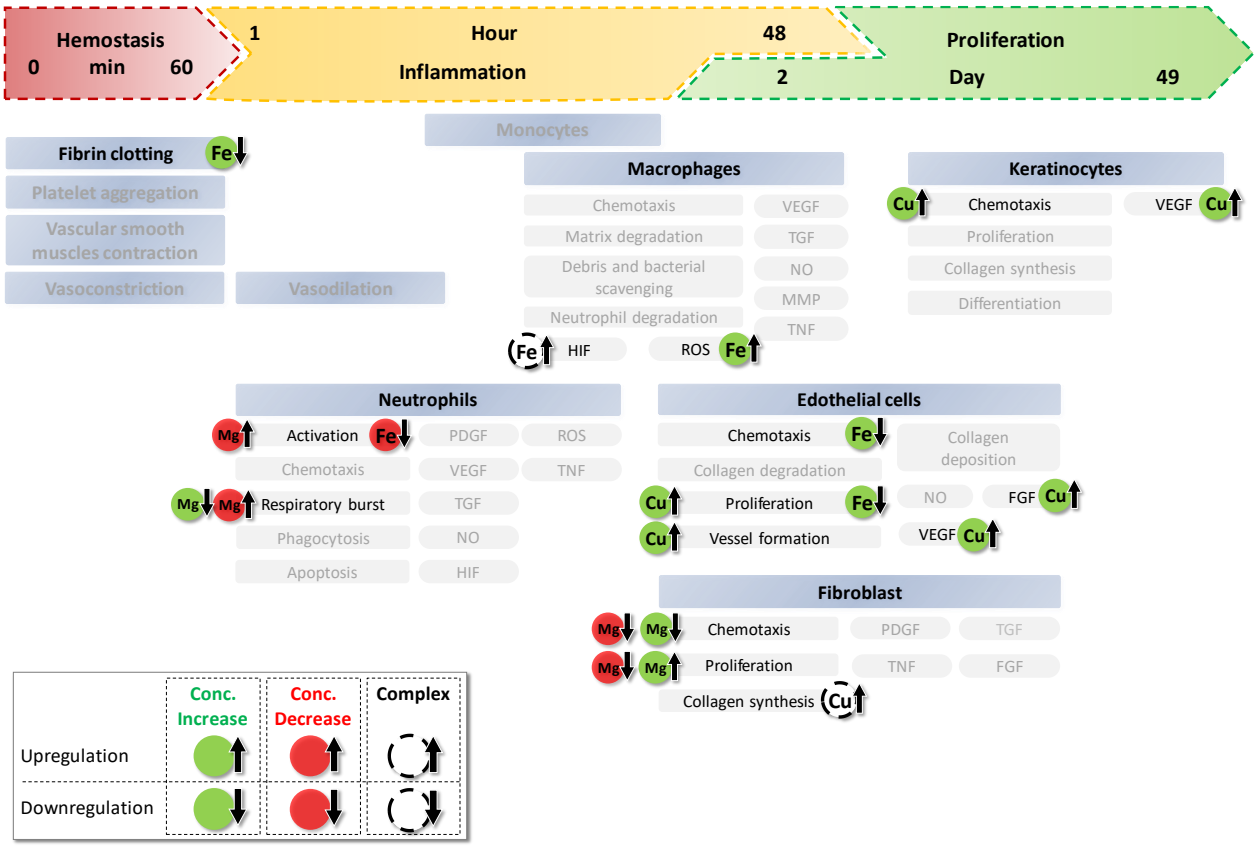

Figure 2-6: Summary of literature reported effects of iron, copper and magnesium level changes on cells types and stages of wound healing. Elevated iron is reported to be detrimental to the healing process and can increase the reactive oxygen species (ROS) production. In contrast, Magnesium is reported to decrease the respiratory burst and increase some functions of fibroblast. Copper is reported to mostly affect the proliferation phase.

Protons and Hydroxide ions: pH

pH is the negative logarithm of proton (H^+) concentration. Protons intervene in cell metabolism through proton pumps and is involved in many reactions and mechanism (NADPH/NADP+ conversion, Energy production through the ATP mechanism, etc.). pH influences molecular conformation and therefore their activity, and can change protein and enzyme activity. [340] In the

case of wound healing, proteases and metalloproteases are the most prevalent enzymes affected by pH.[341, 342] It also directly influences the chelation properties of metallic ions: most chelating agents are unstable at low pH, whereas at high pH metals tend to form insoluble hydroxides which are less accessible to chelating agents. [343]

Physiologically skin surface has an acidic pH (4 to 6). [344] After wounding, the pH of the wound evolves: in acute wounds the pH is acidic (<6), whereas in chronic wounds the pH is usually found to be near neutral (>7.4). In these wounds a local increase of ammonia concentration and dissipation of CO_2 is responsible for a pH increase [344, 345]; such alteration can turn an acute wound into a chronic one. pH modulation of chronic wounds has a potent effect on their healing [346]: it has been reported that acidification of wounds was found beneficial for chronic wound healing by having an antibacterial effect and neutralizing cytotoxic effects of ammonia [347-349] by keeping it in the less toxic ammonium form. Additionally, low pH causes oxygen release from heme into surrounding tissues [345, 349] and a decrease protease activity slowing degradation of the extracellular matrix. [342, 346] Some reported influences of pH on wound healing process are summarized in ***Figure 2-7***.

Inflammation

Neutrophil activity of is pH related. The respiratory burst is optimal at pH 7.2 and will decrease with higher or lower pH. Hydrogen peroxide production can be increase by an alkalization of the extracellular space as well as their apoptosis. [350] On the contrary, low pH delays neutrophil death, inhibiting reactive oxygen species production and enhancing neutrophil endocytosis while diminishing their killing ability. [350-352] Similarly, an increase in pH causes an augmented production of hydrogen peroxide by monocytes. [353]

Extracellular acidification can cause macrophages to activate caspase-1 and release pro-inflammatory cytokines thus aggravating inflammation. [354] It is to be noted that different types of pH changes, for example metabolic acidosis caused by different sources (Lactic acid, HCl) exhibit different results on immune functions: Lactic acid will exhibit anti-inflammatory effects and pro-inflammatory for HCl. [355]

Proliferation

pH influences proliferation and migration of fibroblasts. It as demonstrate in *in vitro* scratch wound model that a pH increase from 7.4 to 8.4 causes a decrease in fibroblast proliferation and migration. [356] In contrast, it was shown that alkaline pH (pH= 8.5) could increase proliferation while decreasing the migration. [357] pH also affects keratinocyte behavior, it has been shown that lowering the pH was deleterious to the cells for both proliferation and migration. [358] Proliferation was found to be optimal in slightly alkaline environment, but migration decrease in both acidic and alkaline. [357]

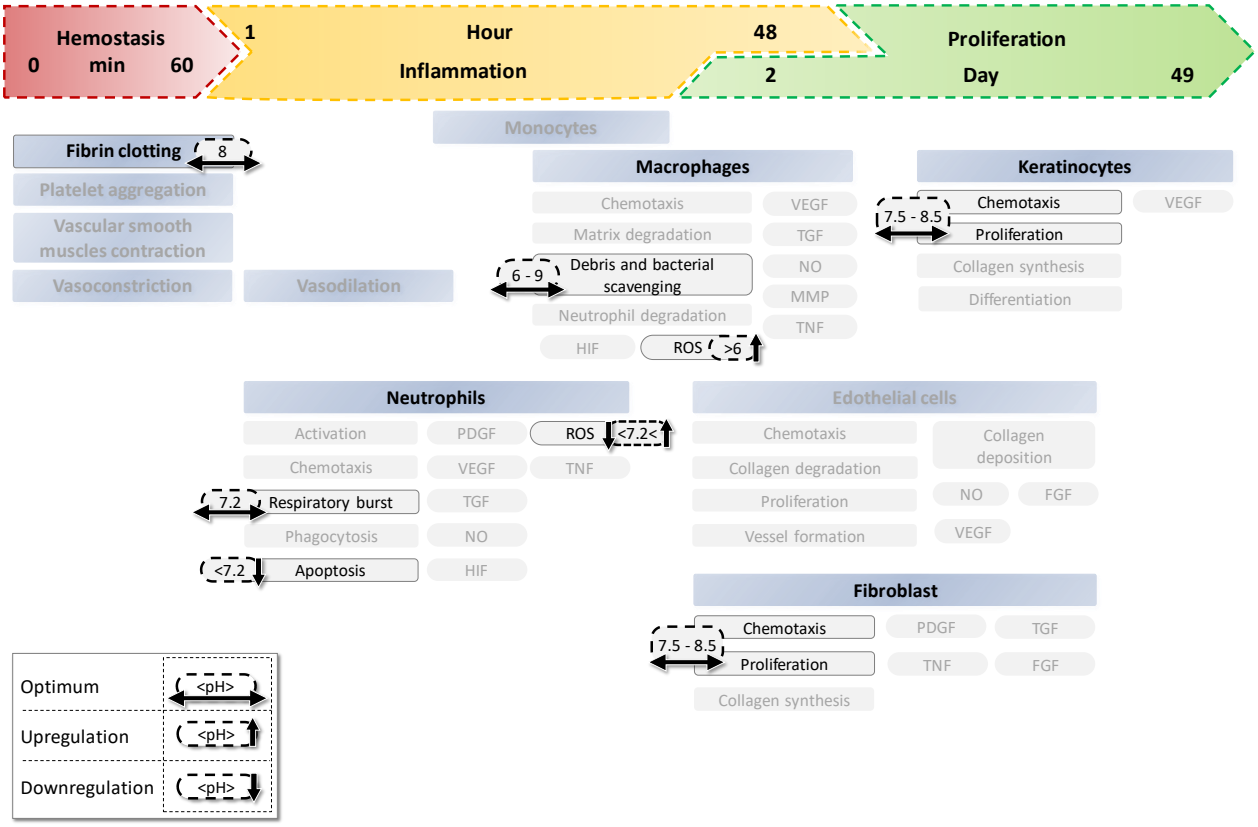

Figure 2-7: Summary of literature reported effects of pH changes on cells types and stages of wound healing.

<u>pH modification in wound healing</u>

The use of dressings in wound care alters the wound bed pH. Many available dressings (Fibers, hydrogels, hydrocolloids, and polymers) can absorb wounds exudate thus modifying the pH of the wounds as an unintended side effect. [359-361] Acidification has been used to improve impaired wound healing and has been found to be beneficial for wound closure in most cases for both clinical and clinical studies. [341, 342, 345-349, 362] Direct application of an acidic dressing on a wound is not sufficient to maintain its pH for a long period, [349] but different types of materials such as manuka honey, sodium carboxymethylcellulose fibers (pH 4.51), protease modulating collagen

cellulose (pH 2.3), Poly(ethylene glycol)/Alginate hydrogel dressings loaded with acrylic acid, have been shown to actively modulate the buffering properties of dressings. [363, 364]

In contradiction to the animal and clinical studies, Kruse et al. have shown using titanium chambers implanted in rats that contain culture medium buffered at different pH [357, 365] that prolonged exposure of the wound to an acidic environment prevents wound closure and re-epithelialization, whereas an alkaline environment did not have a negative impact on wound closure or re-epithelialization when compared to physiological pH. This suggests that a temporary exposure to acidic conditions is more beneficial to heeling than full time exposure and that a on term exposure to an alkaline pH (8.5) would be beneficial for wound healing.

Honey has long been used as a wound dressing [366, 367], and has been demonstrated to be beneficial to wound healing. [368, 369] Its acidic pH (3.2 to 4.5) [370] is involved in its anti-bacterial activity and wound healing properties. [371, 372] It is however a complex mixture and it is difficult to ascertain to what extent its pH is responsible for its healing. Indeed, its high osmotic potential, the high glucose content and its ability to produce hydrogen peroxide [373] could all also potentially account for a part of the alleged effects. In contrast, other reports have found that there was insufficient evidence that honey was beneficial to healing [374] it had no significant effect on legs ulcers [375] or even appeared to delay healing of burns when compared to surgical treatment. [376]

Carbon Dioxide, Bicarbonates and Carbonates

Carbon dioxide is an end product of cellular respiration in organisms that obtain energy by breaking down sugars, fats and amino acids with oxygen as part of their metabolism.

Carbon dioxide is soluble in water, in which it reversibly forms H_2CO_3 (carbonic acid, a weak acid), the relative concentrations of CO_2, H_2CO_3, and the deprotonated forms HCO_3^- (bicarbonate) and CO_3^{2-}(carbonate) depends on the pH. In solution, it can easily bind with metals such as calcium, magnesium, lithium, etc., to form carbonated compounds with a limited solubility. Not often studied as it is usually associated with metals (Ca, Li) that have physiological effects. The buffering effects of carbonates also augment the difficulty of the studies as it can induce a pH change. HCO_3^- is involved in cell homeostasis through different chlorine/sodium transporters and cotransporters and will serve to regulate pH. Acute carbon dioxide exposure resulting in elevated levels in the blood (hypercapnia) is accompanied by respiratory acidosis which decreases serum pH. Oxygen binding to haemoglobin is influenced by carbon dioxide concentration (Bohr effect)[377]: the carbon dioxide entering the tissue capillaries promotes the release of oxygen by decreasing the blood oxygen affinity, and the corresponding decrease in pH promote the release as well. Influences of carbon dioxide, bicarbonates and carbonates level alteration on the wound healing process are summarized in ***Figure 2-8***.

Haemostasis

Sodium bicarbonate ($NaHCO_3$) have been demonstrated to amplify platelet aggregation [378], the use of solid carbon dioxide (-80°C) appear to shortened haemostasis time [379, 380] (mainly due to freezing).

Inflammation

It augments the synthesis of nitric oxide in alveolar macrophages [381] and also reversibly inhibit TNF and interleukin-6 expression in different macrophages, known for their ability to promote

inflammation and their association with tissue injury, suggesting that hypercapnia would have anti-inflammatory effects. [382]

Proliferation

Exposure of fibroblasts to elevated levels of carbon dioxide to 7.5 and 15% (5% is the usual concentration *in vitro*) did not appear to promote cell death but decreased cell metabolism. [383] It was suggested that hypercapnia may contribute to the promotion of vascular regeneration and tissue repair therefore wound healing [384], depending on its level, timing, and duration, as well as the prevailing oxygen tension: a 10% carbon dioxide exposure up to 48 hours has been shown to supports the maintenance of endothelial integrity and homeostasis by promoting wound repair that has been compromised under hypoxic conditions. carbon dioxide also augments the synthesis of nitric oxide in cerebral endothelial cells. [385]

Carbon Dioxide, Bicarbonates and Carbonates in wound healing

Often associated with calcium (under $CaCO_3$ form), the direct effects of carbonates are rarely investigated but utilization of carbonate salts (discussed previously) has resulted in interesting positive results on wound closure [152], or as haemostatic agents. [386] Carbonate enriched hot water has been demonstrated to significantly improve wound closure [387] however the influence of the other ions potentially beneficial to the healing (e.g.: calcium) was not studied. Local delivery of carbon dioxide releasing microparticles has been shown to improve blood flow, wound closure as well as micro-vessel formation and collagen deposition. [388]

Nitric Oxide (NO)

Nitric oxide or nitrogen monoxide (NO) is a gas. It is one of the principal oxides of nitrogen and is a free radical with a short half-life that will react with water and oxygen to form nitrite and nitrite. [389] It is enzymatically produced by different human and animal cells from L-arginine through different calcium dependent Nitric oxide synthase isoforms (neuronal NOS (nNOS) in nervous tissues [390], endothelial NOS (eNOS) by the endothelium [391]) an calcium independent inducible NOS (iNOS) by the immune system. [392] The synthesized NO is involved in many different physiological and pathophysiological processes, its effects on vasodilation have been extensively studied [393, 394], it be used as a neurotransmitter, and has anti-neoplastic, anti-microbial, and anti-proliferative effects. [395] ***Figure 2-8*** summarizes some of the literature indicating which phase of wound healing may be affected by altering nitric oxide levels.

Haemostasis

During haemostasis, NO can act as an inhibitor of platelet and leukocyte aggregation [393, 396] in response to different stimuli, and impairs platelet adhesion to the endothelial cell. [397] It has been demonstrated that the inhalation of NO was resulting in a prolongation of the bleeding time in animals [398], and was controlling blood fluidity in humans. [399] Intravenous injection of NO generating agent resulted in a decreased inactivation of tissue-plasminogen activator by plasminogen activator inhibitors and an increased fibrinolysis. [399]

Inflammation

During the inflammation phase human PMN produce NO [392, 400] to modulate different physiological functions and its effect are well documented. [401, 402] NO inhibition significantly enhances neutrophils chemotaxis in a dose dependent manner and downregulate neutrophils

recruitment. [403, 404] NO reduces neutrophil adhesion to endothelial cell, [403] plays a key role during the respiratory burst as a messenger and as a reactive specie [405] ; it regulates as well their apoptosis through caspase 8 cleavage and caspase 9 activation that are enhance in presence of NO. [406] Similarly, macrophages are capable to sustain and high NO release initiated by inflammatory cytokines for a cytostatic or cytotoxic purpose against bacteria, fungi, tumor cells, etc. [407] NO also up and down regulates expression of different cytokines, chemokines and growth factors. [408]

Proliferation

The effects of NO on endothelial functions has been extensively studied. [394, 409] On one hand, increased levels of NO were associated with an inhibition of vascular smooth muscle proliferation, decreased VEGF production [410] and decreased collagen I and III production in endothelial cells [411] ; a suppression of NO formation on the other hand was associated with an increased smooth muscle cells proliferation. [409]

VEGF stimulates production of NO from rabbit and human endothelial cells via upregulation of eNOS [412], and also stimulates proliferation of postcapillary endothelial cells through the production of NO. [413] Results regarding NO stimulation or inhibition of VEGF production remains debated as there is reports of different NO-generating agents having contradictory effects.[414]

Fibroblasts can be stimulated by cytokines to synthesize NO, while wound-derived fibroblasts synthesize NO spontaneously. [415] NO has been however associated with a decrease fibroblast cytotaxis and an increase of collagen production. [416] NO releasing nanoparticles applied to a fibroblast *in vitro* scratch model resulted in an improved fibroblast migration and collagen type I and III deposition. [417] Similar results were observed with NO-releasing gel. [418] Keratinocytes respond to NO-donating agents in a biphasic manner with increased proliferation and decrease

differentiation at low concentration, and increased cytostasis and increased differentiation at high NO concentrations. [419, 420]

Nitric oxide modulation in wound healing

On wounds, the use of NOS inhibitors topically applied or by intraperitoneal injection, resulted in decreased epithelial proliferation in different models (i.e.: photodamaged skin [421], of skin excisions [422] and burns [423]).

NO production can be increased by up regulating NOS using statins. Oral treatment [424], topical application as a cream [424] or a dressing [425] or a combination of both cream and oral [424] in normal rats or in type I diabetic rat model has been shown increase NO level in the wounds and resulted in improved healing speed.

L-Arginine is the substrate for NOS, and has been shown to improve collagen deposition and wound strength in both animals and humans. [199] L-arginine supplementation resulted in an increased NO level in the fluids of incisional wounds in normal and diabetic mice and was associated with increased breaking strength and collagen deposition. [426, 427] Similar results were observed in wound healing following trauma/haemorrhagic shock. [428] On burn wound model in rats L-Arginine supplementation affect positively epithelialization and accelerated the synthesis of reparative collagen in a dose dependent manner. [429] On the contrary, L-arginine supplementation had no effect in NOS-Knock out mice. [427] A comparison between oral and topical delivery demonstrated that a systemic application was leading to more VEGF and NO expression and was resulting in healing improvement in incisional diabetic wound model in rats.[430] In humans, L-arginine supplementation has been shown to promote pressure ulcer healing in diabetic patients, in a dose independent manner. [431]

Nitric oxide delivery in wound healing

NO release has been achieved using different NO-generating agents that will fit different uses depending on the purpose of the NO release (intravenous injections, topical application, etc.). They have been associated to different carriers to help the localization and timing of delivery with dendrimers, polymers, nanocarriers, liposomes, etc.; or associate to applicators to applicators for topical applications (gels, pastes, oil, etc.). [432, 433] On wounds the use of NO-releasing dressings (gels, nanoparticles) on excisional wounds [418, 434-436] or ischemic excisional wounds [437], resulted in lower amount of inflammatory cells, a faster wound closure related to an acceleration of collagen deposition and granulation tissue formation. [418, 434, 435, 437] However *Schanuel et al*. reported having a higher number of inflammatory cells with NO-generating agents related to an excess of NO despite observing a similar healing then the other studies.[435] NO increase also has been associated with an increase blood vessel density in wounds [417, 435, 436] as well as epithelialization. [417, 434, 437] Currently commercially available NO-generating use nitroglycerine and are dedicated to preventing chest pain caused by angina (Nitro-bid, Nitrol), or to relieve pain in anal fissure (Rectiv).

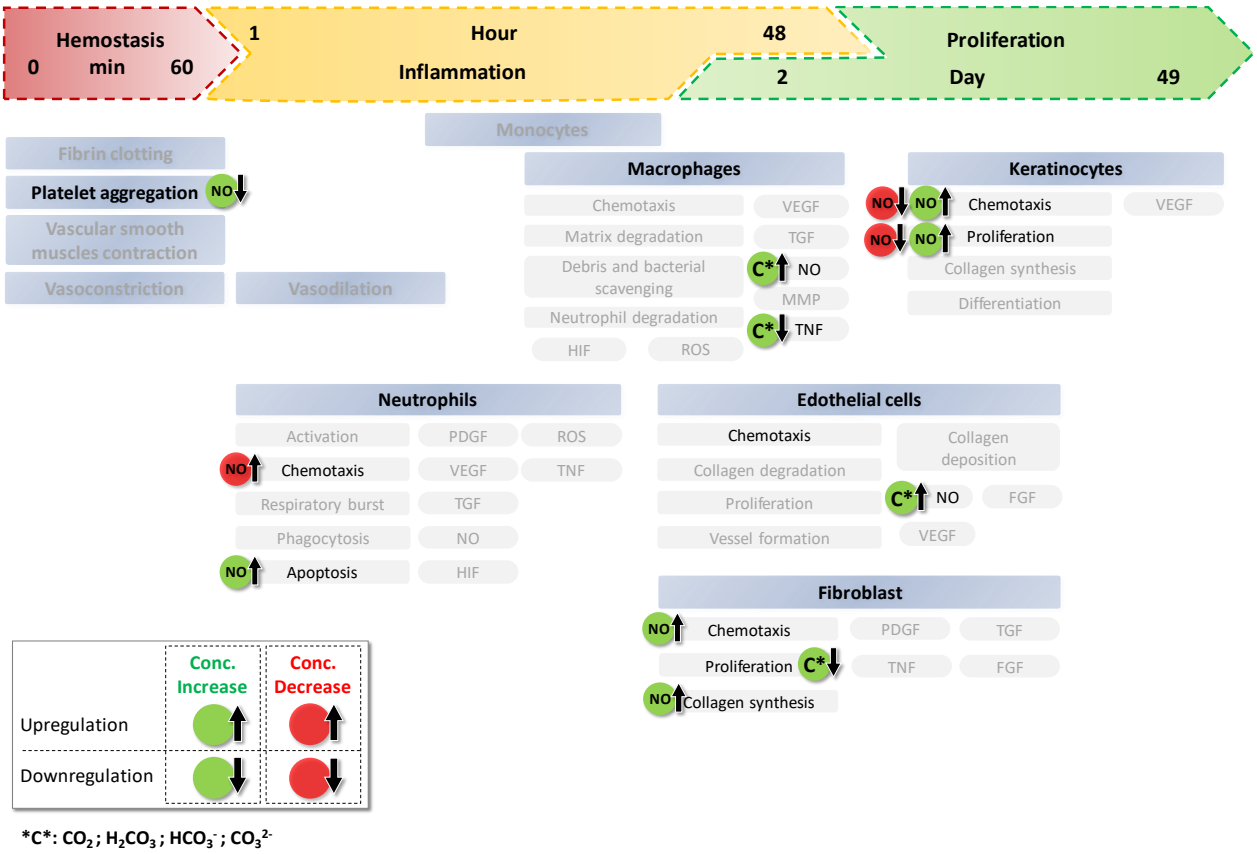

Figure 2-8: Summary of reported effects of changes in levels of carbon dioxide, bicarbonates, carbonates and nitric oxide level changes on cells types and stages of wound healing. Carbon dioxide, bicarbonates, carbonates are linked to NO production which affects all phases of the process.

Manganese

Like other metals, manganese can complex organic molecules and influence the activity of several enzymes. It is relatively non-toxic, and is essential to human health as it acts as a co-factor of enzymes, is required for nerve and immune cells normal development and maintenance, and regulation of blood sugar and vitamins. [438] Its antioxidant properties are due to Fenton-Haber-

Weiss type reaction, modulating ROS [439, 440] in a free form. When bound to superoxide dismutase it can affect collagen contraction and has been found to accelerate wound healing in diabetic mice. [441, 442] Like magnesium, manganese can accelerates factor-VII induced coagulation [323] only in the presence of calcium ions. It also has been found to enhance keratinocytes migration through the modulation of integrins expression. [214, 242, 243]

Concluding remarks

Wound dressings and the healing enhancement (increasing healing speed and quality) are two components of wound care that lead to a proper healing. Wound care today consists mostly in removing waste and necrotic tissues from a wound, preventing infections, and keeping the wounds adequately moist. There are many types of wounds, and the wide variety of wound dressings available makes it relatively easy to find a fitting dressing that would achieve these goals. Creation of an adequate environment is however challenging because of the local disruption of vascularization the local environment is lacking oxygen, nutrients, and has a modified ionic and molecule concentration which may limit the healing process. The ionic concentration change may affect ionic pumps, energy production, chemotaxis, etc. If re-establishing oxygen has been considered and partially achieved using oxygen permeable dressings and other medical devices, re-establishing the ionic balance has not been thoroughly investigated. Some studies have shown that in normal wounds the exudate content of some ions was not significantly different than plasma. [443, 444] If similar results were observed in chronic wounds, the study was limited in number of patients and did not differentiated between the types of chronic wounds. Furthermore, of the ions presented in this review, only calcium, magnesium and bicarbonates have been quantified. [444]

Paying attention to wounds and wound exudates inorganic content in correlation with the types of wounds and re-establishing the "balance" could possibly allow to further improve wound healing outcomes.

Today's research is mostly focusing in enhancing healing using cells [445], growth factors [446], pro-regenerative dressings [447, 448], etc. to increase cells recruitment, migration and proliferation; and meet different grades of success. Most of these "healing enhancers" are not available clinically and are under *in vitro/ in vivo* evaluation and will have challenging regulatory requirements. Some however are commercially available like Dynamatrix® products, containing FGF-2 and TGF-β, and dedicated to being used for bone regeneration and healing of periodontal defects, for gingival augmentation, to maintain or enhance alveolar ridges.

Review of the literature shows that Bioinorganic could be an opportunity to enhance healing to an extent (***Table 2-2***), using inorganic compounds for which some are already approved for different use (Calcium alginate as a dressing, systemic Deferoxamine Mesylate injections, Copper sulphate as food additive, etc.). Due to the overlapping nature of wound healing phases, finding a single inorganic agent to improve wound healing is unrealistic: the outcome of the healing process is the sum of all its stages, and a constant addition or removal of a molecule or ion all along the process may not be in the best interest of the process as an alteration of one step might interfere with another one by slowing it down or even impairing it. There are a great number of factors that can impair wound healing, some of which can be predicted and targeted to improve healing outcomes. For example, Bevacizumab is a chemotherapeutic agent that targets VEGF and impairs angiogenesis and often results in surgical wound complications. [449] Similarly, glucocorticoids have a negative impact on fibroblast proliferation and collagen production which could be answered by pH

modification or the use of oxygen. [449] Considering this precondition, adding to post-surgical wound care bioinorganic compounds that will promote VEGF-producing cells (calcium) and/or VEGF release (Boron, Hydrogen peroxide, etc.) may one day allow to preparation of personalized dressing formulations.

Different wound types and different wound healing stages mean that it is hard to say that changing one ionic concentration consistently throughout will have a beneficial outcome. This review has not discussed infection a risk associated with large and long-term open wounds, and there is a revival of interest in pre-antibiotic era antimicrobials such as silver and peroxides [450]now that antibiotic resistant bacteria are becoming a prevalent problem.

Most researchers envisage a topical application yet in a poorly vascularized wound it is not clear how effective delivery can be. There have been studies considering systemic injection notably the work of Kawai et al [152] in which calcium chloride and calcium carbonate were delivered systemically, but for practical day to day application the risk and difficulty associated with such an injection limits the approach. As described in this review, some pre-existing dressings already have been adapted to deliver ions. [254, 364, 418, 435, 451].

Although some studies have shown the promising effects of bioinorganics in wound care, the level of clinical and even preclinical evidence to support their use is low. Some of the main advantages of such dressings are their relatively low cost, long term stability, and relatively well-established safety profiles: zinc oxide, copper sulphate, magnesium chloride, etc. are already approved by health authorities for consumption and some for topical application.

Many materials that are used in wound care already alter inorganic ion concentration even though that is not their intended mode of action, for example absorbents based on alginate hydrogels can either complex or release cations and chitosans have a high affinity for some cations and anions. [452]

An important role of biomaterials science might be to produce test systems that can isolate interrelated physical and chemical effects, for example by using bioglasses, to understand better how materials can have a beneficial biological effect on wound healing. The encouraging work of Sun et al [447, 448] reported that burns could regenerate a mature epithelial structure with hair follicles and sebaceous glands simply by treatment with a proangiogenic hydrogel with degradation profiles appropriate for each stage of healing. Biomaterials have historically sequentially been limited first by availability of natural substances and then by a lack of understanding of wound biology and more recently by an incomplete understanding of how materials can modify the body's normal progression of wound repair. By better appreciating how ions either deliberately released or as degradation products may be used to manipulate healing environments it may be possible to further improve healing with cytokine, growth factors, and cell-free materials offering simplified treatments to a growing healthcare problem.

Table 2-2: Summary of literature reported effects of the different ion concentration changes presented in this review on the different cell types involved in wound healing (C*: carbon dioxide, bicarbonates and carbonates; concentration increase: ↑ upregulate, ↓ downregulate; concentration decrease: ↑ upregulate, ↓ downregulate; Ion chelation/blocker: ↑ upregulate, ↓ downregulate; ↕ intermitent changes; ⟷ Optimum range.)

	Neutrophils	Macrophages	Endothelial cells	Fibroblasts	Keratinocytes
Ca	↑↓ Respiratory burst [131, 132]	↑ Chemotaxis [134, 135]	↑ Chemotaxis; proliferation [141] ↑ Chemotaxis; proliferation; collagen deposition; NO production [142-149]	↑↓ Chemotaxis; proliferation [137]	↑ Differentiation [137] ↓ Proliferation [137]
H_2O_2	↑ ROS production [172]	↑ VEGF expression [177]			↑ Chemotaxis; proliferation; VEGF production [160, 173-176]
O_2	↓ Respiratory burst; VEGF production [188, 189] ↕ TGF; VEGF; TNF production [189]		↓ NO production [198]	↑ Proliferation [191, 192] 6 - 9 ⟷ Collagen synthesis [197]	↑ Chemotaxis, Proliferation, differentiation [191, 193] ↑ Chemotaxis [173]
Zn	↑ Chemotaxis; phagocytosis [240] ↓ ROS production [240]				↑ Chemotaxis [242, 243]

B			↓ Vessel formation [216]	↑ Chemotaxis; Proliferation; Collagen synthesis; PDGF; TNF; TGF; FGF production [210]	↑ Chemotaxis; Collagen production [214, 215]
Fe	↓ Activation [265]	↑ ROS production [267] ↑ HIF expression [271, 272]	↓ Chemotaxis; proliferation [274]		
Cu			↑ Proliferation; Vessel formation; VEGF ; FGF production [298]	↑ Collagen synthesis [315]	↑ Chemotaxis; VEGF production [242, 299, 303]
Mg	↑ Activation [327] ↓↑ Respiratory burst [328]			↑↓ Chemotaxis; proliferation [329-333]	
pH	7.2 ↔ Respiratory burst [350] ↓ 7.2 Apoptosis [350] ↓ 7.2 < ↑ Respiratory burst [350-352]	6 - 9 ↔ Debris and bacterial scavenging [342] ↓ >6 ROS production [353]		7.5 – 8.5 ↔ Chemotaxis; proliferation [357, 365]	7.5 – 8.5 ↔ Chemotaxis; proliferation [357, 365]
C*		↑ NO production[381] ↓ TNF production [382]	↑ NO production[385]	↓ Proliferation[383]	
NO	↑ Apoptosis [406] ↑ Chemotaxis [403, 404]			↑ Chemotaxis, Collagen synthesis [416, 417]	↑↓ Chemotaxis; proliferation [419, 420]

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada, Discovery Grant (JB) (NSERC)

Conflict of Interest

The authors declare no conflict

Chapter 3 - Anti-necrotic oxygen releasing wound dressings

Anti-necrotic oxygen releasing wound dressings

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Abstract

Insufficient or damaged vascularization is a contributing factor to delayed wound healing in commonly encountered clinical conditions that impair healing and may occur due to ageing, diabetes, radiotherapy, etc).[93] Presently treatment options for ischemic wounds are limited and eventual loss of tissue is often unavoidable. Indeed, tissue necrosis may occur at the center of large ischemic wounds and spread to the surrounding healthy tissue causing tissue loss greater than the original wound. Targeting this regenerative roadblock, we developed a degradable biomaterial gel system to deliver oxygen directly to tissues. This study aimed to evaluate preclinical efficacy of this oxygen-producing dressing [49] (named O2patch) to promote healing and prevent necrosis in large ischemic wounds. The use of the O2patch significantly improved wound closure in wounds with restricted blood flow ($P<0.05$). The exposed cartilage rapidly became necrotic in the control group, but oxygen delivery allowed to significantly retain its viability. The necrotic tissue amount was significantly different between both groups ($P<0.01$) suggesting that by locally delivering oxygen, it was possible to limit secondary necrosis while the healing process is taking place. This first study provides compelling proof of concept that *in situ* oxygen delivery with biomaterials can augment conventional treatments and improve tissue survival.

Introduction

Skin is the largest organ of the human body. When the skin integrity is disrupted, a tightly regulated cascade of external and internal stimuli and biochemical events is set into motion to repair the damage (i.e.: hemostasis, inflammation, proliferation and maturation), referred to as wound healing. [92, 93] Disruption of the normal healing process can result in non-healing wounds or delayed healing (chronic wounds). This impaired healing is often a consequence of the patient pre-condition (e.g.: diabetes, age, ischemia) or of external factors (e.g.: infections) [96]. In the U.S. alone, 6.5 million suffer from chronic skin ulcers [92], making restoration or enhancement of wound healing one of the major challenges in healthcare.

In ischemic wounds, reduction or cessation of blood flow limits the supply of oxygen and nutrients and wastes removal thus delaying the healing process or even preventing it. Similarly, surgical wounds from the incisions made during surgery can progress to chronic wounds if the blood supply was damaged or the wound care inadequate. Reestablishment of vasculature through capillary regrowth takes time during which necrosis can occur.

In blood, the glucose concentration is regulated between 1.4mmol/L and 6.2mmol/L, [38] and oxygen concentration 104 to 146µmol/L.[7] According to the overall reaction of ATP production, during ischemia the first limiting factor to energy production in cells is oxygen.

The concept of delivering oxygen to poorly vascularized tissues either as gas or an oxygenated perfused solution is well known and life-saving but requires a functioning vascular network or the recreation of vascular conduits. Hyperbaric oxygen therapy (HBOT) which consists in the medical use of oxygen at a higher pressure than of the atmospheric pressure to increase systemic oxygen levels in the body, has been widely clinically used and associated with more rapid chronic wound healing [53-55] but is not always found to bring statistically significant improvements to healing.

[56] HBOT can also have serious drawbacks like barotrauma, pneumothorax, oxygen toxicity, seizures, etc. [200], and its effect is temporary as oxygen levels are systemically elevated by HBOT for the 1-2 hour treatment time, but returns to baseline within minutes after leaving the chamber. [50] Previously reported local oxygen delivery using perfluorocarbons or inorganic peroxides and percarbonates either enabled the temporary delay of necrosis appearance in ischemic skin flap models [69], or increased wound closure, re-epithelialization, epidermal thickness, collagen content of dermis and neovascularization [201]. Recently polymer/PFC-based oxygen loaded particles have been prepared as a mean of oxygen delivery [202], but only increased oxygen level in the blood during 2h *in vitro*. The use of inorganic peroxide is not straightforward since they react very rapidly when in contact with water and can result in both the production of oxygen and hydrogen peroxide that is potentially cytotoxic. Traditionally, inorganic peroxides have been used in agriculture to prevent root rot and in aquaculture to sustain fish in high density caging or as remediation after oxygen-reducing pollution events.

Hydrogen peroxide has long been clinically used in wound management for its bactericidal properties, fungicidal and sporicidal [180] and as an hemostatic agent. [169] It was shown to facilitate healing in full thickness wound model in mice [171, 181] when used at low concentrations (10 to 50 mM), it was not found beneficial when used at a higher concentrations (166 to 975mM) [182] and even retarded wound healing as a result of its cytotoxicity. Clinical studies on hydrogen peroxide [183] and topical ointments containing hydrogen peroxide [184] have been developed to treat cutaneous infections and were found to be beneficial to wound healing and skin graft ‘take’ in the cases of burns and ulcers.

An ischemic wound model that can replicate some features of diabetic ulcers was developed by Ahn & Mustoe (1990) [67, 453] and consists of four full thickness 6mm diameter skin wounds on an ear in which blood flow is compromised. While adequate for quantifying healing and closure

rates the wound is not big enough to develop necrosis. We modified it first by increasing the size of the four defects to 15mm. While necrosis was reproducibly induced, we observed that healing varied depending on which side of the ear was measured, presumably because the inhomogeneous blood flow. We further modified the model by placing the three 15mm wounds along the center line of the ear and found this minimized variability and allowed better comparisons. We developed a new oxygen delivery bioinorganic dressing able to maintain oxygen delivery for three days and measured oxygen in the wound bed at the interface between the skin and the dressing. Experimental treatment maintained cartilage viability and promoted wound healing, and more importantly greatly reduced necrosis in this model of impaired wound healing.

Methods

O2patch preparation

O2patches were prepared by preparing a suspension of 1g CaO_2 and 4g Fe_3O_4 in 10% (w/v) PCL solution in chloroform. The resulting mixture was cast into 3x3cm molds and left to dry for 24h. O2patches were immersed in ethanol for 30 minutes and left to dry under a sterile hood, they were then embedded with 6mL of a sterile 3% (w/v) sodium alginate and crosslinked with 6mL of sterilized $CaCl_2$ (1M).

Oxygen & Hydrogen peroxide measurements

In vitro release of oxygen and hydrogen peroxide was performed by immersing O2patches in 40mL water at 37°C and compared to a non-oxygenating alginate patch. Measures were performed at 24, 48 and 72h (N=6 per group). Oxygen release was measured on the surface of the O2patches using an oxygen probe (AL300 Oxygen Sensor Probe, Ocean Optics) calibrated using water flushed with

0, 21, 50% dissolved oxygen at 37°C. Hydrogen peroxide was measured using a peroxide measurement kit (Pierce™ Quantitative Peroxide Assay Kit (Aqueous), Thermofisher, Canada).

Surgical methods

Five New Zealand white rabbit (3.5 to 4 kg) were allowed to acclimatize for 7 days prior to intervention. Anesthesia pre-medication was induced with I.M. injection of 5 mg/kg of xylazine and 0.75mg/kg of acepromazine followed by I.M injection of 20-35mg/kg of ketamine. An isoflurane mask was used for anesthesia induction. The rabbits received 0.12mg/kg of Buprenorphine slow release prior to the surgery. Following induction, the rabbits were intubated, and anesthesia was maintained with isoflurane 1-5% endotracheal intubation inhalation. A single dose of Baytril (5-10mg/kg) was given pre-operatively. The same dose was given post-op to prevent wound infection. Aseptic procedures were carried out after the animal showed signs of being fully anesthetized. The animals were placed in sternal recumbency, the ear shaved, and the cutaneous surface was disinfected with a chlorhexidine, Lidocaine/Bupivacaine (dilute to 2 %, 20 mg/ml) was administered prior the surgery (during preparation). Ischemia was induced by arterial ligation and defects were performed following a modified protocol previously described in literature [453]. Briefly, to simulate ischemia, three vertical incisions were made closely to each of the 3 main ear bundles (composed of the artery, vein and nerves) at a distance of 1cm from the base of the ear. The central artery was ligated but venous circulation was preserved. Both the artery and vein of cranial bundle were ligated. The caudal bundle was left untouched. Connectives tissues in between the bundles were sectioned to remove peripheral vessels. Vicryl 4-0 non-resorbable suture material was used to ligate the vessels. Three 15mm full-thickness skin wounds were created on each ear. Care was taken to remove both the skin and the perichondrium to expose the cartilage. The wounds were then properly cleaned using saline and dressed. For each rabbit, ears received

either O2patch or a non-oxygenating alginate patch as control (3 defects per ear, 5 ears per group). The dressings were changed every 3 to 4 days up to 17 days, the rabbits were anesthetized using a combination of butorphanol 0.2mg/kg and acepromazine 1mg/kg for induction, followed by with isoflurane 1-5%. Bandages were then removed, and the wound cleaned using saline and gauzes. Wound vascularization was assessed using injections of a 10% fluorescein solution (15mg/kg) and a black light (λ=395nm), pictures were taken 15min after injection.

Sacrifice was performed after sedation of the animals using intramuscular injection of xylazine and 0.75mg/kg of acepromazine followed by an intravenous overdose of Sodium pentobarbital (Euthanyl) after sedation is confirmed.

Dressing preparation and change

Prior the wound dressing, O2patches were washed with saline. The wounds were cleaned, and the dressing applied directly on the wounds, then covered with a Tegaderm dressing. 3M surgical tape was used to completely recover the membrane and Vaseline was applied on the whole dressing. A dressing wrap was used to keep the dressing in place.

Wound size measurement

At each dressing change pictures of the ear were taken using standard lighting, zoom and distance with a scale included in the image. Dimensions of the wounds were calculated from the pictures at day 17 after euthanasia using ImageJ software.

Histology and Histomorphometry

After sacrifice, were individually collected and fixed in 4% paraformaldehyde for 24h. Samples were cut in half to expose the center of the wound and underwent paraffin embedding and H&E

staining, pictures were taken under microscope. Area of necrosis, epithelialization and granulation tissue were measured using ImageJ software.

Results

O2patches allowed increasing significantly the dissolved oxygen concentration of 40mL water from 263±4µmol/L (~21% dissolved oxygen) to a maximum of 513±59µmol/L (~42%) at day 2 and a minimum of 457±22µmol/L (~37% dissolved oxygen) at day 3. Hydrogen peroxide release peaked at day 1 with a maximum concentration of 1101±89µmol/L (***Figure 3-1***).

Oxygen concentration at the interface of the ear and the gel was measured after dressing the wound at 564±146µmol/L for O2patch and 219±28µmol/L for alginate (N=25 and 10 respectively, P<0.01). Oxygen was measured before removal of the dressing at 117±37µmol/L for O2-gel and 55±59µmol/L for alginate (N=21 and 10 respectively, P<0.01).

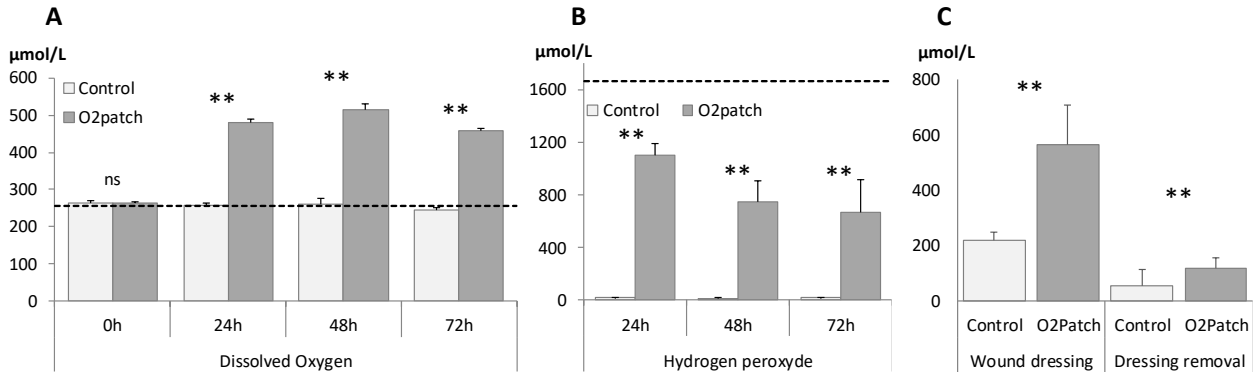

Figure 3-1: *In vitro* dissolved oxygen (A) and hydrogen peroxide (B) released from O2patches and non-oxygenating alginate dressing measured in 40mL deionized water at 25°C. The dotted line in (A) represents the standard oxygen concentration in water at 25°C, and (B) the potentially harmful concentration of hydrogen peroxide. (C) Represent the oxygen concentration at the interface between the wound and the O2patch after dressing of the wound and before its removal. (ns= not significant, **P<0.01)

Wound closure at day 17 was 54±29% for the group receiving O2patch versus 31±23% for the group receiving alginate only (***Figure 3-2***). For the alginate group, the exposed cartilage appeared to be thinner as soon as day 4 and sign of full thickness necrosis could be observed at day 7, whereas in the O2patch group this was rarely observed.

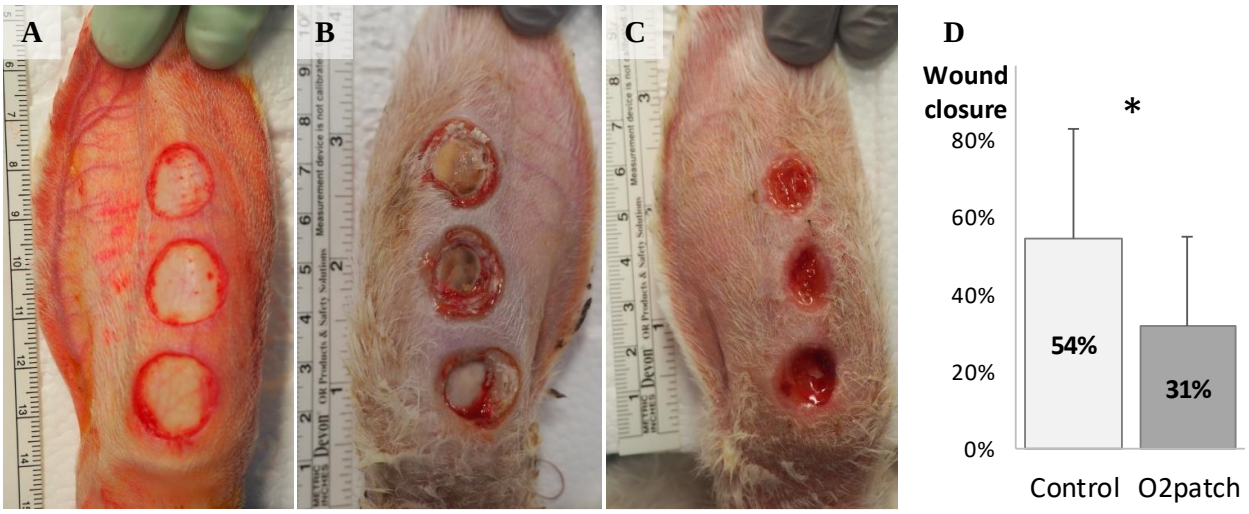

Figure 3-2: Representative photograph of the defects (A) after surgery and (B) and (C) dressed 17 days with alginate or O2patch, respectively. (D) Wound closure at 17 days, mean ± SD, expressed as area of the wound divided by its initial area (N=15 for each group; *$p<0.05$).

Fluorescein injections at day 0 and 4 gave rise to weak or no fluorescence on the edges of the wounds for both groups. At days 7, 11, 14 and 17 the surface of fluorescence of the wounds increased but was still limited to the edges in the control group, whereas the fluorescence was partially covering the wounds in the experimental group as soon as day 7. The quantification the fluorescent surface exhibited significant differences between the two groups (***Figure 3-3***).

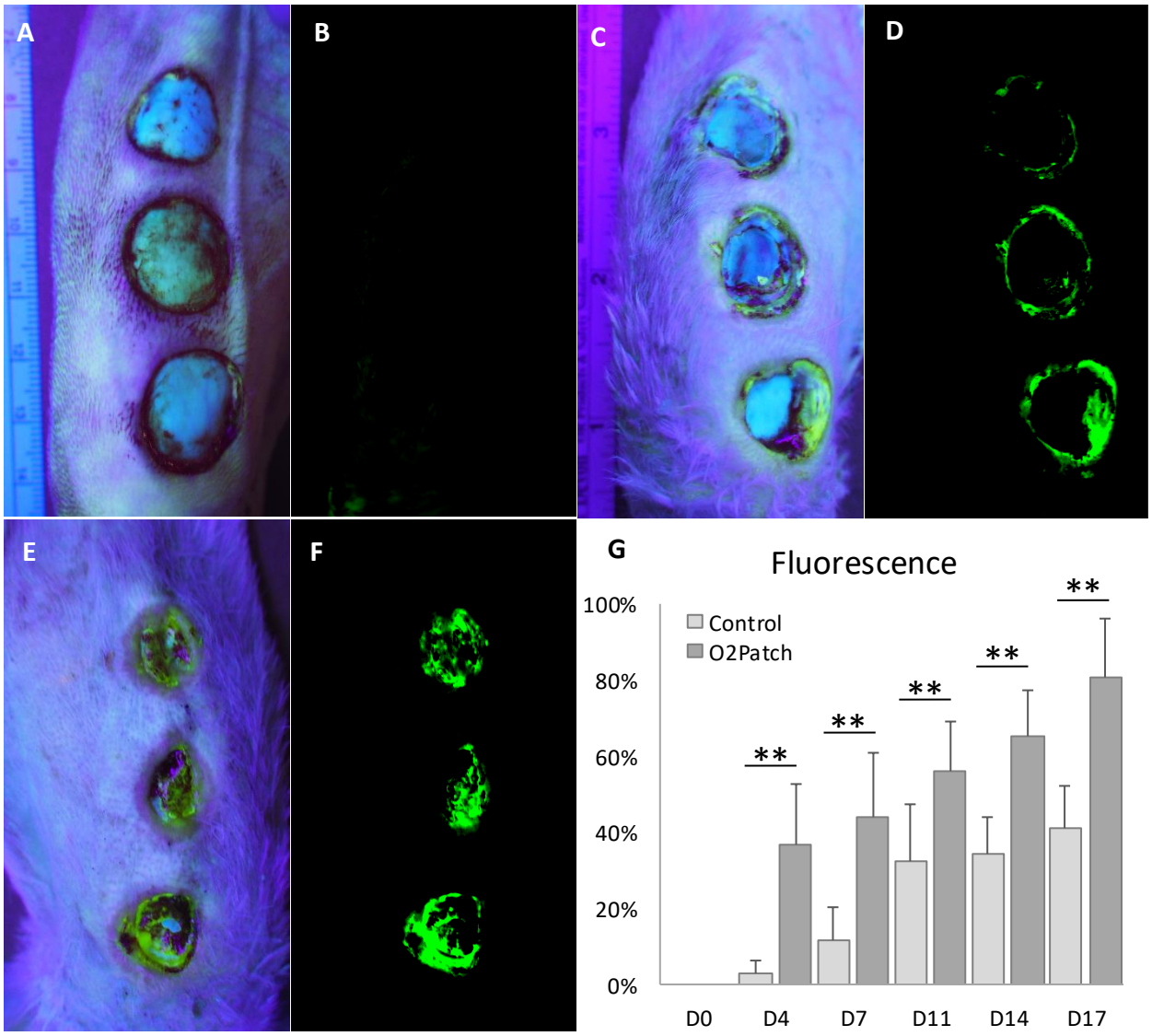

Figure 3-3: Ear photograph under fluorescent light (395nm) 15min after injection of a 10% fluorescein solution (15mg/kg): (A) Post surgery and (B) digitally extracted fluorescence; and after 17 days treated with control (C, D) or O2patch (E, F). (G) Fluorescence from day 0 to 17, expressed as the visible fluorescence in the wound divided by the wound total size (**P<0.01).

At day 7, in the control group, 87% of the wounds had visibly detectable full thickness necrosis and only 40% (N=15 for each group) with O2patch. H&E staining of the paraffin embedder sections (**Figure 3-4**) of the center of the wounds exhibited significant differences between the two

groups. On the outside of the ear, on the edge of the wounds, granulation tissue was visible in most of the samples for both groups. In the experimental group, a thin coherent epithelial layer was covering the granulation tissue, whereas in the control group wounds exhibited a wide range of epithelialization levels, from almost no epithelialization to normal or large clumps of epithelialized tissues. On the inside of the ear, signs of ischemia were visible in both groups. In the center of the wounds, in the control group the cartilage was exposed on the outside of the ear and was showing clear signs of full thickness necrotic skin and cartilage (14 out of 15 wounds). In most wounds of the experimental group, the outside of the ear was covered with granulation tissue and the cartilage had disappeared, however some wounds exhibited full thickness necrosis (4 out of 15 wounds).

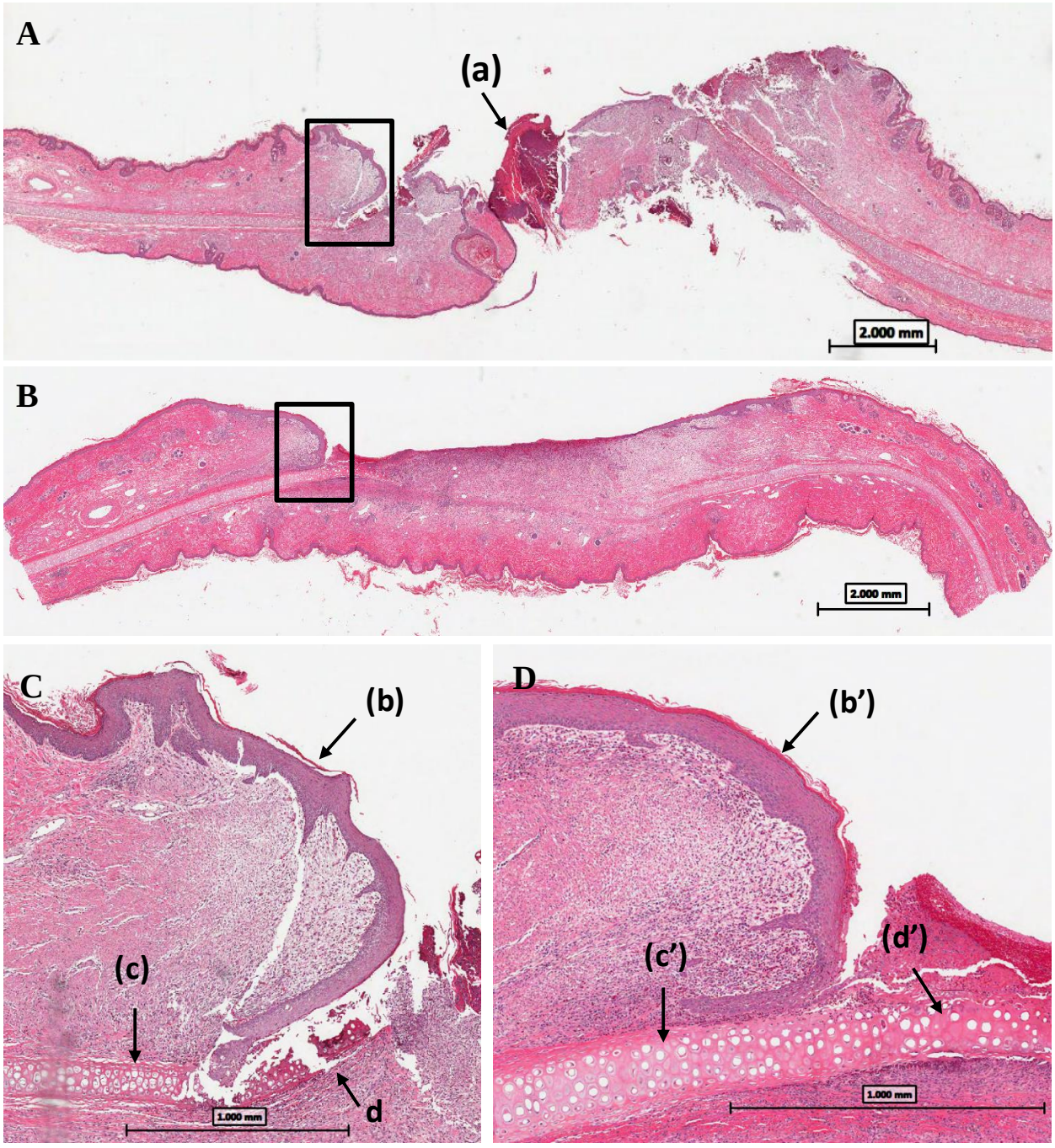

Figure 3-4: Representative histological sections (H&E staining) of wounds centers for the control group (A) and the O2patch treated group (B). Arrow (a) shows full thickness necrosis. Black rectangles are magnifications displayed in (C) and (D) (respectively control and O2patch groups). Arrows (b) and (b') exhibit the epithelial layer, (c) and (c') live cartilage and (d) and (d') dead cartilage.

Histomorphometric analysis was performed on the H&E stained sections. Each wound section was split in two parts: top and bottom (***Figure 3-5***, A). On the top part the amount of necrotic tissue was not significantly different between both groups. The amount of granulation tissue in the wound was significantly higher in the wounds treated with O2patch than in the control group (43.7±25.5% and 27.3±13.9% respectively). Similarly, the epithelialized surface was significantly higher in the experimental group than in the control (0.85±0.4mm² and 0.47±0.22mm² respectively). In the bottom part, the amount of necrotic tissues and necrotic cartilage was significantly higher in the control than in the oxygen group (41.7±32.7% and 16.6±19.4% for issue necrosis and 56.5±11.3% and 32.8±24.5% respectively).

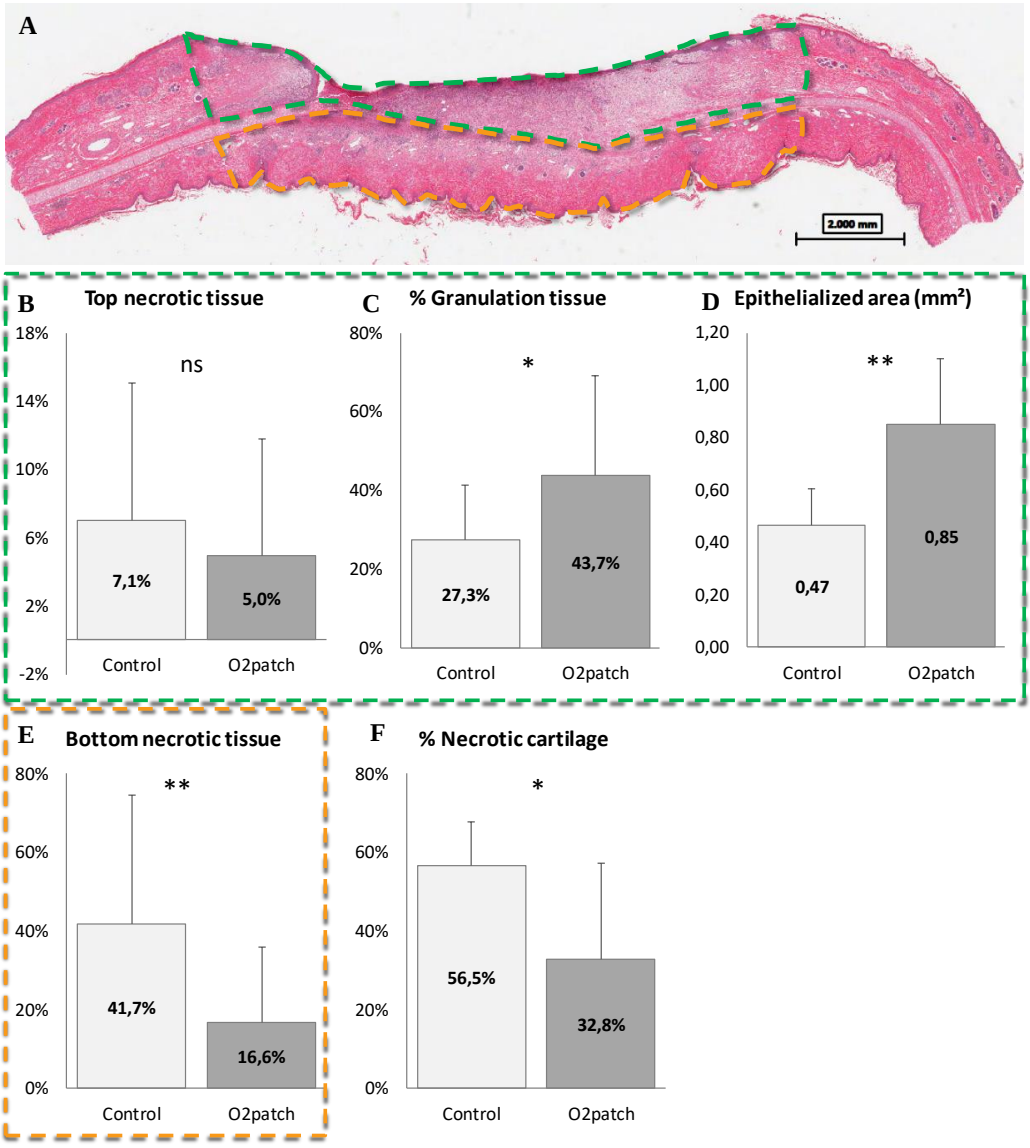

Figure 3-5: (A) Representative histological section (H&E staining) of wounds centers for the O2patch treated group, the green rectangle illustrates the top part and the bottom part in orange. (B) Histogram exhibiting the amount of necrotic tissue surface expressed as necrotic area over total tissue in the top part. (C) Percentage of granulation tissue expressed as area of granulation tissue over total volume of the wound. (D) Epithelialized area of the top part expressed in mm². (E) Amount of necrotic tissue surface expressed as necrotic area over total tissue in the bottom part. (F) Percentage of necrotic cartilage in the wounds relative the total amount of cartilage observed in the H&E section. (ns= not significant, *P<0.05, **P<0.01)

Discussion

Local oxygen concentration increased was significant over 3 days *in vitro* as well as *in vivo*. HOBT only increases arterial oxygen concentration to maximum of 700µmol/L with several exposures a day and decrease within minutes when the treatment is over.[50] With O2patch dressing it was possible to continuously increase significantly oxygen concentration *in vivo* and *in vitro* to within the range of HBOT treatments. Furthermore, hydrogen peroxide release was found to be below concentrations potentially harmful to the wound healing processes (166 to 975mM). [182]

In this animal model initially developed by Ahn & Mustoe (1990) [67], healing proceeds with a minimal contraction and all the new granulation tissue comes from the edges of the wound, signifying that the increased wound closure observed with the O2patch treatment is due to an augmented tissue formation.

Fluorescein injection allowed clear visualization of the formation of a new functional vascularization from the edges to the center of the wounds and therefore the formation of granulation tissue. With this technique it was possible to observe a tissue granulation surface coverage during the whole length of the experiment. Results observed at day 7 by Ahn & Mustoe [67] for their ischemic control with a 6mm diameter wound displayed a similar wound surface coverage by granulation tissue observed in this study with a 15mm wound. Histomorphometric measurements of the granulation tissue area performed by Chen et al [454] in a similar animal model (6mm wound) showed that the control group was 40% of the value obtained for the experimental group at day 7, within the range of what was observed by fluorescence in this study with a control group exhibiting a fluorescent area value of 30% of the experimental group

fluorescent area. In a study by Howard and al [455] (6mm wound) the ischemic control has shown about 5% of granulation tissue coverage and about 55% at 17 days, similar to what was observed under fluorescence in this study with 12±9% and 41±11% at day 7 and 17 respectively for a 15mm wound.

Histomorphometry analysis showed the amount of necrotic tissue in the upper part of the wound was not different between experimental and control groups. The amount of granulation tissue is higher in the oxygen group, this suggests an increase in proliferation and migration from the cells forming the granulation tissue composed mostly of fibroblast and endothelial cells.[456] Increasing oxygen levels in the skin through HBO has been shown to stimulate the reconstruction of an epidermis by enhancing fibroblast proliferation (*in vitro* and *in vivo*) [191, 192] as well as proliferation, migration, and differentiation keratinocytes. [191, 193] In addition, the work Gordillo et al, showed that normobaric topical oxygen treatment was associated with higher VEGF expression in the wound edge tissue in humans. [56] This would correlate with the higher and faster amount of granulation tissue found in the experimental group. Sub-lethal concentrations of hydrogen peroxide (up to 500µM) have been also shown to increase VEGF expression in keratinocytes [175, 176], macrophages [177] and vascular smooth muscle cells [178] through mRNA expression. Increased VEGF level are also observed *in vivo* with full thickness wound models in mice. [171] Furthermore, endothelial cells chemotaxis has been reported to be mediated through hydrogen peroxide and cyclooxygenase-2 interactions that play an important role in the promotion of wound repair. [179] These effects combined may have participated to a faster formation of blood vessels and an improved healing.

Cartilage is avascular; it receives oxygen and nutrients by diffusion of extracellular fluid from surrounding blood vessels in the skin. Cartilage consumption of oxygen/nutrients is relatively low when compared to other tissues. [457] For both groups, observing the wounds under black light after fluorescein injections exhibited that up to 7 to 11 days more than 50% of the cartilage surface in the center of the wound does not receive blood which is sufficient to bring it to its ischemic limit [458, 459], and signs of full thickness necrosis were visible in the wounds at day 7. This suggests that supplying oxygen directly to the surface of the wound allowed to maintain cartilage viability while the healing process was taking place, thus preventing cartilage necrosis.

Once necrosis appears in tissues, the cellular content is released into the extracellular space and is potentially harmful to surrounding cells, which in turn causes secondary necrosis and lead to its spreading. [460] In wound care, secondary necrosis can be prevented by debriding the wounds and removing the necrotic parts. In this model the necrotic cartilage was not removed, and necrosis was left to spread to the bottom part of the ear that should not show signs of necrosis, thus resulting in a non-healing wound. The necrotic tissues amount was significantly different ($P<0.01$) between both groups suggesting that by limiting necrosis occurrence with oxygen, it was possible to also limit secondary necrosis while the healing process is taking place.

Conclusion

There is no consensus today on the best wound dressings available. Wound care today consists of wound cleansing, disinfection, closure if needed and dressing. Wound dressings mostly consist in providing optimum parameters for the healing process that is patient-dependent. Today's research consist in creating modern dressings designed to account for the cause and type of wound and allow modification of the physical parameters of the wound environment, or are bioactive by either

playing an important role in healing process or delivering bioactive molecules enhancing migration and proliferation of cells, etc., and is still at an early stage.[461]

Topical oxygen delivery into the wound bed and has been shown to improve healing in chronic wounds.[56, 58, 59] The different technologies developed and commercialized like portable oxygen chambers (O2Boot®, O2Sacral ®, TWO2®) are intrusive. Oxygen concentrators (EPIFLO®, NATROX®, TransCuO2®) require to wear an oxygen generating source but have been shown to improve ulcer healing in patients.[60, 61] Newer oxygen pre-loaded wound dressings like Oxyband™ has been reported to improve healing in human burns. [62] More recently an experimental sodium percarbonate/calcium peroxide based oxygen releasing wound dressing [64] has also been shown to improve full thickness surgical wound healing. However, topical oxygen delivery to chronic wounds in clinical settings is not always sufficient to prevent necrosis [65, 66], and its prevention also rely on the re-establishment of a nutrient supply and wound's homeostasis. Studies are focusing on improving healing in patients with impaired healing and not on salvaging tissues that will otherwise likely undergo necrosis. A local and sustained supply of oxygen, one of several essential nutrients for most cell types, should in theory prolong ischemic survival and thereby prevent to onset of tissue loss.

Here we report a modified the method of Ahn & Mustoe [67] to create necrotic ischemic wounds and the use of oxygen releasing wound dressing with proprietary calcium peroxide formulation able to maintain tissue viability and prevent necrosis while maintaining the healing process. To our knowledge this has not been attempted previously and the results are highly encouraging as they bring a new tool for wound care.

Chapter 4 - Pilot study of efficacy of oxygen delivery biomaterial for ischemic skin preservation

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Abstract

Impaired or inadequate blood supply (e.g. in a wound, or an avascular graft) can result in tissue ischemia. As revascularization can be a slow process, the limited supply of oxygen and nutrients and the lack of waste removal may induce necrosis, depending of the extent of the wound. Secondary necrosis can spread to surrounding healthy tissue and treatment options are limited such that loss of tissue in ischemic limbs or wounds is considered unavoidable. Here we developed a biomaterial implant able to deliver oxygen directly to tissues and evaluated its preclinical efficacy in preventing ischemic necrosis and spreading in full thickness random skin flaps. Necrosis of the distal portion of the skin flap was delayed, but not prevented. Secondary necrosis in the middle part of the flap however was prevented indicating prolonged survival of skin with restricted blood flow for sufficiently long for the native vascular bed to re-establish blood supply. This study indicates that topical oxygen delivery alone cannot completely mitigate necrosis. Further experimentation is warranted to develop materials that can completely prevent necrosis. Nonetheless it provides a compelling proof of concept that materials can improve survival of tissue at least to augment conventional treatments or to gain time until surgical intervention.

Introduction

The skin flap is an important approach to reconstructive wound repairs. When performing a skin flap a part of the vascularization of the skin is sectioned which may lead to an inadequate blood supply and induce ischemia. In addition, comorbidities like diabetes, radiotherapy, etc., may worsen the outcomes of the procedure. Ischemia is a restriction of blood supply to tissues causing a shortage of oxygen and nutrients that are needed for cellular metabolism [1] and results in damage or dysfunction of tissue when delivery fails to meet metabolic requirements. In blood, the glucose concentration is regulated between 1.4 mmol/L and 6.2 mmol/L, [38] and oxygen concentration is 104 to 146 µmol/L.[7] Without oxygen the adenosine triphosphate (ATP) production cycle is limited to a yield of 2 moles of ATP for 1 mole of glucose. In absence oxidative phosphorylation, glucose is turned into lactates through glycolysis and the intracellular pH increases. The lack of ATP leads to various ATPase dysfunctions and a Na^+, water and Ca^{2+} accumulation, cell membrane depolarization, protease activation as well as an increased reactive oxygen species (ROS) production[40] that will damage cells and lead to necrosis. In addition, in this harmful environment, the mitochondrial membrane is disrupted and opens its permeability transition pore which further decreases the ATP production and the releases of apoptotic factors and initiate the apoptotic cascade.[1, 41] These alterations and thus the degree of tissue injury varies with the extent and duration of the ischemic period. At the same time, secondary necrosis can spread to surrounding healthy tissue, and treatment options are still currently so limited that loss of tissue in ischemic limb or wounds is considered unavoidable.[462]

We developed an implantable biomaterial to deliver oxygen directly to tissues. This study aimed to evaluate preclinical efficacy of this oxygen-producing biomaterial (O2-implant) to prevent ischemic necrosis and its spreading in full thickness random skin flaps.

Methods

Scaffold preparation

700mg PCL was dissolved at 15% (w/v) in chloroform (Fisher Scientific, Canada) and 500mg calcium peroxide (CaO_2; Aldrich, USA) and 1.1g Fe_3O_4 nanoparticles (50-100nm; Sigma-Aldrich, Canada) were suspended in the solution. The resulting mixture was cast into a 7x2cm rectangle, and was decontaminated by immersion into ethanol for 24h, then left to dry before the surgery.

Surgical methods

Wistar rats (male, 5 to 6-month-old, 500 to 600g, Charles River Laboratories Inc. Montreal, QC, Canada) were randomized into 2 groups. The control group received no biomaterial, the oxygen group received the oxygen releasing biomaterial (N=8 per group). All procedures were performed in accordance with the animal care and use committee. Animals received carprofen (10mg/kg) 30 min prior the surgery, all surgeries were performed under general anesthesia using 2% isoflurane. Full depth skin flaps of 9x2 cm in size were created on the back.[463] A silicone sheet was placed over the muscle to prevent revascularization and reperfusion of the flap from the underlying tissue, then the biomaterial was positioned, and the skin replaced on top of it and sutured. Animals received carprofen (10mg/kg) every 24h for 3 days post-surgery then slow release buprenorphine ever three days until the end of the experiment. Animals were allowed free access to food and water and housed in a 12 h day/night cycle. Five animals were sacrificed at days 6, three animals at day 10 using CO_2.

Oxygen and lactate measurements

Oxygen release of the scaffolds was assessed using an oxygen probe (AL300 Oxygen Sensor Probe, Ocean Optics. For *in vitro* measurements, the scaffolds were immersed in 40mL phosphate-

buffered saline (PBS) at 25°C in an open beaker (Ø=5cm; 19.65cm² interface, 2cm liquid depth) and oxygen content was measured in PBS. For *in vivo* measurements, the probe was inserted under the skin flap at 3 different positions namely proximally centrally and distally. Measurements were performed under anesthesia just after the surgery (day 0) and at day 1, 2, 4, 6, 8, 10. At the moment of euthanasia sections of the flap were frozen at -80°C and processed using previously described methods[69] to measure the lactate content of the tissues using a Lactate assay kit (Sigma-Aldrich).

Results

O2-Implants were able to sustain oxygen delivery (***Figure 4-1***) over 14 days *in vitro* causing a significant increase in the dissolved oxygen concentration of 40mL PBS from 256±11µmol/L (~20% dissolved oxygen) to 427±45µmol/L after 15 minutes and kept increasing to 586±10 ETC µmol/L and 637±38µmol/L at 1 and 2 days respectively. The concentration decreased to 614±26µmol/L, 606±47µmol/L, 501±8µmol/L, 513±51µmol/L and 544±5µmol/L at days 4, 6, 8, 10 and 14 respectively.

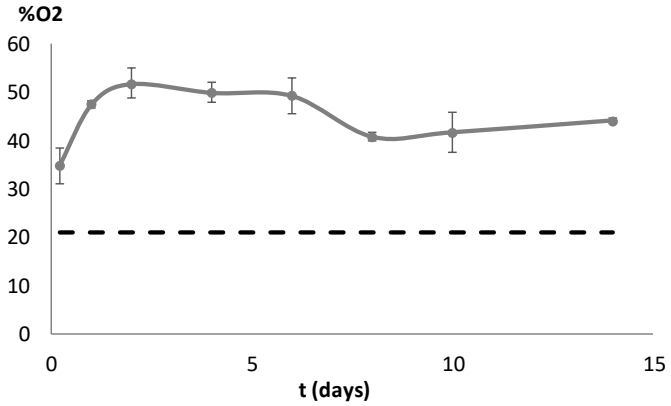

Figure 4-1: Dissolved oxygen released from O2-implant measured in 40mL PBS at 25°C. The dotted line represents normal PBS oxygen content for comparison.

Immediately after the surgery the distal part of the flaps appeared slightly blue (**Figure *4-2***, A, F), and dark blue at day 1 with some necrosis at the extremity (black color and hard leathery texture). At day 2, necrosis was visible (black color and hard leathery texture) on the distal portion of the flap for both groups (***Figure 4-2***, B, G). At day 4 the dark blue portion that did not became necrotic turned brown, and slowly turned necrotic up to day 10. The visible necrotic surface was significantly higher ($P<0.05$) in the control group than in the experimental at day 6, 8 and 10. (**Figure *4-2***, K). In the control group, the necrotic surface area was stable at around 41±12% of necrotic surface from day 4 to day 8 and increased to 48±2% at day 10. In the experimental group a similar trend was observed, with a stabilization between 30±11% and 32±4% from day 4 to 10.

Subcutaneous oxygen concentration was measured after the surgery and at days 1, 2, 4, 6, 8, 10 in the middle of the three sections. In the control group, no significant differences ($P>0.05$) in subcutaneous oxygen level was observed from day 0 to day 10 in the proximal, middle and distal sections (***Figure 4-3***). A similar observation was done for the experimental group. The subcutaneous oxygen concentration was found to be significantly higher in the oxygen group than in the control, and near physoxic concentration for each section of the flap.

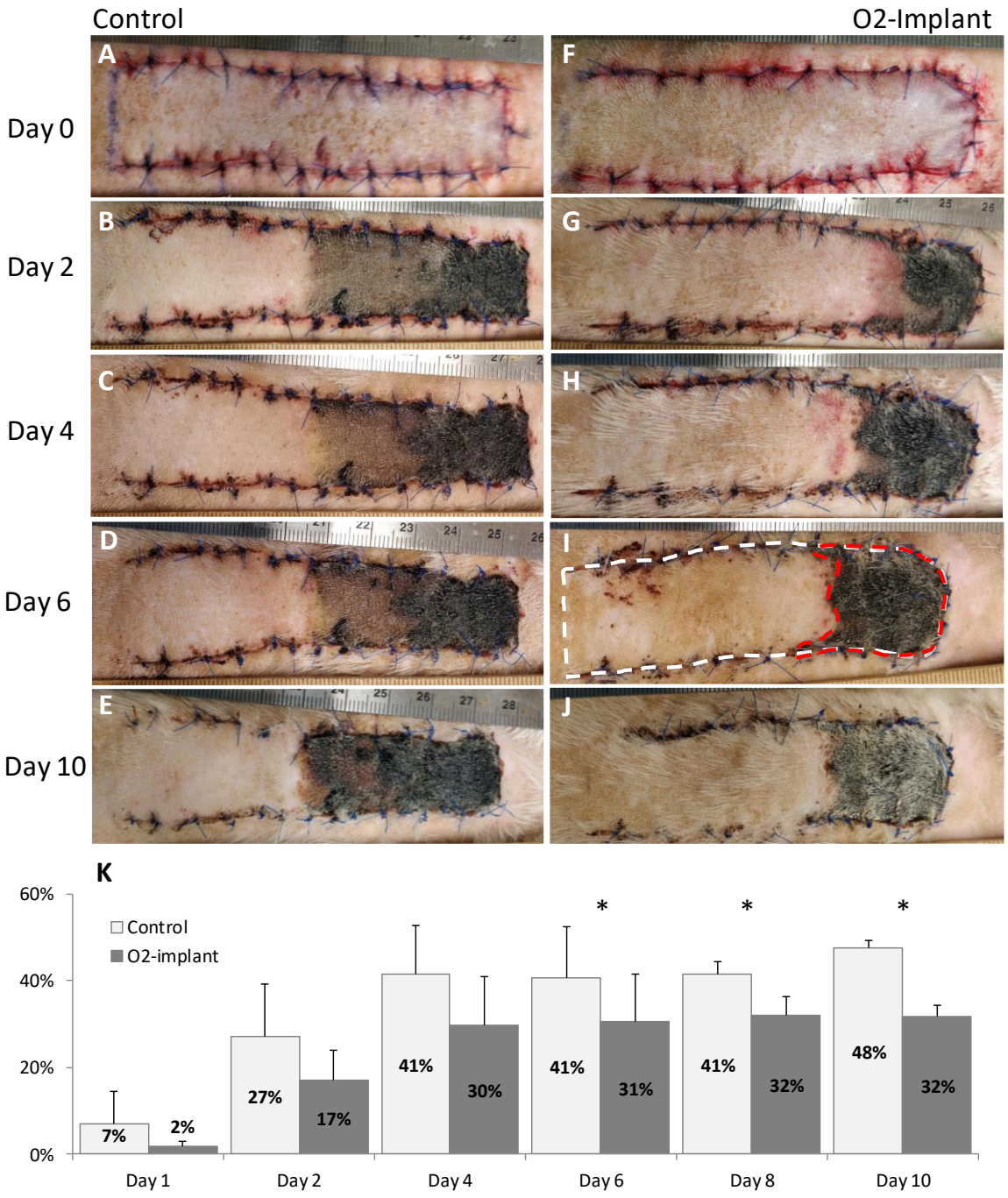

Figure 4-2: Representative photograph of the skin flap control group at days 0 (A), 2 (B), 4 (C), 6 (D), 10 (E); and for the O2-implant group (F, G, H, I, J). (K) Histogram representing the visible relative necrotic area over time, expressed as necrotic area (red dotted line in picture (I)) over total visible flap area (white dotted line in (E)) (*P<0.05).

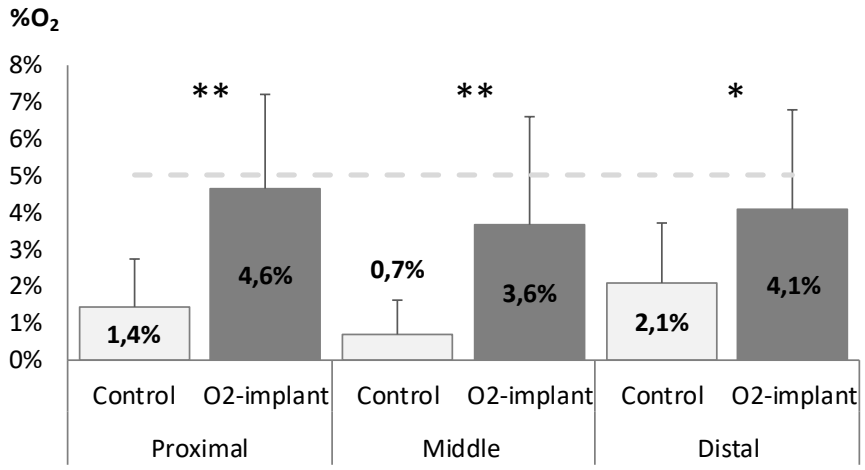

Figure 4-3: Histogram representing the subcutaneous oxygen concentration of the sin flap for the proximal, middle and distal part of the flap for both the control and the experimental group all time points combined. The dotted line represents physoxic oxygen concentration for comparison (5±2%) (*P<0.05; **P<0.01).

Lactate mesaurements for each section of the flap did not exhibited significant differences between the groups at days 6 and day 10 (***Figure 4-4***)

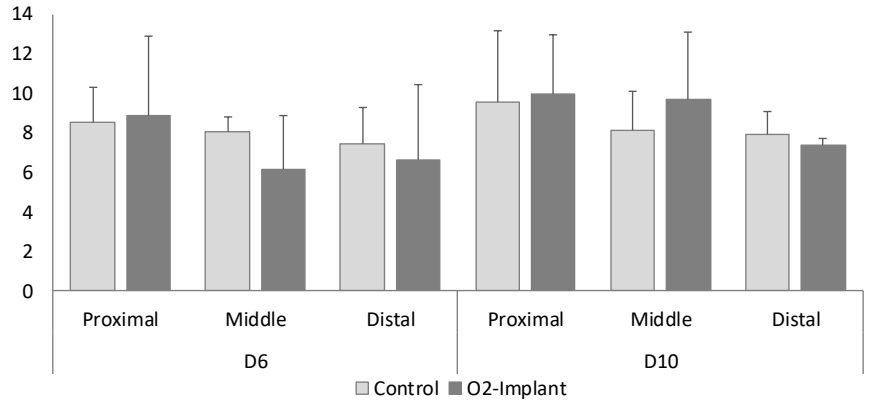

Figure 4-4: Histogram representing lactate quantification of each section of the flap for control and experimental group at days 6 and 10. Results are expressed as absorbance per milligram of tissue and were not significantly different between groups (P=0.05).

Flaps were separated into sections (proximal, middle and distal) and then cut in half lenghtwise parafin embeded and stained with hematoxylin and eosin (H&E) and hematopoietic progenitor cell antigen CD34 to identify blood vessels. Little diference was visible in the tissue architecture between both groups in the proximal section when observed under H&E staining (***Figure 4-5***, A, B; ***Figure 4-6***, A, B). In the middle sections the control group was exhibiting a larger necrotic area than the experimental (***Figure 4-5***, C and ***Figure 4-6***, C). At the interface between the necrotic and healthy tissue polymorphonuclear neutrophils (PMN) were visible (***Figure 4-5***, E; ***Figure 4-6***, F), and less nuclei were discernable as the distance from the proximal section inceased. In the necrotic protion of the flap ghost cells were visible around the appendages identified as preserved cell outlines without nuclei (***Figure 4-5*** D, G; ***Figure 4-6*** G). For both groups the distal section mostly consisted of necrosed tissues resembling some of the aspects of coagulative necrosis with the diapearance of the nuclei and appendages. (For clarity puroposes both groups are displayed together in **Figure *9-1***)

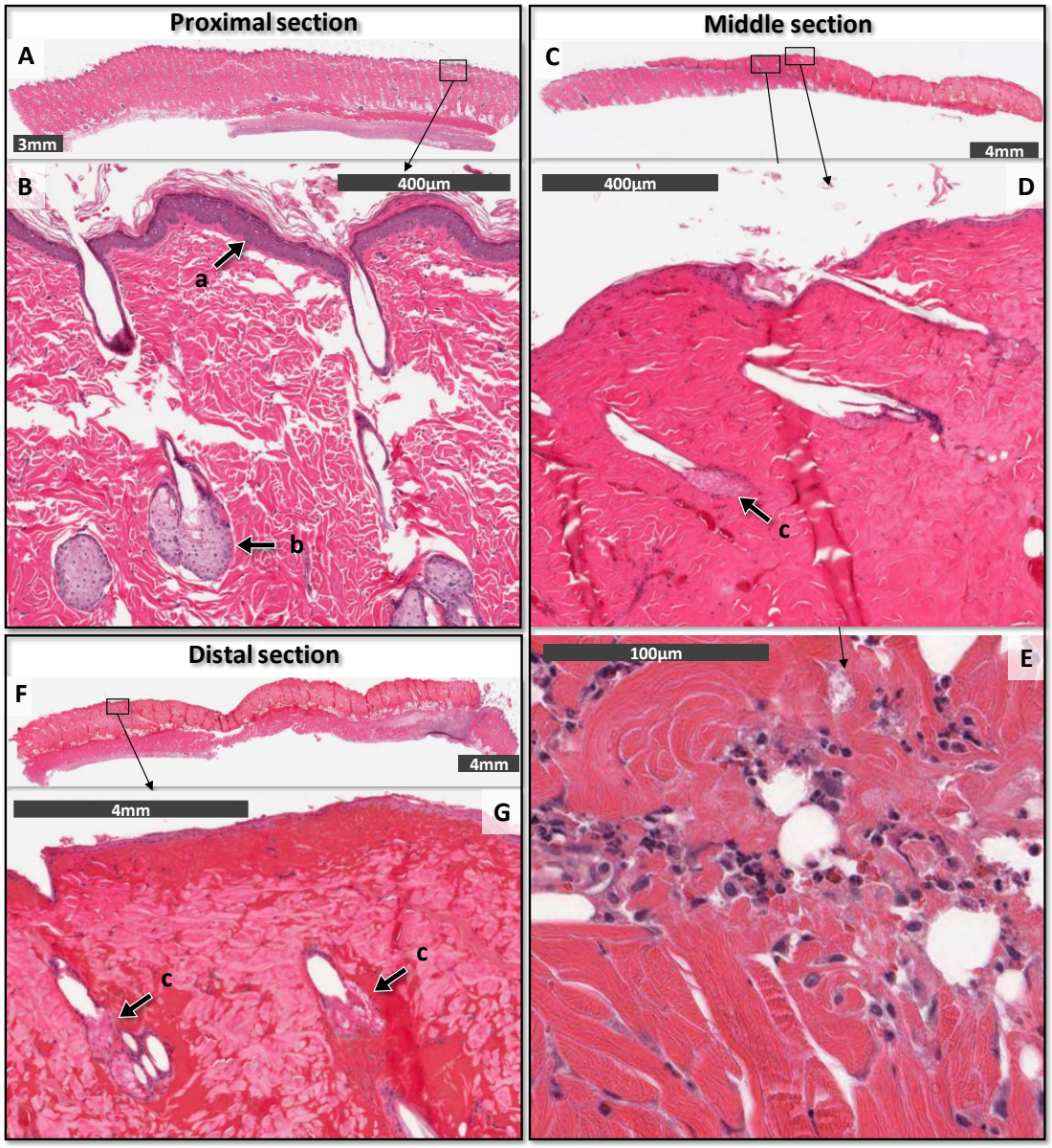

Figure 4-5: Representative histological sections (H&E staining) of skin flap sections for the control group: (A) proximal section of the flap and (B) magnified picture exhibiting live epidermis (a) and appendages (b). (C) middle section of the flap (proximal direction to the left) and (D) its high magnification exhibiting ghost cells around the appendages (c); (E) high magnification of (C) exhibiting inflammatory cells where? at the interface between necrotic and non-necrotic tissue. (F) shows the distal section and (G) its high magnification also exhibiting ghost cells around the appendages (c).

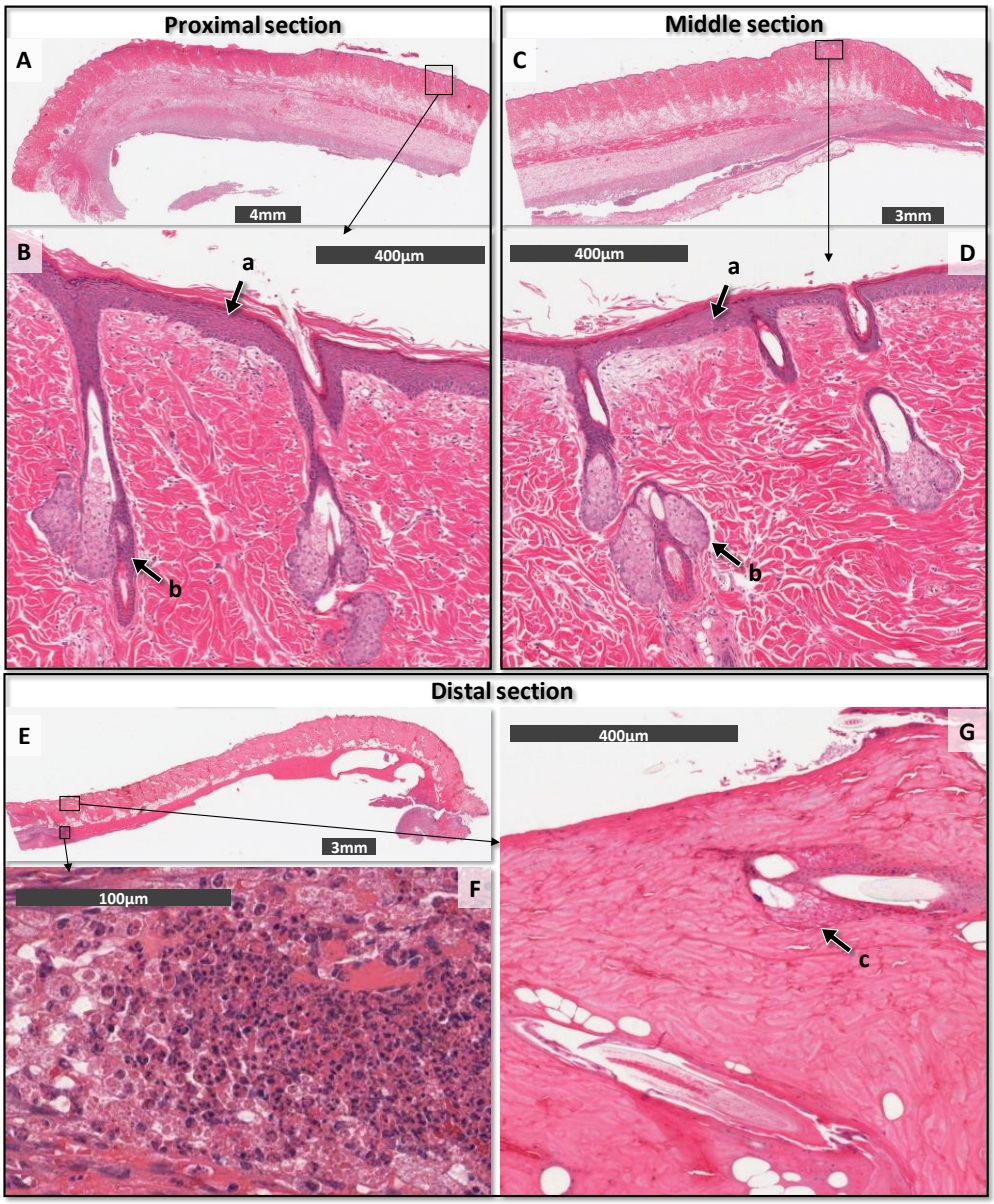

Figure 4-6: Representative histological sections (H&E staining) of skin flap sections for the experimental group: (A) proximal section of the flap and (B) magnified picture exhibiting viable epidermis (a) and appendages (b). (C) display the middle section (proximal direction to the left) and (D) its high magnification also exhibiting live epidermis (a) and appendages (b). (E) Distal section of the flap and (F) its magnification exhibiting ghost cells around the appendages (c); (G) is a high magnification of (E) exhibiting inflammatory cells at the interface between necrotic and non-necrotic tissue.

Histomorphometric measurement of the relative necrotic area in the skin flaps (epidermis, dermis and hypodermis) was performed on the H&E stained sections (***Figure 4-7***). At day 6 the amount of necrotic tissues in the proximal (0% for both groups) and distal section (100±0% and 86±11% for control and O2-implant group respectively) of the flaps was not significantly different. In the middle section necrosis reached 49±37%of necrotic area in the control group and 19±10% for the O2-implant (P=0.11, N=5 for both groups). At day 10 the amount of necrotic tissues in the proximal (0% for both groups) and distal section (100±0% and 87 ±13% for control and O2-implant group respectively) of the flaps was not significantly different. In the middle section necrosis was significantly higher in the control group with 31 ±14% of necrotic area in the control group versus 6±6% for the O2-implant. (N=3 for both groups).

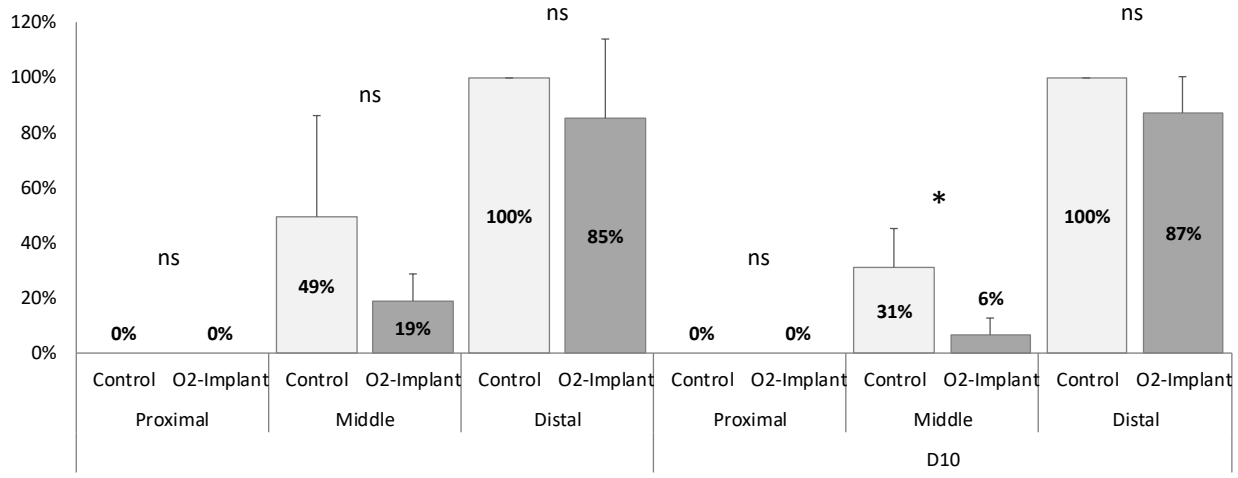

Figure 4-7: Histogram representing relative necrotic area observed histological sections (H&E staining) of the different skin flap sections for the control and O2-implant groups at day 6 and day 10, expressed as necrotic area over total flap area (ns= not significant, *P<0.05).

Immunohistochemistry and CD34 stainting (***Figure 4-8***; ***Figure 4-9***) exhibited no significant difference in blood vessel surface bewteen both groups. Measurements of the of blood vessel density (mm^2 of blood vessels per mm^2 of tissue; **Figure 4-10**) revealed no significant difference between the proximal (0.239±0.034mm^2 and 0.032±0.013mm^2 for control and O2-implant group respectively) and distal section (0.047±0.004mm^2 and 0.146±0.05mm^2 for control and O2-implant group respectively). In the middle blood vessel density was 0.076±0.036mm^2 in the control group and 0.146±0.05mm^2 for the O2-implant.

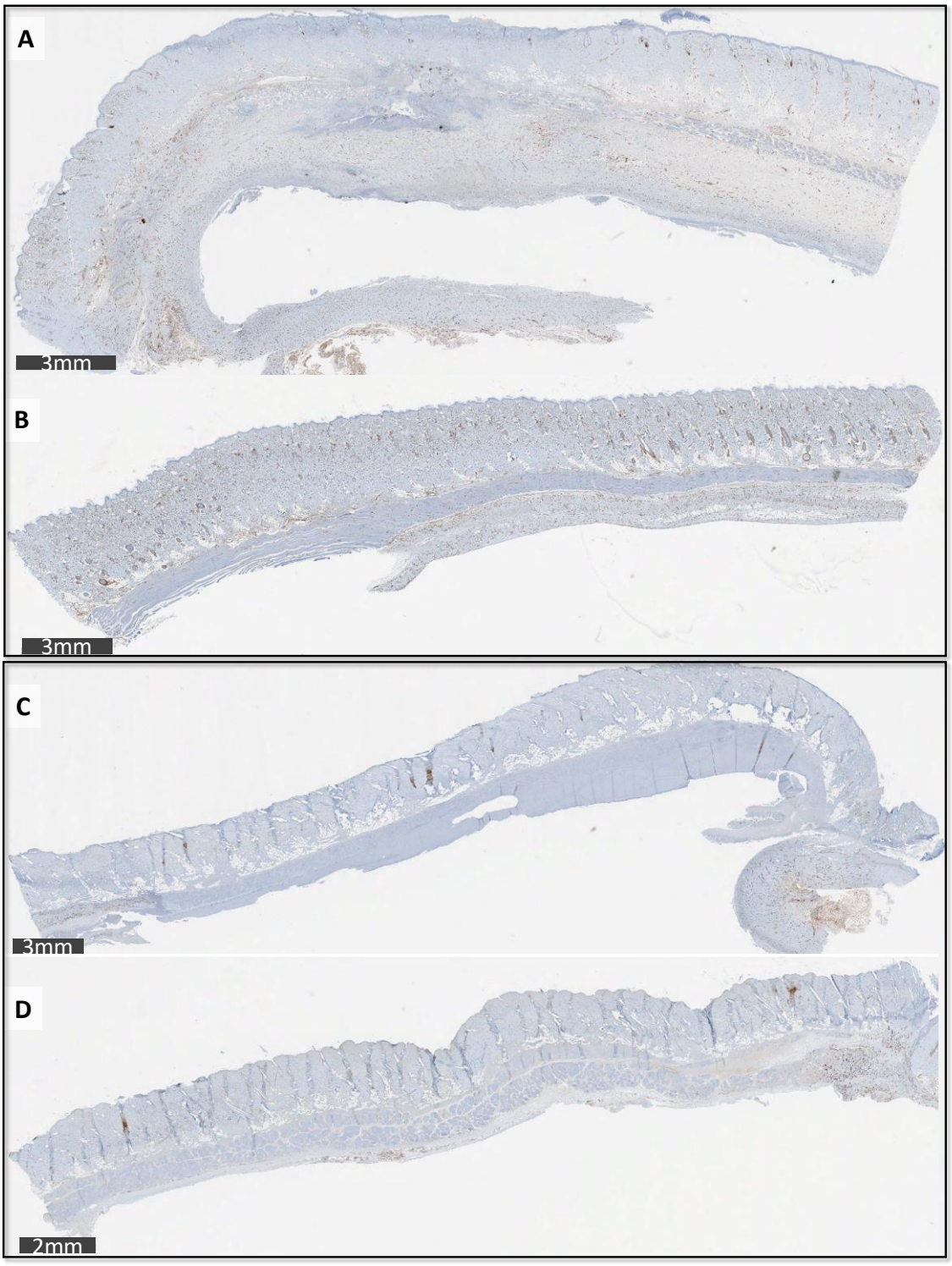

Figure 4-8: Representative histological sections (CD34 staining) of skin flap sections for: upper Image proximal section (A) O2-implant group, (B) control group, lower image distal section (proximal direction to the left, (C) O2-implant , (D) control group).

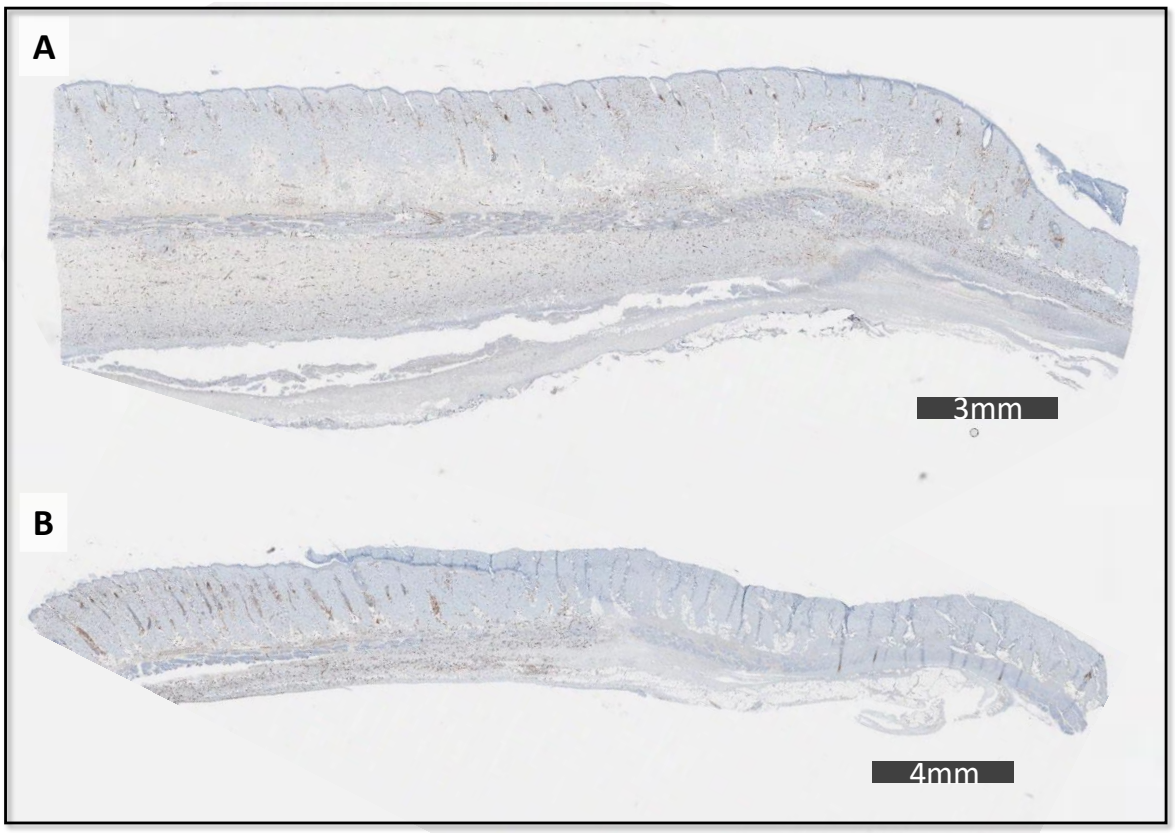

Figure 4-9: Representative histological sections (CD34 staining) of skin flap sections for the middle section of the flap (proximal direction to the left, (A) O2-implant group, (B) control group).

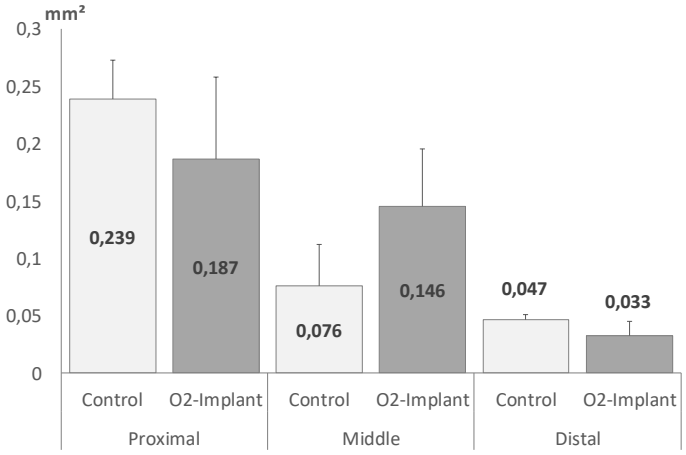

Figure 4-10: Histogram representing relative blood vessel density in epidermis, dermis and hypodermis, expressed as mm² of blood vessels per mm² of tissue for of the different skin flap sections for the control and O2-implant groups at day 6. Results were not significant ($P<0.05$).

Discussion

Many different technologies have been developed to address issues encountered when oxygen is insufficient *in vitro and in vivo* [83] like HOBT, perfluorocarbon (PFC) technologies, Hemoglobin based carriers, etc. The particular advantage of the use of peroxide like hydrogen peroxide, sodium percarbonate and calcium peroxide is to produce oxygen and carry it. That makes peroxides more suitable for implant design, indeed to produce one liter of pure oxygen less than gram is required for peroxides, were it would require several hundredth of gram of red blood cells, several kilograms of PFC or plasma.[464] In this study, the oxygen generating implant was able to release oxygen using the reaction between calcium peroxide and water. Skin oxygen consumption is approximately 0.38µl/h/mg (~0.017µmol/h/mg)[465] equivalent to 2.6g of CaO_2 for a 7.5g skin flap over 6 days. Such amount of peroxide would drastically change the subcutaneous pH and result in skin damage. Hyperbaric oxygen therapy (HOBT) has been shown to improve groin[466, 467] and dorsal[468] skin flap survival in rats and swine[469] as well as in humans[470], and can increases arterial oxygen up to 300mmhg (422µmol/L). [50] With 500mg CaO_2 it was possible to maintain in an open beaker of PBS at concentration significantly higher than its normal content (from 256µmol/L to a minimum of 520µmol/L) for up to 10 days, demonstrating that the implant is a steady source of oxygen.

In vivo implantation allowed the delay and partial prevention flap necrosis. *In vivo* oxygen measurements show that with the implant, a near physoxic subcutaneous oxygen concentration was re-established. However, the lactate concentration, a marker of tissue hypoxia, inside each section of the flap was not significantly different between both groups. This suggests that oxygen delivery alone was not sufficient to maintain aerobic mechanism in the whole skin thickness.

The coagulative necrosis observed corresponds to what is typically observed in organs under severe ischemia: tissue is firm, and architecture is maintained days after cell death, preserved cell outlines without nuclei and apparition of ghost cells.[471]

Histomorphometric analysis of the histological sections at 6 days did not show a significant difference in total necrotic area but a trend was visible with 41±14% and 27±12% (P= 0.13, N=5) for the control group and the oxygen implant respectively, similar to what was observed on the top of the skin during the experiment. At day 10 the total necrotic area was significantly different with 36±4% and 25±5% for the control group and the oxygen implant respectively, lower that wat was observed on the top of the skin. This may be explained by the shrinkage of the necrotic sections over time.

This study demonstrates the potential benefits of oxygen delivery, and results are in accordance with previously published work consisting in increasing oxygen in the flap with normobaric hyperoxia therapy[472], HOBT.[466-470] The only previous work on subcutaneous implants for oxygen delivery to skin flaps published by Harrison et al (2007)[69] used sodium percarbonate in PLGA able to release oxygen over 3 days. The authors have shown the necrosis was significantly reduced at day 3, but not significantly different from the control at day 7. In our study we were able to deliver oxygen subcutaneously for 10 days and we have shown significant improvement in skin flap survival over those 10 says, yet it was not sufficient to prevent necrosis. The results obtained for the control group in this study are consistent with other studies in literature using a similar model and a similar size.[473-476]

Oxygen diffusion distance through tissues is rarely more than 200µm[477], yet the thickness of the skin was several millimetres, implying that a part of the flap was not receiving oxygen from the implant. This is confirmed by the non significant difference that was found between the skin lactate

content of both groups. In that regards, it is possible that the use of split thickness skin flap could result in improved viability.

Another limitation of this experiment was evidenced by the skin's blue color post-surgery, indicating a venous stasis, a pathology that commonly occurs in skin flaps and that may have participated in necrosis occurrence and spreading [478] and may have hindered partially the potential benefits of the implant. This pathology is usually addressed clinically using for example compression methods[479], antithrombotics[480] or leeches.[481, 482] One can easily envisage a combination of O2implants to other techniques to extend further skin flap survival. The use of leeches have been shown to improve epigastric flap survival during venous congestion[483], and in a similar model the use of some antithrombotic has also been shown to improve skin flap viability.[484] Beyond the prevention of venous congestion, other methods have been shown to increase skin flap survival, like N-acetylcycstein[485, 486], an antioxidant scavenging the radicals formed during the ischemic cascade, or nitric oxide producing agents like nitroglycerin[487, 488] that will act as vasodilators.

Conclusion

Here we report the fabrication and use of a non-biodegradable oxygen delivery implant able to sustain the release of a large amount of oxygen over 14 days. Although we demonstrate its efficacy to prevent necrosis, the study is inherently limited by the oxygen amount delivered, by the ischemic model used as oxygen is not the only factor of necrosis onset and venous congestion that may have played a non-negligible role. This situation in not uncommon and clinically relevant, and oxygen delivery is here shown as a tool to help preventing necrosis onset.

Chapter 5 - Self-oxygenating bioinks

Introduction

A clear clinical need exists for the development of technologies such as tissue engineering strategies, facilitating the on demand regeneration of damaged tissues. [489] Numerous tissues types would benefit from this type of technologies (muscles bones, liver pancreas, etc.), however the clinical applications have remained limited to tissues with low metabolic demand (e.g. skin, cartilage and bladder). Tissues with higher metabolic demand (muscle, myocardium, liver, etc.) are sensitive to oxygen and nutrient levels and are highly vulnerable to ischemia [490], hindering the survival of clinically relevant volumes during *in vitro* culture or after implantation *in vivo*. Indeed, oxygen and nutrients supply in bioreactors is only possible through the movement of the culture medium through their structure and thus limited by diffusion.[77] and such construct do not possess a functioning blood vessel network that could anastomose to the host vasculature upon implantation. Without an integrated vascularization their *in vivo* survival depends on the host ability to vascularize the graft and previous researches have shown that the complete vascularization of a 3mm tissue engineered construct could take between one and two weeks.[83]

3D-Bioprinting has the potential to deliver a mixture of cells, biomaterials mimicking extracellular matrix environment to support their adhesion, proliferation, migration, etc., and bioactive agents. Also known as bioinks, these mixtures are allowing some degree of spatial control of cell position to facilitate the creation of tissue engineered structure, but do not yet match the required resolution to build complex organs with vascular networks. [81] *In vitro*, techniques like PFC, HOBT, define haemoglobin-based carriers have allowed to improve oxygen delivery to increase size and quality of said constructs.[83] However, these means of delivery are extrinsic to the construct, therefore

also require conduits and are subjected to the laws of diffusion. *In vitro*, many different well documented approaches have been developed to induce rapid vascularization *in vivo* or direct vascular integration *in vivo* by creating *in vitro* vascularized tissue [491-493], from immobilization of angiogenic factors, to structured based approaches using 3D bioprinting with different bioinks. However, anastomosis of a pre-vascularized construct to the host vascularization after implantation can take a few days [494], and partial tissue death may occur during this time. Indeed, upon implantation the cellular scaffold becomes isolated and the oxygen delivery inside the construct relies solely on diffusion of oxygen and nutrients from the surrounding tissues and vessel. But in those conditions oxygen diffusion distance is rarely more than 200µm.[477] Direct surgical anastomosis to perfuse immediately vascularized tissues has only been demonstrated using vascular explants but requires multiple surgeries to harvest the vascular bed. [495, 496]

This limit both the size of the scaffolds that can be build or implanted as well as their cellular density.

Tissue engineered constructs would greatly benefit from technologies delaying cell death by mimicking the primary functions of blood (i.e.: oxygen and nutrient delivery, waste removal) in order to build larger constructs with biologically relevant cellular densities i.e. high density cultures (10^7 to 5×10^8cells/mL)[71]. As oxygen concentration is the major limiting factors for cell survival (its concentration is about 50 times lower than glucose blood [8, 497] and is used 6 times more), achieving *in situ* oxygen delivery in cellular constructs is the first step towards building self sustaining system.

We developed oxygen releasing microparticles (OµP) that could be incorporated in bioinks to deliver oxygen locally in order to prolong cell survival. Material oxygen release was first assessed, then its cytotoxicity as well as its ability to deliver oxygen to cells under anoxic condition and re-

establish primary functions such as proliferation of two different cells types (Madin-Darby Canine Kidney (MDCK) and Chinese hamster ovary (CHO) cells) in two dimensions.

High density three-dimensional cellular constructs were then created under a bead format (ø 1.7mm) with a cell density (CHO cells, 1×10^8 cells/ml) close to those found in organ ($1\text{-}5\times10^8$ cells/ml)[73] and cultured first in medium to observe the changes in viability to simulate conditions where the construct would be surrounded by blood vessels after implantation. The medium was then replaced by PBS to simulate absence of surrounding blood vessels, then OμP and glucose were incorporated to the bioink to sustain the cells.

Method

Microparticles and bioink preparation

Oxygen releasing microparticles (OμP) were produced by phase separation method.[498] Briefly, 1g PCL (Mw 70,000-90,000, Aldrich, USA) was dissolved at 10% in chloroform (Fisher Scientific, Canada) and 100mg calcium peroxide (CaO_2; Aldrich, USA) and 1g Fe_3O_4 nanoparticles (50-100nm ; Sigma-Aldrich, Canada) were suspended in the solution. The resulting mixture was slowly added to glycerol 1% PVA under agitation to form the microparticles. Once dried, the particles were collected by centrifugation and washed with ethanol then dried.

The bioink was prepared by mixing OμP with 1% sodium alginate solution and extruded through a 20G needle in a 0.1M calcium chloride solution to form 20μL beads.

Characterization: Morphology, Oxygen and hydrogen peroxide release measurements

Scanning electron microscopy (FE-SEM, FEI Inspect F-50, FEI, USA) was used to examine the morphology OμP. Oxygen release was measured on the surface of the bioink immersed in 1mL PBS using an oxygen probe (AL300 Oxygen Sensor Probe, Ocean Optics), hydrogen peroxide

released from the bioink in the water was measured using a peroxide measurement kit (Pierce™ Quantitative Peroxide Assay Kit (Aqueous), Thermofisher, Canada).

Cytotoxicity and proliferation assay

Bioink cytotoxicity was assessed using MDCK cells: cells were seeded at 2500 cell/cm² in 2mL DMEM high glucose and left to attach the plate for 2h. The bioink was then supplemented with a loading of 0, 5, 10, 25mg/mL of microparticles and incubated 24h in DMEM under 21% air and 5% CO2. Cells were the detached using trypsin and counted using trypan blue. Anoxia cytotoxicity was assessed by keeping the cultured cells in a sealed jar flushed with 95% N_2 and 5% CO_2 kept in a desiccator constantly flushed with N_2 at 37 °C for 24h. Anoxic conditions were confirmed by measuring the using an oxygen content of the medium at the end of the experiment using the oxygen probe.

Proliferation assays under anoxic conditions culture was carried out by seeding MDCK or CHO cells in similar conditions than previously described with the bioink in a sealed jar flushed with 95% N_2 and 5% CO_2 kept in a desiccator which was constantly flushed with N_2 at 37 °C. After 3 days anoxic conditions were confirmed as previously described. Live/dead (Thermofischer scientific, Canada) assay and Hoechst 33258 (Thermofischer scientific, Canada) staining were used to observe the cultures under a fluorescent microscope. Cells were then detached using trypsin and counted using trypan blue

Three-dimensional cellular constructs

A 100µL suspension CHO cells at $2x10^8$ cells/mL were thoroughly mixed to 100µL of 2% sodium alginate solution then extruded through a 20G needle in a 0.1M calcium chloride solution to form 20µL beads (ø 1.7mm). Beads containing cells alone were cultured either in 1mL DMEM high

glucose (DMEM group) or in 1mL PBS (PBS group). Beads were prepared containing glucose (225mg/mL, resulting in 4.5g/L when cultured in 1mL PBS; Glucose group); 25mg/mL OμP (OμP group) or with glucose (225mg/mL) and OμP (25mg/mL, Bioink group) were cultured in cultured in 1mL PBS. At days 1, 2 and 3 alginate beads were dissolved using a sodium citrate/ sodium chloride solution (55mM / 90mM)[499] and live cell number was assessed using MTT assay (Vybrant® MTT Cell Proliferation Assay Kit; Invitrogen, Canada). ***Figure 5-1*** summarizes the different experiment and groups.

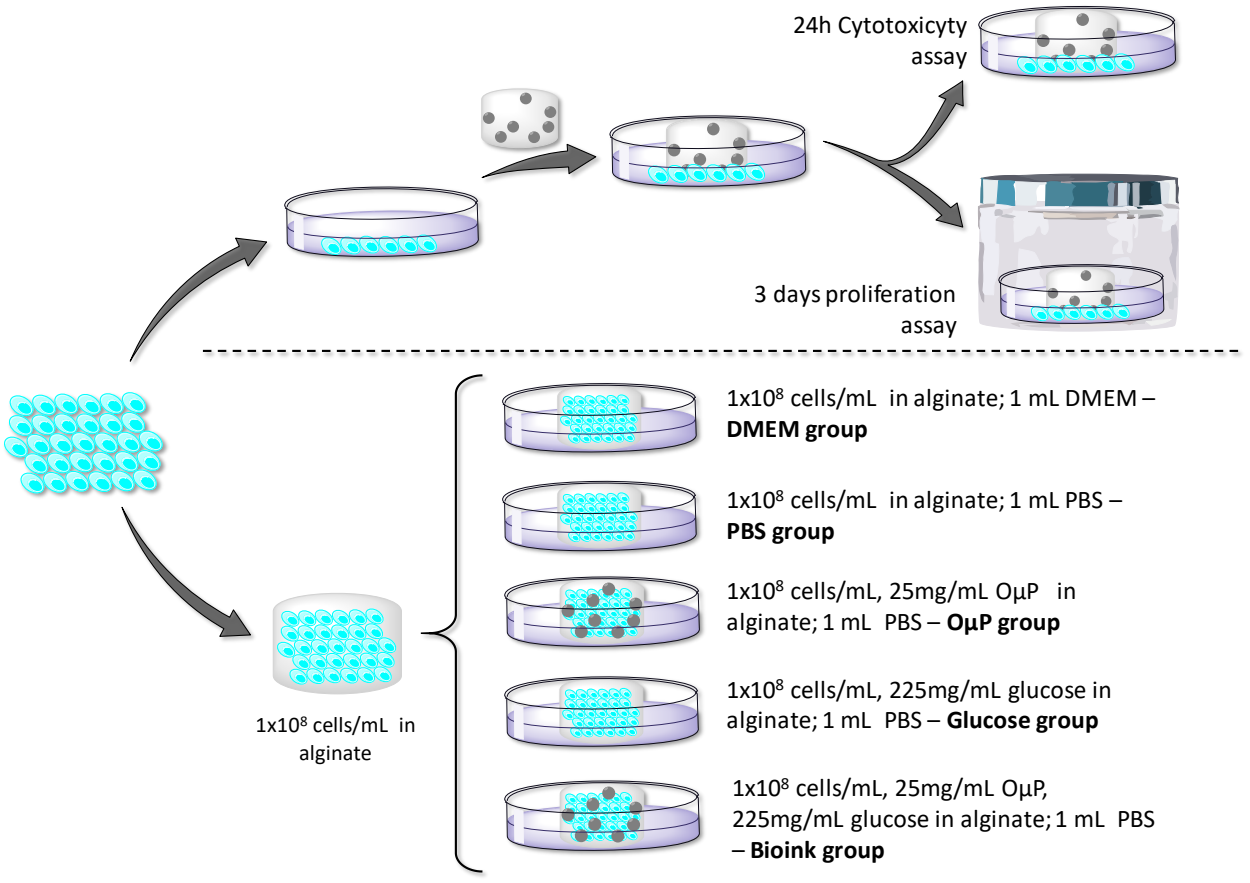

Figure 5-1: Scheme summarizing performed experiments

Results

Microparticles characterization

Scanning electron microscopy revealed that the oxygen-release microparticles were roughly spheroidal with a diameter ranging from 10-200 µm (**Figure *5-2***). The bioink sustained a release of oxygen and hydrogen peroxide over 3 days, a trend as visible as the microparticle loading increased both release increased. Oxygen release from the bioink loaded with 5 and 10mg/mL OµP was not significantly different over the course of the experiment (286.3±6.5µmol/L and 297±7.5µmol/L; 320.3±7µmol/L and 317±0µmol/L; 298.6±14.2µmol/L and 275.9±21.7µmol/L for 5 and 10mg/mL at day 1, 2 and 3 respectively). A similar observation was made between 25 and 50mg/mL OµP (320.7±5.6µmol/L and 328.1±5µmol/L; 340.4±3.3µmol/L and 338.3±5.1µmol/L; 294.1±1.4µmol/L and 284.3±18.4µmol/L for 25 and 50mg/mL at day 1, 2, 3 respectively). Oxygen release from 5 and 10mg/mL loading was significantly lower than 25 and 50mg/mL at day 1 and 2 but no significant difference was measured at day 3 between those four groups. As expected 100mg/mL loading released significantly more oxygen than lower loadings on the first day (346.5±18.42µmol/L) and was not significantly different than 25 and 50mg/mL ones at day 2 (344.1±5.63µmol/L).

Hydrogen peroxide release from the bioink loaded with 5 and 10mg/mL OµP was not significantly different over the course of the experiment (9.2±2.6µmol/L and 11±2.2µmol/L; 6.5±1.8µmol/L and 6.2±0µmol/L; 7.6±0.5µmol/L and 5.8±.0.6µmol/L for 5 and 10mg/mL at day 1, 2 and 3 respectively (P=1 at day 1, 2 and 3; N=6). Hydrogen peroxide release at day 1 the release was significantly higher for 25, 50 and 100mg/mL (43.9±0.3µmol/L; 101.7±11.7µmol/L; 365.9±24.8µmol/L respectively). Hydrogen peroxide content at day 2 sharply decreased for 25 and 50mg/L to 6.68±0.7µmol/L and 5±0.5µmol/L respectively and remained stable at day 3

(5.81±0.49µmol/L and 5.5±0.7µmol/L respectively). Hydrogen peroxide content at day 2 and 3 for 100mg/mL remained significantly higher with 301±37.7µmol/L and 279±30.2µmol/L.

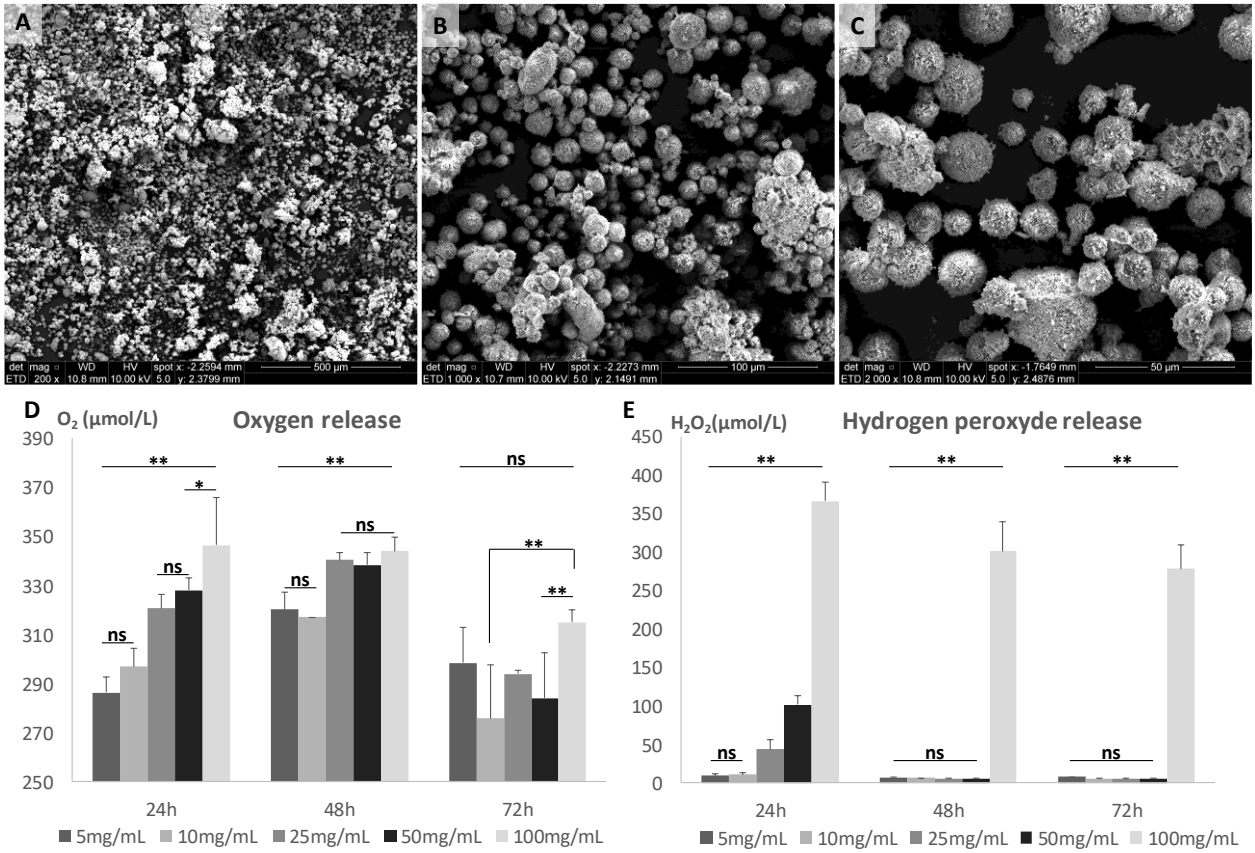

Figure 5-2: Representative SEM pictures of the oxygen releasing micoparticles at different magnifications (A, B, C). (D) Bioink oxygen release graph over time measured in 1mL PBS for 20µL beads of bioink with different OµP concentrations (in mg per mL of sodium alginate 1%) (E) Bioink hydrogen peroxide cumulative release graph over time for 20µL beads of bioink with different OµP concentrations. (ns=not significant, *P<0.05, **P<0.01).

Cytotoxicity and proliferation assay

24h cytotoxic assay showed no significant differences between normoxic and anoxic conditions (***Table 5-1***). Similarly, the bioink did not show any significant difference up to 25mg/mL microparticle loading.

Table 5-1: Cell viability of MDCK cells seeded at 2500 cell/cm² in 2mL DMEM high glucose after 24h incubation and expressed as number of live cells over total number of cells.

	Normoxia	**Anoxia**	**5mg**	**10mg**	**25mg**
Viability	91.6±6.9%	92±2.6%	89.6±4.5%	92.4±3.2%	85.8±5%

Proliferation assay over 3 days demonstrated that anoxia was significantly reducing both MDCK and CHO cell's proliferation (***Figure 5-3***). In addition, MDCK cells under anoxia did not exhibit a swollen morphology when compared to normonic culture. Addition of the bioink at 5 and 10mg/mL OμP did not allowed re-establishment of cell proliferation in MDCK anoxic culture. Similar results were obtained for CHO anoxic culture. A loading of 25mg/mL of OμP allowed partially re-establishment of proliferation for both cell types.

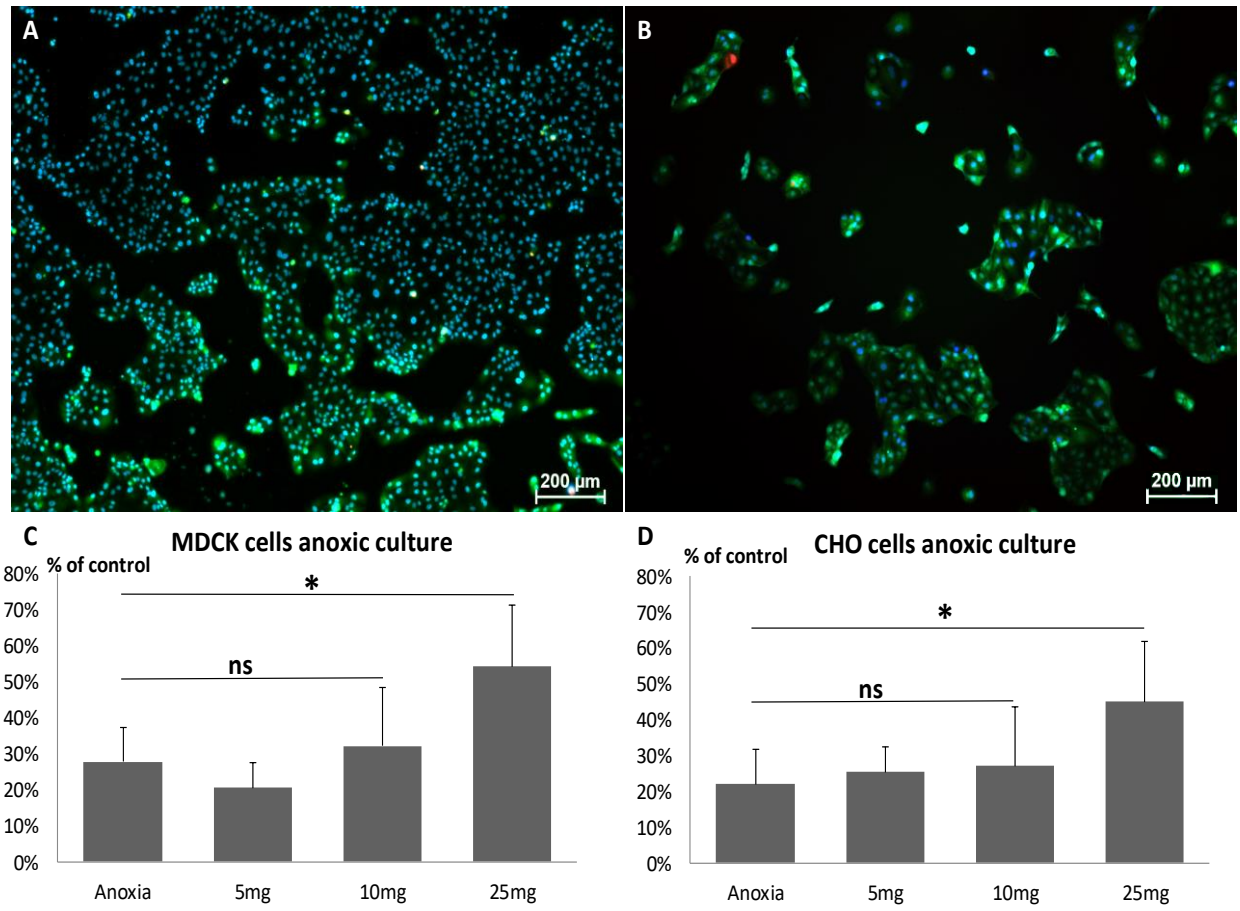

Figure 5-3: Fluorescent images of MDCK cells stained with live/dead assay (live cells cytoplasm shows in green, dead cells nuclei in red) and Hoechst (cells nuclei shows in blue), after 3 days in normoxic conditions (A) and anoxic conditions (B). Graph (C) represents MDCK cell number as a percentage of the normoxic culture, expressed as the mean number of cells in the sample divided by the mean number of cells in the normoxic culture. Graph (D) represent the CHO cell number as a percentage of the normoxic culture. (ns=not significant, *$p<0.05$).

Three-dimensional cellular constructs

Fluorescent imaging of the DMEM, bioink and glucose beads at day 1 exhibited mostly live cells on the surface, the glucose group exhibited the most dead cells (**Figure *5-4***). At day 3, DMEM

group was contained mostly live cells whereas bionink and glucose groups exhibited mostly dead cells.

Beads were seeded at $1x10^8$ cell/mL, equivalent to 2×10^6 cell /beads. Beads cultured in DMEM exhibited an increase in cell population up to $2.42\pm0.31\times10^6$ cell /beads over the first day, then a decrease in cell number for day 2 and 3 to $2.2\pm0.34\times10^6$ and $1.95\pm0.01\times10^6$ respectively. PBS, OμP, glucose and bioink groups exhibited a sharp decrease in cell population to $0.51\pm0.28\times10^6$, $0.27\pm0.04\times10^6$, $0.78\pm0.27\times10^6$ and $1.42\pm0.34\times10^6$ respectively at day 1. For those group the decrease continued to $0.12\pm0.08\times10^6$, $0.57\pm0.12\times10^6$ and $1.24\pm0.17\times10^6$ at day 2 and $0.03\pm0.09\times10^6$, $0.006\pm0.11\times10^6$ and $1.12\pm0.08\times10^6$ at day 3.

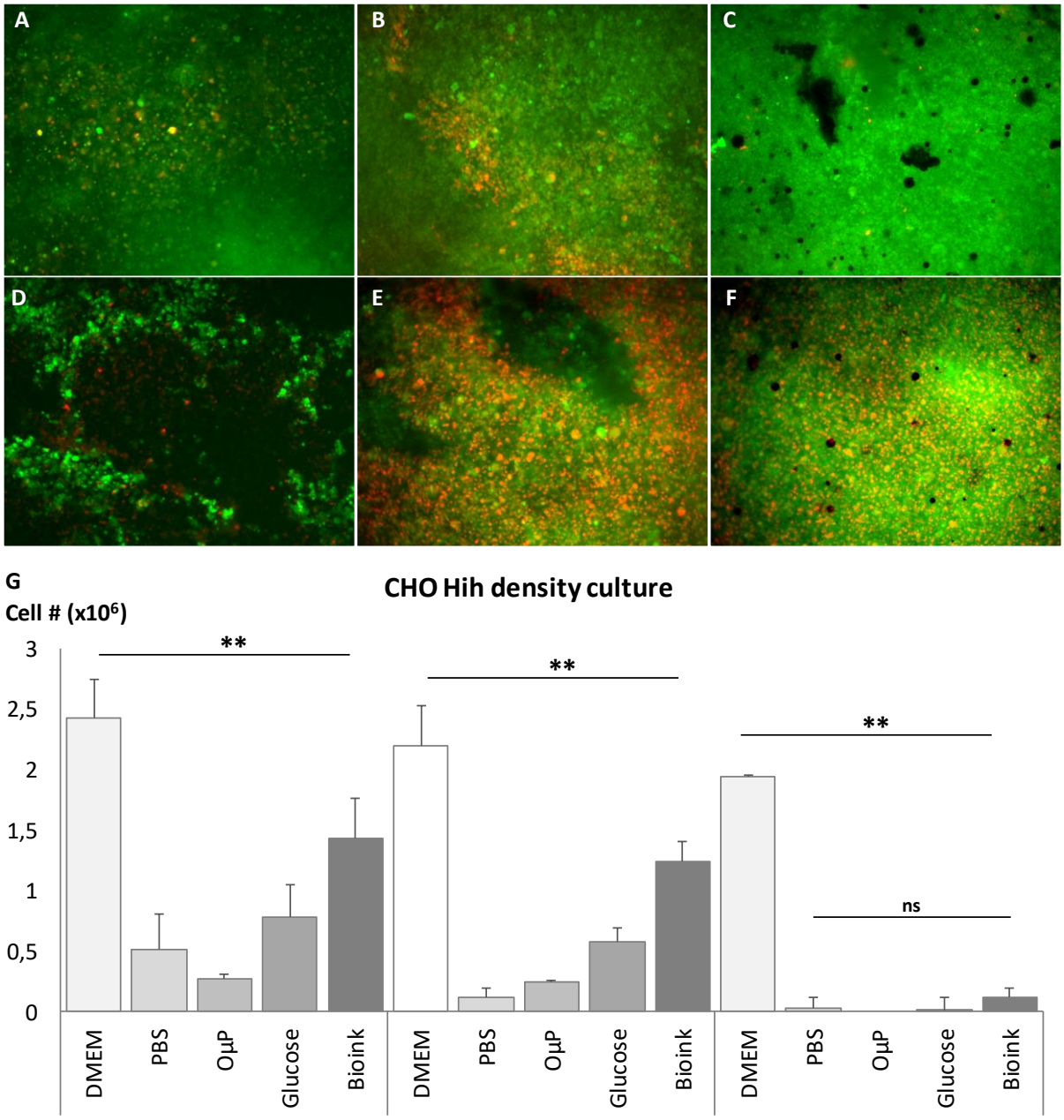

Figure 5-4: Representative Fluorescent images of the CHO cell beads stained with live/dead assay (live cells cytoplasm shows in green, dead cells nuclei in red) of the high-density CHO cell beads for the DMEM group at day 1 (A) and day 3 (D); glucose group at day 1 (B) and day 3 (E); Bioink group at day 1 (C) and day 3 (F); live cells cytoplasm shows in green, dead cells nuclei in red. Graph (G) represents cells number for the DMEM, PBS, OμP, Glucose and Bioink groups over three days. (ns=not significant, **P<0.01)

Discussion

This study was designed to partially simulate conditions to which tissue engineered construct would undergo after implantation *in vivo* with no vascularization and create a bioink able to sustain cells viability while vascularization is taking place.

Oxygen generating microparticles were successfully created. Oxygen release was achieved by reacting calcium peroxide and water to produce oxygen and hydrogen peroxide. Because of the rapid reaction of CaO_2 and water, a hydrophobic polymer was used to tune the reaction rate. To harvest the potential of hydrogen peroxide to produce oxygen and reduce its concentration to prevent a potential toxicity, magnetite (Fe_3O_4) was used to catalyze H_2O_2 into oxygen and water (Fenton-Haber-Weiss catalytic reaction [51, 52]).

When in contact with water the particles released both oxygen and hydrogen peroxide. Oxygen release from the microparticles when incorporated in a hydrogel rapidly reached a plateau despite the increasing microparticle loading. This may be explained by the formation of micro bubbles in the gel appearing as CaO_2 decomposes.

At 50mg/mL microparticle loading, H_2O_2 release was above the cytotoxic concentration described in literature [167, 500] and cytotoxicity was confirmed during cytotoxicity experiment (data not shown). Because 25mg/mL allowed the release of a maximum of oxygen without significant cytotoxic effects, this loading was used for further experiments.

In this experiment, anoxic culture of MDCK and CHO cells did not result in significant cytotoxicity at 24h and 72h, however proliferation of the cells was significantly reduced. This is in accordance with previously published work where MDCK and CHO cells have ben shown to have a certain resilience to hypoxic/anoxic conditions *in vitro* [501-503]. Although the bioink allowed partial re-

establishment of proliferation for both MDCK and CHO cells, the amount of oxygen delivered in those conditions was not sufficient to sustain it as it would be in a normoxic culture.

Anoxic/hypoxic conditions can be achieved using chemicals or special chambers. [504] Chemicals like $CoCl_2$ or deferoxamine can simulate hypoxia by inducing HIF-a expression [505], while others like cyanide and 2-deoxyglucose will inhibit ATP production by mitochondria [506], thus resulting in a simulation of hypoxia or anoxia. But those compounds may also regulate other genes and therefore are not adapted for all cell types or experiment. In this study the choice of the hypoxic chambers was made because unlike chemical hypoxia, it has the advantage of not using drugs altering cell behavior with no regards of the of the oxygen tension.

The three-dimensional cellular constructs had a cell density of 1×10^8 cells/ml, a volume of 20µL and an approximate diameter of 1.7mm. Such high cell density is close to some organs cell density ($1\text{-}5\times10^8$ cells/ml) [507] and without vascularization oxygen and nutrient enter the beads from the outside and their diffusion towards the inside is governed by the laws of diffusion. They are at the same time consumed by the cells, thus producing wastes that undergo a similar diffusion process toward the outside, and if the diffusion process is too slow, cells in the core of the system maybe found lacking oxygen and nutrients and face a waste build-up. If oxygen diffusion distance no more than 200µm at physiological cell densities [477] this suggest that 45% of the bead will not receive a proper oxygen supply. When cultured in DMEM medium the beads exhibited first an increase in cell number at day 1 followed by a decrease ad day 2 and day 3. Addition of OµP in the beads cultured in DMEM did not show significant difference with the DMEM group (data not shown) suggesting that oxygen shortage was not the major detrimental facto to cell survival.

When placed in PBS, most of the cells died within 24h, and incorporating OμP alone did not improved viability. Adding glucose in the beads allowed to sustain a higher viability up to 48h and addition a combination with the oxygen releasing material allowed to further increase the viability by two-fold at 48h. These experiments suggest that although supplying oxygen and glucose to an "isolated" system may allow to maintain cell viability to an extent, it is not sufficient.

When in anoxia/hypoxia, cells have the ability to switch their energy production mechanism from aerobic to anaerobic, thus producing lactates, from which an accumulation which may result in toxicity if its diffusion outside the bead is too slow. [508] CHO can keep their viability during an anoxic period of 3 days, suggesting that the decrease in viability observed in the DMEM group is either due to poor glucose diffusion or a high content of lactates.

Different studies have shown the potential of using peroxide in tissue engineered construct *in vivo*.[509] They however rely on catalase to eliminate hydrogen peroxide [510-512], but have limited applications *in vivo* due to its limited half-life.[513, 514] In this work we demonstrate the potential of using peroxide-based and catalase free oxygen releasing biomaterials to help maintain cell survival. However, blood does not deliver only deliver oxygen, it also brings nutrient and remove wastes.

Going further in this work these experiments will be performed in anoxia in order to complete the model. In addition, metabolic waste like lactates quantification in the media and in the construct, and the measure of pH will be necessary to assess how *in situ* oxygen delivery impacts cell viability. Developing a self-sustaining bioink systems will also require developing glucose releasing biomaterials either based on hydrophobic embedding or using glycogen and glycogen debranching enzyme.

3-Dimensional bioprinting can allow to print complex structures containing different cell types [515, 516], and recent work have shown the possibility to create constructs that will be fully perfused scaffolds by blood in five days.[517] Combining such techniques with fully developed self-sustaining bioinks may allow to create larger scaffolds with a higher cell density.

Chapter 6 - General discussion

Current methods to deliver oxygen do not allow to perform a safe long-term delivery of oxygen. As demonstrated in chapters 3, 4 and 5, we succeeded in designing safe long-term release oxygen delivery biomaterials.

In Chapter 3 we use an oxygen releasing wound dressing to an animal model of wound healing. In this animal model [67], healing proceeds by forming new granulation tissue comes from the edges of the wound, signifying that the increased wound closure observed is due to an augmented tissue formation. Increasing oxygen levels in the skin using HBO may stimulate the reconstruction of an epidermis by enhancing fibroblast proliferation (*in vitro* and *in vivo*) [191, 192] as well as proliferation, migration, and differentiation keratinocytes. [191, 193] Furthermore, Gordillo et al, showed that normobaric topical oxygen treatment may induce higher VEGF expression in the wound edge. [56] This correlates with the higher and faster formation of granulation tissue observed in the experimental group. Hydrogen peroxide at sub-lethal concentrations (up to 500μM) have been also shown to increase VEGF expression in different cells (keratinocytes [175, 176], macrophages [177] and vascular smooth muscle cells [178]) through mRNA expression. It was also associated with increased VEGF level *in vivo* with full thickness wound models in mice. [171] These effects combined may have participated to a faster formation of blood vessels and an improved healing.

At necrosis, the cellular content is released into the extracellular space and in turn causes secondary necrosis and lead to its spreading. [460] In wound care, it can be prevented by debriding the wounds. In this model the necrotic cartilage was not removed, and necrosis was left to spread, thus resulting in a non-healing wound. The difference in necrotic tissues amount was significant

between both groups, suggesting that by limiting necrosis occurrence with oxygen it was possible to also limit its spreading while the healing process is taking place.

In chapter 4, the *in vivo* implantation allowed the delay and partial prevention flap necrosis. *In vivo* oxygen measurements show that the implant allowed to re-established a near physoxic subcutaneous oxygen concentration. However, the lactate concentration inside each section of the flap was not significantly different between both groups, suggesting that oxygen delivery alone was not sufficient to maintain aerobic mechanism in the whole skin thickness. The necrosis observed is and corresponds to what is typically observed in organs under severe ischemia: tissue is firm, maintained architecture days after cell death, cell outlines preserved, disappearance of nuclei and apparition of ghost cells.[471] These experiments demonstrates the potential benefits of oxygen delivery with results are accordance to previously published work consisting in increasing oxygen in the flap with normobaric hyperoxia therapy [472] or HOBT.[466-470] The results also compare to the previous study on subcutaneous implants for oxygen delivery to skin flaps published by Harrison et al (2007)[69]. The authors have shown the necrosis was significantly reduced at day 3, but not significantly different from the control at day 7. In our study we were able to deliver oxygen subcutaneously for a longer time and we demonstrated significant improvement in skin flap survival over those 10 says, yet it was not sufficient to prevent necrosis. Results obtained for the control group are consistent with other studies using a similar model.[473-476] As discussed previously, oxygen diffusion distance through tissues is rarely more than 200μm [477], implying that a part of the flap was not receiving oxygen from the implant as the thickness of the skin was several millimeters. This is confirmed by the non-significant difference that was found between the skin lactate content of both groups. In that regards, it is possible that the use of split thickness skin flap could result in improved viability. Another limitation of this experiment was venous

stasis, evidenced by the skin's blue color post-surgery. This is a pathology commonly occuring in skin flaps and that may participate to necrosis onset and spreading [478] and may have hindered partially the potential benefits of the implant.

In chapter 5, oxygen generating microparticles were successfully created. The oxygen release achieved by reacting calcium peroxide and water was controlled using a hydrophobic polymer, and the Fenton-Haber-Weiss catalytic reaction was used to harvest the potential of hydrogen peroxide to produce oxygen and reduce its concentration thus also preventing a potential toxicity. Different studies have shown the potential of using peroxide in tissue engineered construct *in vivo*. [508] They however rely on catalase to eliminate hydrogen peroxide eficiently [510-512], but have limited applications *in vivo* due to its limited half-life.[513, 514] When in contact with water the particles released oxygen and a non-cytotoxic hydrogen peroxide concentration when in contact with cells, and allow to partially re-establish cell proliferation, the thus demonstrating the potential of using peroxide-based and catalase free oxygen releasing biomaterials to help maintain cell survival.

Biomaterials delivering oxygen safely over an extended period have been prepared in this thesis. Chapter 3, 4 and 5 clearly show the limitation of a system delivering only oxygen. Blood does not deliver only deliver oxygen, it also brings nutrient and remove wastes. If today oxygen delivery can be performed to some extent, nutrient delivery and waste removal cannot. Therefore, the study of oxygen delivery systems on ischemic cells or tissues *in vivo* and *in vitro* is inherently limited by the absence of control over these parameters.

Chapter 7 - Conclusion and future work

The main contributions of this thesis to the *in vivo* and *in vitro* oxygen delivery applications and its limitations include:

1. Topical oxygen delivery can prevent necrosis in ischemic wounds.

As described in chapter 2 and chapter 3, oxygen has the potential to bring significant improvements to wound healing by promoting different aspect of wound healing, but also by preventing ischemia-induced cell death. In chapter 3 we demonstrate that calcium peroxide-based wound dressing can allow to maintain tissue viability and prevent necrosis while maintaining the healing process.

To date topical oxygen is clinically delivered under a gas form, with either a systemic or a topical approach. Systemic oxygen delivery for wound healing has been widely described in the literature and its effects are still debated. The topical approach using chambers has been proven to have beneficial effects in chronic wound healing but are punctual treatments. Concentrators can be used and supply oxygen continuously, but lack convenience and do not fit every dressing and therefore are not adapted to all wounds. A similar observation can be made for commercial oxygen releasing wound dressings. The new dressing investigated is sizable, and only requires water to generate oxygen and could be adapted to a wide variety of wounds, as most wounds are exudating, thus producing water, and non-exudating wounds require moist dressings. Literature have shown that an optimum of oxygen exist for wound healing, and all of the technologies available are hardly controlling the amount of oxygen delivered. In that regards, the oxygen release of the O2patch can easily be tuned by modification of the formulation in order to fit wound's oxygen requirements.

All wounds are by their nature, different, depending on their origin, associated comorbidities, etc., that are difficult to replicate in preclinical studies. In addition to the difficulty to replicate those

wounds, there is a lack of consensus on the models that are used, making any comparison between wound healing studies difficult.

Furthermore, most prior studies focused on improving healing and not salvaging tissues that will otherwise likely undergo necrosis. As observed in many clinical studies, despite delivering oxygen some wounds still develop necrosis. Studying necrosis prevention in those wounds may allow prevention of irreversible damage to tissues. Chapter 3 focused on the prevention of necrosis using oxygen and examined to which extent damage can be prevented. Going further in this work, studying the delivery of nutrient and stabilize wound's homeostasis may bring improvement to necrosis prevention in impaired wounds.

2. Oxygen delivery implants

Increasing the oxygen concentration of the extracellular fluid of an otherwise ischemic tissue has been achieved mainly by using vasodilators or hyperbaric oxygen therapy. However, these systemic approaches are limited if the vasculature itself is poorly functioning. Topical approaches can only be used for ischemic skin, but its effects are limited by the skin's lack of oxygen permeability. In the literature many different approaches have been taken to tackle oxygen delivery but are not suitable for implantation due to their inherent toxicity and the fact that the sustained oxygen release and level is so low that neither tissues function, nor survival are possible, consequently there have been only two studies investigating implantable oxygen delivery systems. Of all delivery systems, peroxides can generate the most oxygen per unit volume, and as such can act as miniaturized implantable "oxygen tanks". In Chapter 4 we demonstrate that such compounds designed as biomaterials could generate oxygen when implanted and prevent necrosis. The efficiency of such devices may be limited by the total amount of oxygen delivered and its diffusion in tissues. Our recent work on this material tries to address these limitations by developing an

injectable and biodegradable version of the biomaterial that could be injected in multiple points and different moment in time to ensure to maintain a uniform and steady supply of oxygen.

The study is inherently limited by the ischemic model used as the lack of oxygen is not the only factor of necrosis; venous congestion also played a non-negligible role in necrosis. This situation in not uncommon and clinically relevant, and methods have been studied in literature to decrease venous-congestion induced necrosis using anti-thrombotic.

Other methods have demonstrated a potential in preventing necrosis and could be used in addition to oxygen. In our recent experiments we started to study the combination of oxygen delivery and nitroglycerin (vasodilator), but results are for now inconclusive and we have no evidence to suggest an additive effect. It is also possible that nitroglycerin may have both a positive and a negative effect as it increases arterial flow, but may decrease venous return, thus worsening the effects of venous stasis. Further work might combine antithrombotics or antioxidants like N-acetylcysteine as additive therapy that may help to differentially assess the different types of damage that will lead to necrosis in this model. Indeed, like for wound healing, there is no consensus in literature regarding the skin flaps model in term of size, location and orientation.

3. Self-sustaining bioinks

Delivering oxygen homogenously inside tissue engineered constructs would potentially allow to build larger constructs and an increase the variety of *in vivo* applications but remains challenging. The delivery is limited by limited by oxygen diffusion and building mimics of functioning vascularization inside the construct is first limited by bioprinting's current constraints, but also by the fragility of the constructs that limit medium flow through the construct.

In situ oxygen delivery may allow a temporary mimic of oxygen delivery by small vessels, but to date it has been limited by the potential toxicity of peroxides to cell. This has been addressed *in*

vitro using catalase, however applications *in vivo* are limited due catalase half time limited to only a few hours. In this work this issue was tackled using catalysis to turn hydrogen peroxide into oxygen and decrease the inherent toxicity.

In chapter 5 we demonstrated that delivering oxygen in presence of glucose to a three-dimensional tissue construct with a high cell density may allow maintenance of cell viability.

In this chapter we also showed that oxygen deficiency does not affect cell viability, suggesting that its decrease may be due to a lack of glucose or accumulation of wastes in at the center of the construct. Blood's function is not limited to oxygen delivery and developing slow nutrient release systems are as challenging as for oxygen. Going further in this work we will develop systems able to sustain a longer oxygen release.

4. Concluding remarks

In this thesis we demonstrate the potential applications of oxygen delivery systems to prevent necrosis, or to allow cells to maintain their functions. Different pathologies and different applications require an optimization of the amount and length of the delivery, and the technology presented in this thesis is easily tunable to fit those needs. Oxygen is no panacea, and in clinical settings, most pathologies an oxygen shortage is not the only factor leading to tissue damage. As such, oxygen delivery material is a tool to improve outcomes and should be used in addition to other treatments.

Chapter 8 - References

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Chapter 9 - Annexes

Annex 1 - Representative histological sections (H&E staining) of skin flap sections for the experimental and control group

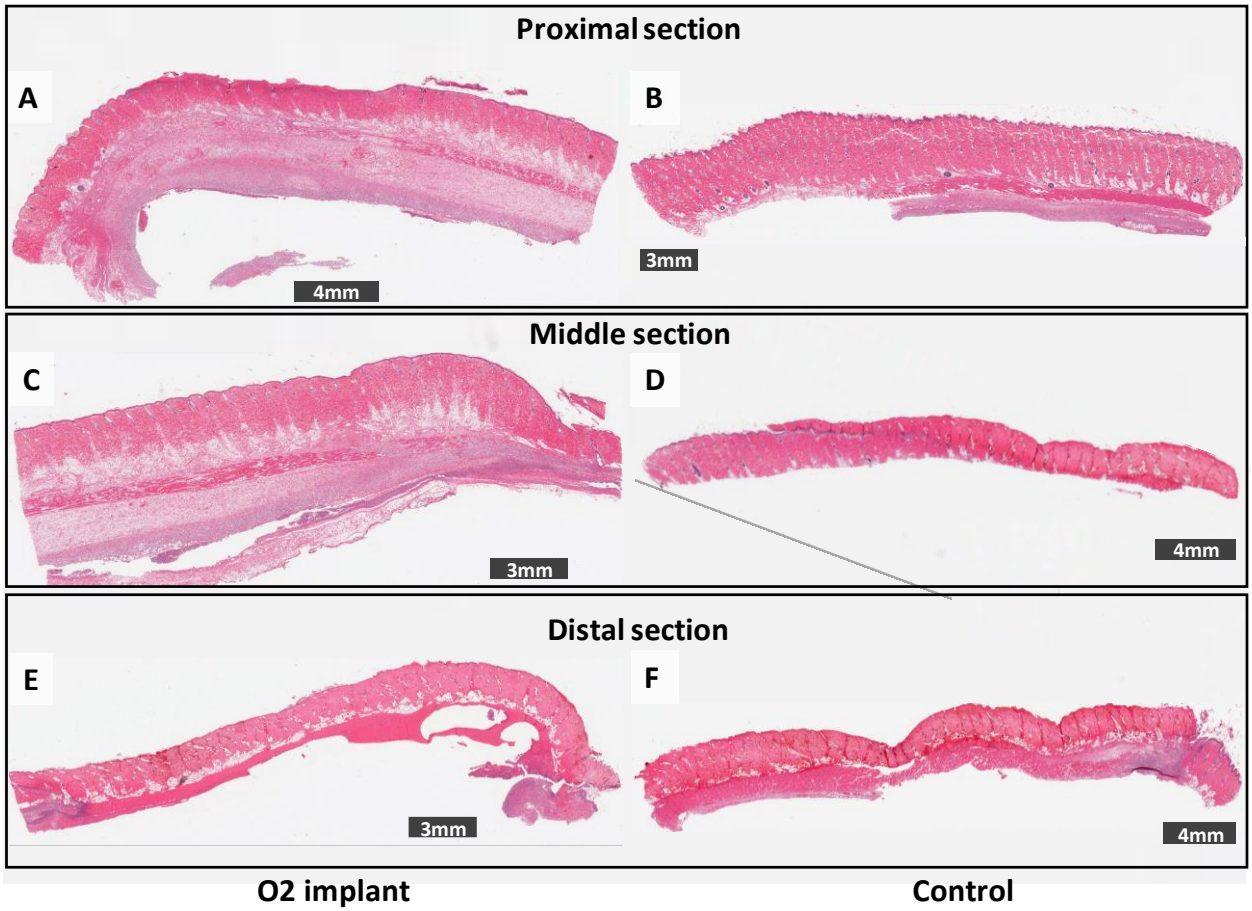

Figure 9-1: Representative histological sections (H&E staining) of skin flap sections: (A), (B) middle section of the flap for the experimental and control group respectively; : (C), (D) proximal section of the flap for the experimental and control group respectively; : (E), (F) distal section of the flap for the experimental and control group respectively.