Effects of gold nanoparticles on vascular endothelial growth factor-A-induced melanoma cell growth and angiogenesis

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Executive Summary

Melanoma is a skin cancer that relies on angiogenesis for growth and progression. Angiogenesis is the growth of new vessels from existing vessels and follows a number of steps that include endothelial cell growth, migration and tubulogenesis. Current anti-angiogenic drugs are not effective in the treatment of melanomas due to serious side effects such as hypertension and the development of resistance. On the other hand, gold nanoparticles (AuNPs) have been reported to be biocompatible in preclinical models. Furthermore, AuNPs were shown to be cytotoxic to prostate cancer cells. In human umbilical vein endothelial cells, AuNPs inhibited the angiogenic protein, vascular endothelial growth factor-A (VEGF-A). Therefore, the study aimed to investigate the possible cytotoxic effects of AuNPs (1.2–3.2 nM) on melanoma cells and angiogenesis parameters (endothelial cell growth and migration) as well as on the levels of angiogenesis promoting proteins, VEGF-A and placental growth factor (PIGF). Melanoma (B16-F10) cells and tumour-derived endothelial (sEnd.2) cells were maintained in an incubator in a humidified atmosphere containing 5% CO_2 at a temperature of 37°C. To investigate whether AuNPs were cytotoxic to melanoma cells, the effect of the particles on B16-F10 cell survival was measured using the crystal violet assay. To determine the effects of AuNPs on angiogenesis parameters, endothelial cell (EC) growth and migration were investigated using crystal violet assay and the scratch assay respectively. Also, EC morphology was studied using polarisation-optical interference contrast light microscopy. The enzyme-linked immunosorbent assay (ELISA) was used to determine the effects of AuNPs on the levels of VEGF-A and PIGF. The results showed that AuNPs decreased the viability of melanoma and endothelial cells. The scratch assay showed that more ECs migrated in cultured treated with AuNPs ($P < 0.05$). The concentration of VEGF-A and PIGF was reduced significantly following treatment with AuNPs, meaning that the particles exhibited anti-angiogenic properties. This outcome provides a basis for further testing of AuNPs as a potential treatment for melanoma.

Keywords: Gold nanoparticles, tumour angiogenesis, melanoma, VEGF-A, PIGF

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"Tell me I will forget. Teach me I will remember. But, involve me and I will learn".

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List of abbreviations

α5β1:	Integrin alpha 5 (CD49e) and integrin beta 1 (CD29)
αvβ3:	Integrin alpha V and integrin beta 3 (CD61)
αvβ5:	Integrin alpha V and integrin beta 5 (sCD23 molecule)
ANOVA:	Analysis of variance
Ang-2:	Angiopoietin-2
df:	Degrees of freedom
DNA:	Deoxyribonucleic acid
DMSO:	Dimethyl sulphoxide
ddH_2O:	Double-distilled water
DMEM:	Dulbecco's modified Eagle's medium
EC:	Endothelial cell
FBXO22:	F-box only protein 22
FGF-2:	Fibroblast growth factor-2
FGFR-1:	Fibroblast growth factor receptor-1
FBS:	Foetal bovine serum
AuNP:	Gold nanoparticle
g:	Gram

HUVECs: Human umbilical vein endothelial cells

H_2O_2: Hydrogen peroxide

$OH^{\bullet}$: hydroxyl radical

IC_{50}: Inhibitory concentration for 50% of cells

IGF: Insulin-like growth factor

MMPs: Matrix metalloproteases

µg: Microgram

µL: Microlitre

µm: Micrometre

mL: Millilitre

mm: Millimetre

mM: Millimolar

mins: Minutes

M: Molarity

MPC: Monolayer-protected cluster

B16-F10: Mouse melanoma cell line

ng/mL: Nanograms per millilitre

nm: Nanometre

nM: Nanomolar

NRP-1/2: Neurophilin-1/2

NO: Nitric oxide

NOS: Nitric oxide synthase

$ONOO^-$: Peroxynitrite

PBS: Phosphate-buffered saline

pg/mL: Picograms per millilitre

PIGF-1/2: Placental growth factor-1/2

PDGF: Platelet-derived growth factor

PlasDIC: Polarisation-optical interference contrast

pH: Potential of Hydrogen

ROS: Reactive oxygen species

rpm: Revolutions per minute

$^1O_2$: Single oxygen

NaCl: Sodium chloride

cm^2: Square centimetre

SD: Standard deviation

$O^{2-\bullet}$: Superoxide anion

SOD: Superoxide peroxide

Temp: Temperature

$HAuCl_4$: Tetrachloroauric acid

TGF-1: Transforming growth factor-1

sEnd.2: Tumour-derived endothelial cell line

VEGF-A: Vascular endothelial growth factor-A

VEGFR: Vascular endothelial growth factor receptor

Vs: Versus

WHO: World Health Organisation

U/mL: Units per millilitre

US-FDA: United States Food and Drug Administration

V: Volt

Symbols

°C: Degrees Celsius

≤: Less than or equal to

%: Percentage

±: Plus-minus sign

×: Times

CHAPTER 1: LITERATURE REVIEW

1.1 Overview of angiogenesis

The growth of blood vessels is controlled by a multistep process called angiogenesis. It occurs from birth until death, both in health and a pathological state. Angiogenesis is important in physiological processes (embryonic development, wound healing and female reproductive systems) and pathogenesis (tumour growth and metastasis)[5-9]. In normal physiological settings, angiogenesis plays a limited role in wound healing and menstruation. It is controlled by various stimulators (pro-angiogenic factors) and inhibitors (anti-angiogenic factors) to maintain an angiogenic balance[6-7]. An imbalance in various stimulators and inhibitors triggers pathological angiogenesis, resulting in disorders such as ocular neovascularisation, cancer and inflammatory disorders[5,10-11]. Angiogenesis affects multiple cell types, including endothelial, mural, inflammatory and blood-derived cells[10,12], as well as different cytokines, the extracellular matrix and proteolytic enzymes[12].

1.1.1 Tumour angiogenesis

In 1971, Folkman and colleagues hypothesised that solid tumours, including melanomas, cannot grow beyond 2-3 mm in diameter without recruiting their blood supply[13-14]. Indeed, tumour cells can only grow to a specific size independent of new blood vessel recruitment. A tumour with a diameter of 1-2 mm is known as being in the resting stage[15]; at this point the tumour is dormant. As the tumour increases in size, hypoxic conditions arise, which trigger the switching of the tumour into an angiogenic phenotype[16].

Tumour cells interact chemically with endothelial cells (ECs) by secreting pro-angiogenic factors such as vascular endothelial growth factor-A (VEGF-A), fibroblast growth factor-2 (FGF-2) and angiopoietins (Angs), which stimulate angiogenesis[17]. Endogenous anti-angiogenic factors such as endostatin, angiostatin or thrombostatin were found to suppress angiogenesis. Homeostasis is maintained by stability between pro-angiogenic factors and anti-angiogenic factors[6-7]. Therefore, angiogenesis contributes to cancer progression in that the newly formed blood vessels are used as new routes for tumour cells to migrate to different sites in the body. This is known as metastasis[18] (Figure 1.1). More research is now being done into suppressing tumour angiogenesis as a way of indirectly suppressing tumour growth for anticancer therapy.

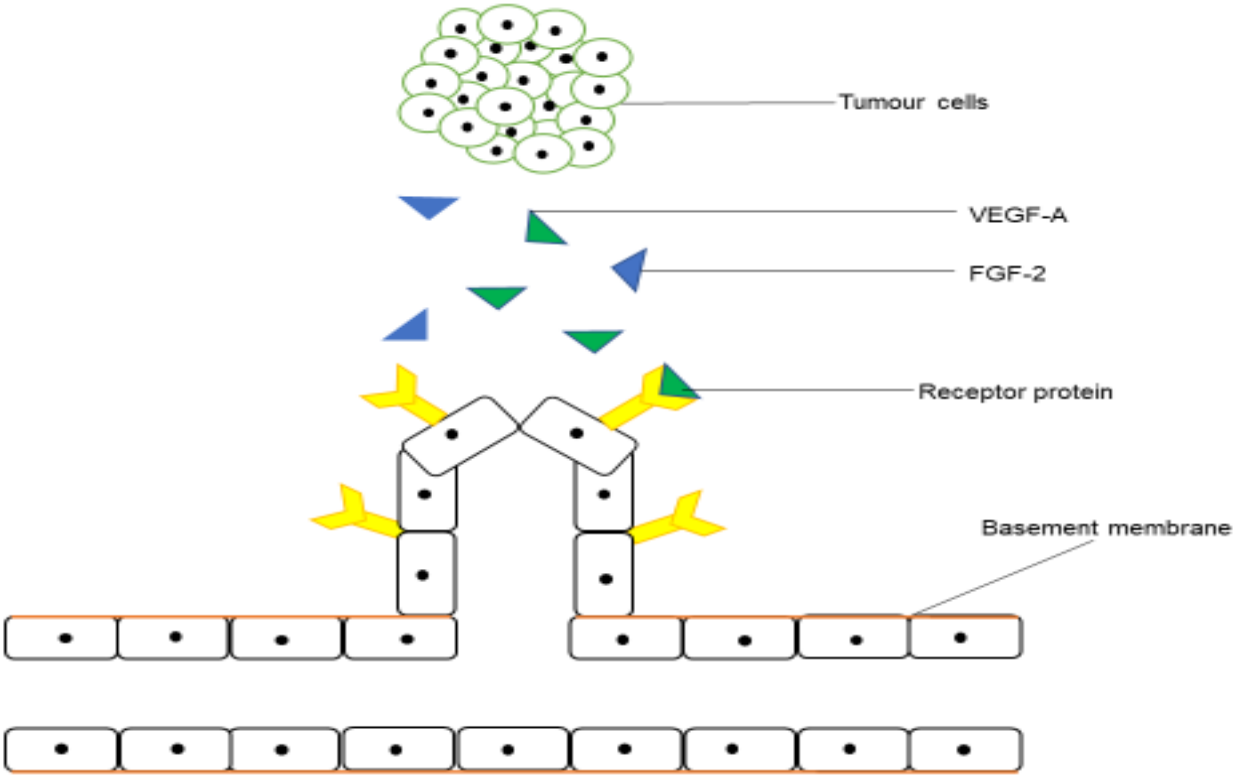

Figure 1.1. Diagram showing the process of tumour angiogenesis. Tumour cells secrete vascular endothelial growth factor-A (VEGF-A) and fibroblast growth factor-2 (FGF-2) to initiate angiogenesis. VEGF-A and FGF-2 bind to the receptors to stimulate endothelial cell growth, thus forming new vasculature. Tumour cells enter the blood circulation to migrate (metastasise) to different sites of the body. Figure drawn by LJ Matutule using Microsoft PowerPoint 2016[3].

1.2 Oxidative stress and angiogenesis

Reactive oxygen species (ROS) are generated by oxygen metabolism which is balanced by the rate of oxidant formation and the rate of oxidant elimination[19]. Oxidative stress occurs when the production of ROS and the antioxidant system is disturbed, which can be a cause and prognosis of many vascular diseases, and thus serve as a biomarker for these diseases[10,12,20]. The formation of ROS is often the main factors resulting in resistant to treatment. It is closely associated with angiogenesis and plays a crucial role in the positive feedback mechanism[12]. Simultaneously, well maintained oxidative stress may promote angiogenesis and tissue repair[12,19]. Furthermore, two signal pathways (i.e. VEGF-A-dependent signal pathway and VEGF-A-independent signal pathway)[21-22] of angiogenesis mediated by oxidative stress have been recognised. Oxidative stress has been proven in several studies to serve as an important stimulus for vessel formation in many cancers, including melanoma[19-20,22-23].

1.2.1 Reactive oxygen species

Reactive oxygen species are oxygen atoms that contain unpaired electrons which can be reduced to four components: superoxide anion ($O^{2-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and a water molecule[24-26]. They are produced by the mitochondrial electron transport chain, arachidonic acid metabolising enzymes, lipoxygenase and cyclooxygenase, cytochrome P450, xanthine oxidase, NAD(P)H oxidases, uncoupled nitric oxide synthase (NOS), peroxidase and hemoproteins[24].

1.2.2 Generation of ROS

Under normal physiological conditions, the production and elimination of ROS are sustained. The production of hydroxyl radicals and singlet oxygen ($^1O_2$) is highly reactive, and

subsequently dangerous of the ROS. Reactive oxygen species must be removed by superoxide dismutase (SOD), peroxidases and redox molecules. Superoxide anion is the main and mostly produced precursor of ROS and occurs through the removal of one electron by the reduction process of the mitochondrial electron transport chain[12,24]. Superoxide is converted to hydrogen peroxide (H_2O_2) by the action of SOD which can be reduced to water, either spontaneously or through the action of catalase[24]. Neutrophil-secreted myeloperoxidase converts H_2O_2 and chloride into highly reactive hypochloride[10,12]. Superoxide anion may react with other radicals such as nitric oxide (NO) to form peroxynitrite ($ONOO^{-\bullet}$).

In biological systems, ROS plays an essential role in migration, differentiation, proliferation, apoptosis, stress adaptation as well as gene expression[4]. Growth factors such as placental growth factor (PIGF) bind to its receptors to cause ROS to initiate transduction signals. Various factors such as deregulation of antioxidant enzymes, change in proliferation and acquisition of metastatic phenotype, mitochondrial dysfunction and disrupted signalling pathways influence the overproduction of ROS in melanoma cells[4,10,22]. An overproduction of ROS before and during tumour formation has been previously reported by numerous authors over the past two decades[4,10,20,22,24,27-28] as well as the significantly increased levels of ROS in melanoma cells. Increased levels of ROS can be reduced by melanocytes, however, melanoma cells do not have the ability to reduce increased ROS levels[4]. Enzymatic antioxidant activity such as catalase, glutathione-S-transferase and manganese superoxide dismutase has a reduced activity in melanoma cells which contribute to low levels of glutathione[4]. Melanoma cells are achieved by an enhancement of superoxide anion levels and a reduction in H_2O_2 levels. This statement is supported by the pro-oxidant intracellular environment and activation of redox-transcription factors to increase the high proliferative rate and drug resistance in melanoma cells[4]. In

addition, sustained exposure to ultraviolet (UV) radiation causes melanocytes to transition into melanoma (Figure 1.2).

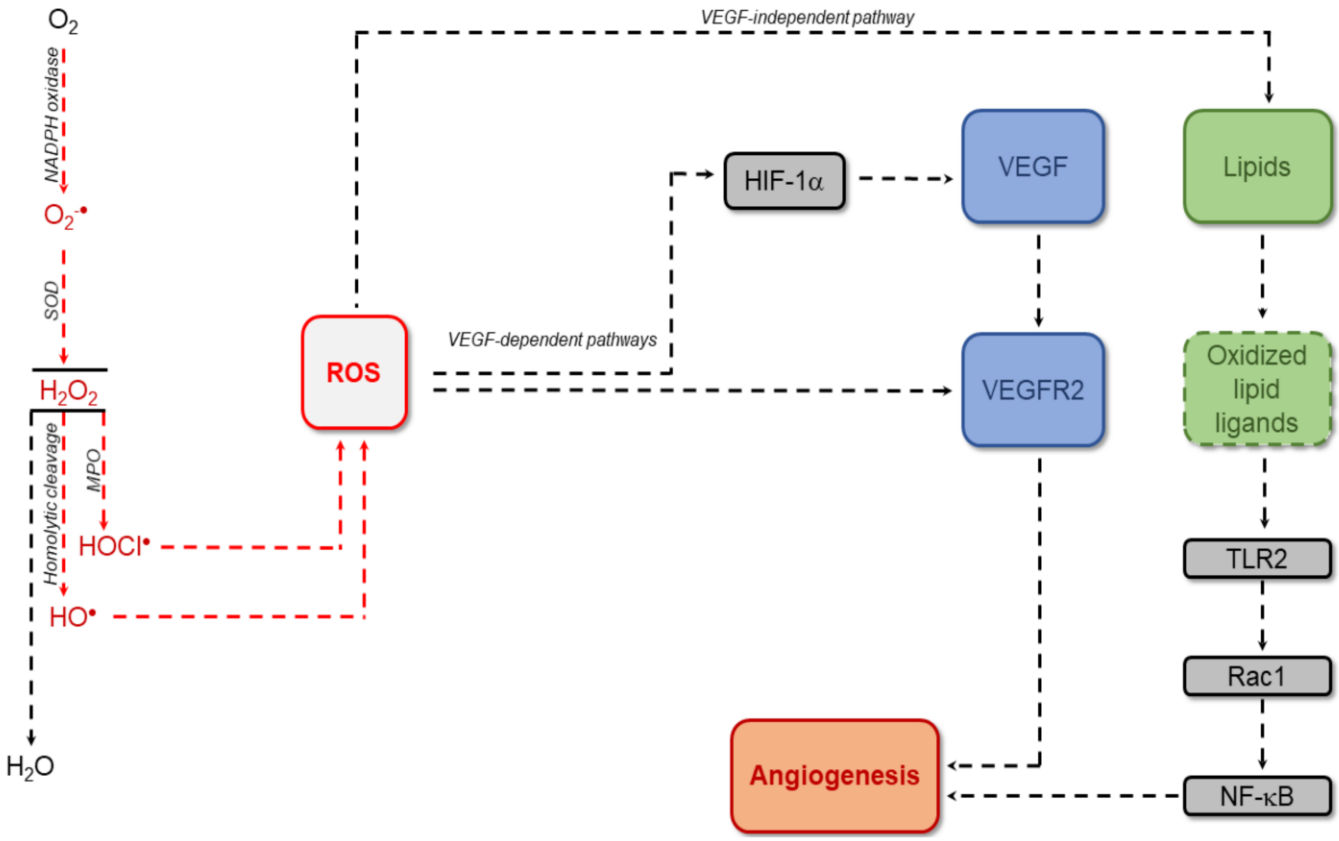

Figure 1.2. Mechanism of oxidative stress and effect in angiogenesis. The generation of ROS and endogenous antioxidant pathways within the mitochondria. Abbreviations: myeloperoxidase (MPO), hypoxia-inducible factor (HIF), toll-like receptor (TLR), cytosolic oxidase component (Rac1), factor nuclear kappa B (NF-$_{k}$B).

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Superoxide anion and H_2O_2 play an important role in angiogenesis to activate diverse pathways to induce either new vascular growth, vascular dysfunction, and destruction[10,24]. Reactive oxygen species are a double-edged sword because high concentrations of ROS are damaging to most tissues, while low concentrations of ROS may activate signalling pathways that promote tissue regeneration and angiogenesis[10,12]. Overproduction of ROS must be continually removed from the cell by an antioxidant defence mechanism.

1.2.3 Antioxidant defence mechanisms

When ROS exceeds the antioxidant defence mechanism, the cell characterises a state of oxidative stress. This causes various pathological conditions such as peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death pathway (apoptosis) and ultimately leading to the death of cells[12,22-24]. In a cellular system, antioxidants are defined as compounds that prevent oxidative reactions by scavenging ROS before cellular and tissue damage can occur[28]. Antioxidants can be classified as a radical scavenger, hydrogen donor, peroxide decomposer, single oxygen quencher, an enzyme inhibitor, synergist, and metal-chelating agent[26,28].

The levels of antioxidant action are classified into three lines of defence. The first line of defence (primary defence) comprises enzymatic antioxidants that suppress the formation of free radicals[24]. The second line of defence (secondary defence), comprises of non-enzymatic oxidants which suppress the chain initiation or the break chain propagation[19,22]. The third line of defence (tertiary defence) comprises of proteolytic enzymes, proteinases, proteases, and peptidases present in the cytosol and mitochondria, thus serve as repair and *de novo* oxidants[28].

1.3 Melanoma

Malignant melanoma has been extensively studied and postulated to serve as an angiogenic tumour type. It is directed by a multistep process involving an interplay of environmental, genetic and host factors[29]. Risk factors that evolve in malignant melanoma include UV radiation, severe sunburn, few viruses and chemical agents, diagnostic melanoma family history, the appearance of dysplastic nevi and immunosuppressive conditions with increased mortality and morbidity rates.

1.3.1 Melanoma pathophysiology

The pathophysiology of melanomas occurs due to an overproduction of melanocytes which transition into melanoma (Figure 1.3). It accounts for 4% of all skin cancer cases, causing 70% of death cases[29-33]. About 5% of melanomas develop in the mucosa, meninges and retina while 95% of melanomas are situated at the skin level[34-36]. This tumour is highly metastatic with poor prognosis and high resistance to treatment. Early detection of melanoma cancer is a crucial key in disease prognosis. If diagnosed at an earlier stage, patients have a five-year survival chance (more than 90% survival rate) and if diagnosed at a later stage, the survival rate is less than 20% within 5 years[34,37]. To date, patient health has been improved by novel drugs such as Vemurafenib and Dabrafenib used independently or in combination with Trametinib[38]. However, due to the refractoriness of the melanoma, the effectiveness of these novel drugs has been challenging.

The World Health Organization (WHO) has reported nearly 132 000 cases of skin cancer that occur annually[39]. In 2015, the global incidence of melanoma was 351 880; it continues to grow[40]

amongst the Caucasian population[32,37] and this is thought to be due to the degree of UV radiation[39]. The incidence of melanoma is elevating than other types of cancer[34].

Currently, the incidence data of melanoma in South Africa is insufficient or outdated because the data have been fixed on a single province or population group. Melanoma incidence data were previously reported for 2000–2004 (inclusive).

1.3.2 Angiogenesis and melanoma

In 1966, Warren and Shubik documented angiogenesis in melanoma by transplanting melanoma cells into the cheek pouches of hamsters[38,41]. Since then, much research has been conducted to explain the underlying mechanisms and clinical significance of melanoma angiogenesis. Melanoma is a crucial part of the vascular growth phase of the disease, occurs when new angiogenesis contributes to the genesis of a mature vascular network within the tumour microenvironment[5]. The growth of melanoma is controlled by secretions from keratinocytes in normal physiological settings[38]. However, uncontrolled regulation of mutations in key genes causes melanocytes to produce abnormal cell growth leading to malignancy[36,38]. Melanomas follow an ordered progression of diseases from atypical melanocytes, via radial growth, to the aggressive vertical growth phase[38,42-43] (Figure 1.3). Substantial exposure to UV radiation leads to the transition of benign nevi into atypical melanocytic proliferations[41]. Thereafter, the process of pathological melanoma angiogenesis is activated.

1.3.2.1 Clinical histological features of melanoma subtypes

Melanoma is categorised according to four subtypes depending on the appearance and localization of the tumour[44]. These are namely from the most lethal to the least lethal, superficial spreading melanoma (60-80% melanoma cases), nodular melanoma contributing to 50-70% of

melanoma cases, lentigo melanoma and acral lentiginous melanoma[44-45] (with the least cases), Figure 1.3. Nodular melanoma and acral lentiginous melanoma are considered to display poor prognosis as a result of their rapid spread. Melanoma is also classified according to four stages, stage 0 representing melanoma in situ which is not dangerous in most cases, (stage I, II, III and IV) classified according to the size and location of the tumour. The premature stages of melanoma (stage I and II) can be successful removed if detected early. According to Breslow's tumour thickness, stage I is classified as a tumour with a depth of $\leq$ 1.0 mm. Stage II has a depth of 1.01-2.0 mm, stage III has a depth of 2.01-4.0 mm and lastly stage IV has a tumour with a depth of $\leq$ 4.0 mm[46]. Stage I and II mean that the tumour cells have not metastasized to nearby lymph nodes or distant organs with fewer chances of ulceration. While stage (III-IV) represents tumour cells that metastasis to different parts of the body leading to higher mortality rates with poor patient health.

Superficial spreading melanoma can be defined as the most common type of melanoma in light-skinned individuals[47]. Major sites affected are in women in areas of the legs while men are mostly affected in the back[47]. The lesions in this area appear flat and variably pigmented. The colour from these lesions usually vary, ranging from brown, red to black with a diameter less than 5 mm to several centimetres, see Figure 1.3. These lesions can potentially become palpable as a result of tumour invasion and aggressive tumours may have nodules and seek ulceration. Lesions such as amelanotic variant have been distinguished and often mistaken for a banal lesion or vitiligo.Nodular melanoma occurs mostly in men than women and usually presents itself at a fifth or sixth decade in life[48-49]. This type of melanoma is reported to grow rapidly by invading deeper into the dermis by the time of diagnosis and commonly appears in the trunk. The histological feature of nodular melanoma is dome-shaped with ulcerates and bleeds very often[47] and relatively symmetrical (Figure 1.3). This type of melanoma commonly

appears darker in colour with tumours that have limited pigmentation, resulting in the poor prognosis of the disease.

Lentigo melanoma often occurs in an elderly population and exposure to UV radiation due to frequent sun burn[50-51]. Lesions in this melanoma appear flat with variable colour and some areas with patches of black and brown or red and pink[47], Figure 1.3. Over time, lentigo melanoma manifests irregular borders which is interesting as the tumour forms single or multiple raised nodules or plaques that appear within the lesion.

Acral lentiginous melanoma occurs most commonly in Asian and African populations[48] and frequently found in old-aged males. This tumour mainly manifests in areas of the foot, the palm of the hand or under the nails. Histological features of this tumour are flat, irregularly large, the variation of the pigmented area from which increased pigment production or ulcerated area may develop (Figure 1.3).

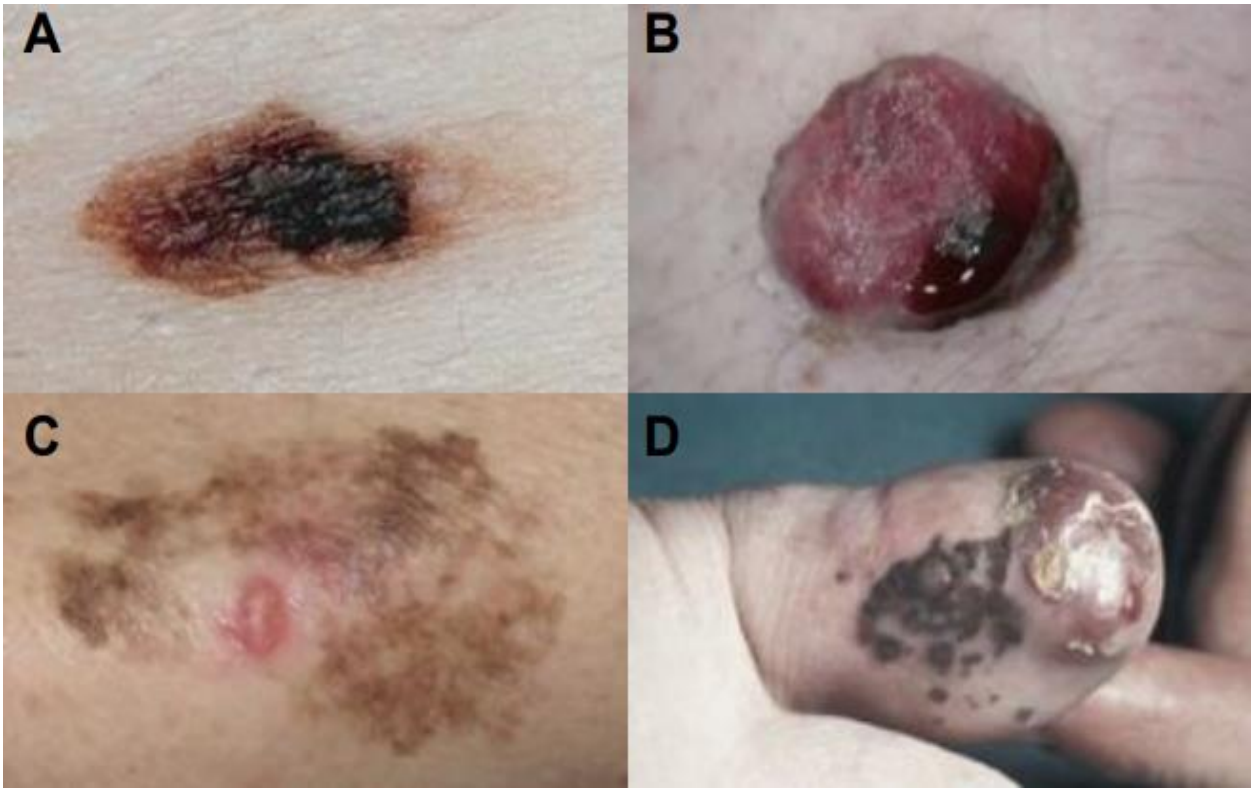

Figure 1.3. Clinical features of four types of melanoma cancer. (A) Superficial spreading melanoma, (B) Nodular melanoma, (C) Lentigo melanoma and (D) Acral lentiginous melanoma characterising various features of melanoma skin cancer.

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1.3.2.2 Stages of melanoma progression

The process begins when atypical melanocyte characterises melanocytic proliferation of atypical moles with undistinctive features of benign or malignant melanoma[41,53-54] and an increased likelihood of melanoma. This occurs due to the overlapping of growth within the dermis and epidermis. However proper diagnosis has been challenging. As the benign or malignant melanoma progress through the radial growth phase (first distinct malignant phase), tumour cells proliferate intraepidermally to evolve as an irregular plaque below the epidermis and invade along the radii of an imperfect circle[41,46] (Figure 1.4). Moreover, angiogenesis plays a

crucial step in promoting the proliferation of melanoma. Lesions in this phase are considered potentially lethal due to undistinctive features of benign or malignant melanoma to metastasie[38].

In the vertical growth phase, tumour cells enlarge vertically and invade the dermis through the basement membrane[41] and subcutaneous tissue (forming a tumour nodule) into the dermal connective tissue with increased angiogenic activity (Figure 1.4). The tumour cells have a capability to evade either vascular or lymphatic channels to metastasise to distant organs such as the liver and lungs[41,46,54]. During this phase, oxygen and nutrient consumption is increased drastically to accommodate the angiogenic switch due to the formation of a rich vascular network essential for melanoma progression[38,41,55]. Moreover, if this switch is not compensated for, local tissue hypoxia directly stimulates hypoxia-inducible factor-1α (HIF-1 α) to upregulate VEGF-A. In turn, VEGF-A increases microvascular permeability within the tumour microenvironment to enhance the proliferation and migration of ECs[41].

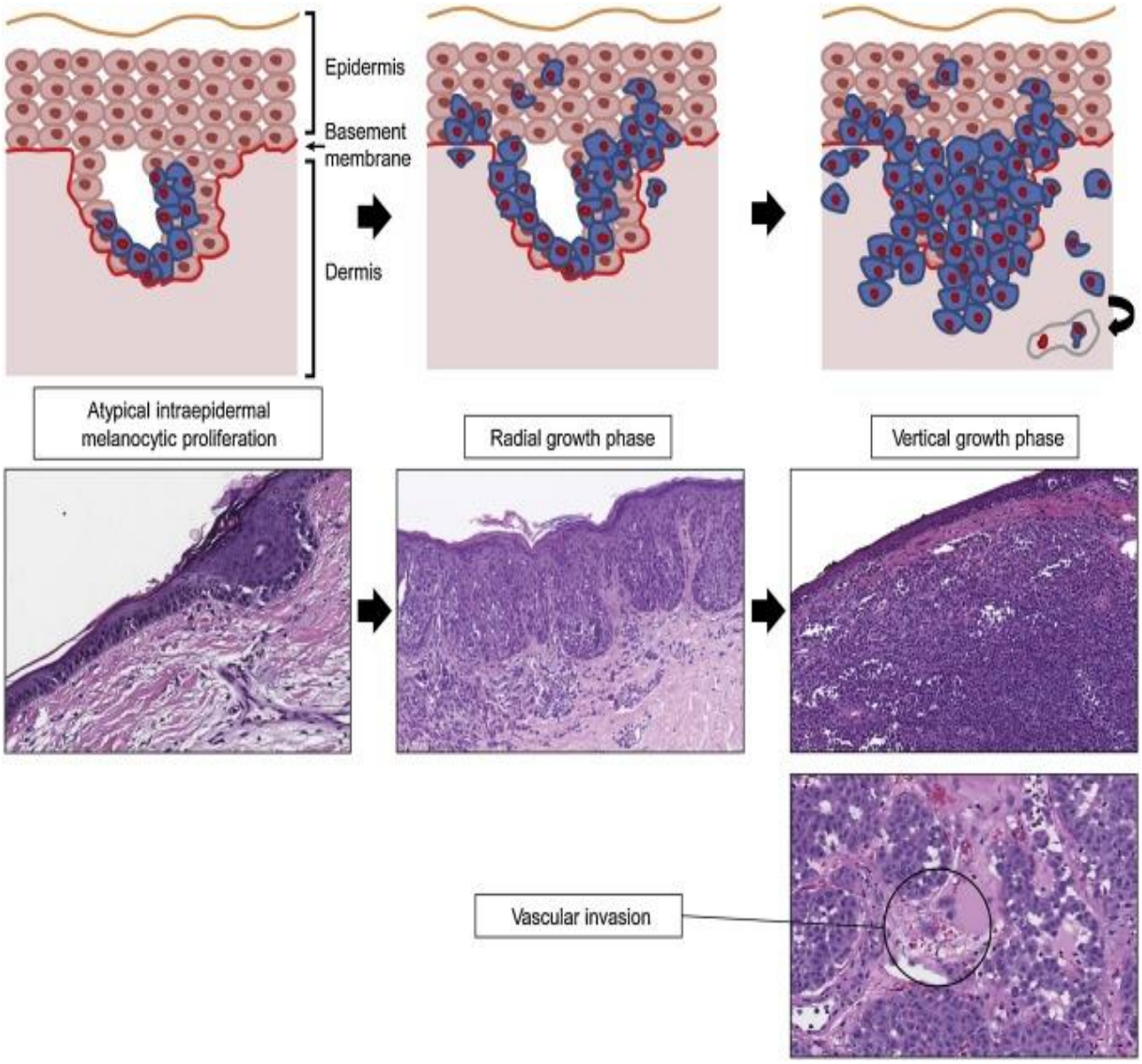

Figure 1.4. Schematic representation of melanoma progression. Melanoma progression from atypical melanocytes, via radial growth to the aggressive vertical growth. See text for details.

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Sustained exposure to UV radiation has been investigated to result in genetic changes in the skin that can regulate the cutaneous immune response and increase the production of a variety of pro-angiogenic factors. This, in turn, results in the uncontrolled proliferation of melanocytes to

acquire oxygen and nutrients due to cell starvation and local hypoxia to meet the angiogenic switch[5].

In addition, within the tumour microenvironment, HIF-1α is directly stimulated by local tissue hypoxia to secrete a potent pro-angiogenic factor VEGF-A[41] that is expressed in almost all solid tumours, including melanomas. Therefore, the angiogenic process is initiated to secrete additional growth factors such as Ang-2.

1.3.3 Angiogenic factors in melanoma angiogenesis

Essential factors such as receptors, growth factors, cytokines and other cellular components play a role in modulating an angiogenic switch by promoting and maintaining angiogenesis in melanoma[5,30]. Some factors will be briefly described in this literature review. These angiogenic factors are initiated during the multistep process of angiogenesis such as cell proliferation, migration and invasion and tubulogenesis[18]. Moreover, blockage and downstream pathways of these factors are known to suppress melanoma angiogenesis and may serve as essential therapeutic targets in melanoma angiogenesis. Other essential growth factors secreted are platelet-derived growth factor (PDGF) and interleukin-8 (IL-8) during tumour transition to the most advanced metastatic phase of melanoma cells (Figure 1.5). Elevated levels of Ang-2, IL-8 and FGF-2 have been associated with poor overall survival and increased risk of occurrence[27,41], while decreasing levels of these factors following systematic treatment seem to be more favourable.

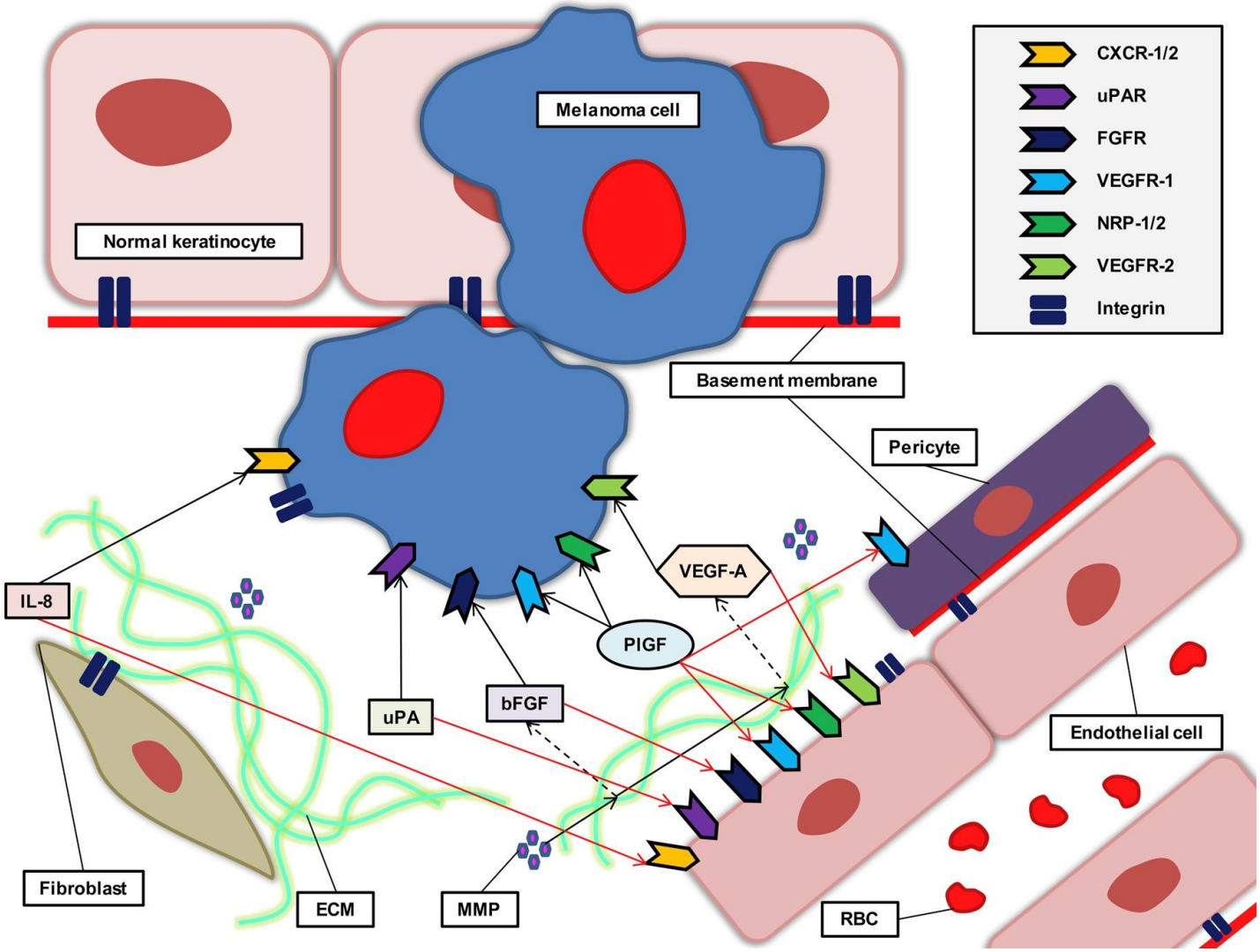

Figure 1.5. Schematic diagram illustrating molecules that are involved in melanoma angiogenesis. Abbreviations: Interleukin-8 (IL-8), urokinase plasminogen activator (uPA), extracellular matrix (ECM), matrix metalloproteinase (MMP), basic fibroblast growth factor (bFGF) or FGF-2, red blood cell (RBC), placental growth factor (PIGF), vascular endothelial growth factor-A (VEGF-A), CXC motif chemokine receptor 1 and 2 (CXCR-1/2), vascular endothelial growth factor receptor-1 (VEGFR-1), neuropilin 1 and 2 (NRP-1/2), fibroblast growth factor receptor (FGFR).

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1.3.3.1 Vascular endothelial growth factor-A

The proliferation of melanomas is known to secrete high amounts of VEGF-A during an ordered progression of diseases from atypical melanocytes via radial growth to the aggressive vertical growth phase. It is postulated that VEGF-A expression by transformed melanocytes plays a more crucial role in mediating melanoma angiogenesis than hypoxia-induced VEGF-A upregulation[29,38].

The VEGF family is a group of secreted glycoproteins that consists of VEGF-A, -B, -C, -D and PIGF, Figure 1.5. Vascular endothelial growth factor-A is the most potent and predominant mediator of angiogenesis (referred to as VEGF-A in this study) and the main mediator of angiogenesis signalling via the class IV tyrosine kinase receptor family of VEGF receptors (VEGFR)[56] on ECs. It binds to two tyrosine kinase receptors, VEGFR-1 and VEGFR-2, expressed on the surfaces of ECs (Figure 1.6). Melanoma cells can develop high-affinity binding to VEGF-A and other pro-angiogenic receptors usually expressed on ECs such as neurophilin-1 and -2 (NRP-1/2)[41]. The VEGFR-1 is associated with angiogenesis, and VEGFR-2 is shown to mediate nearly all known angiogenic responses to VEGF-A associated with EC proliferation, survival and migration[57], as well as melanoma cells.

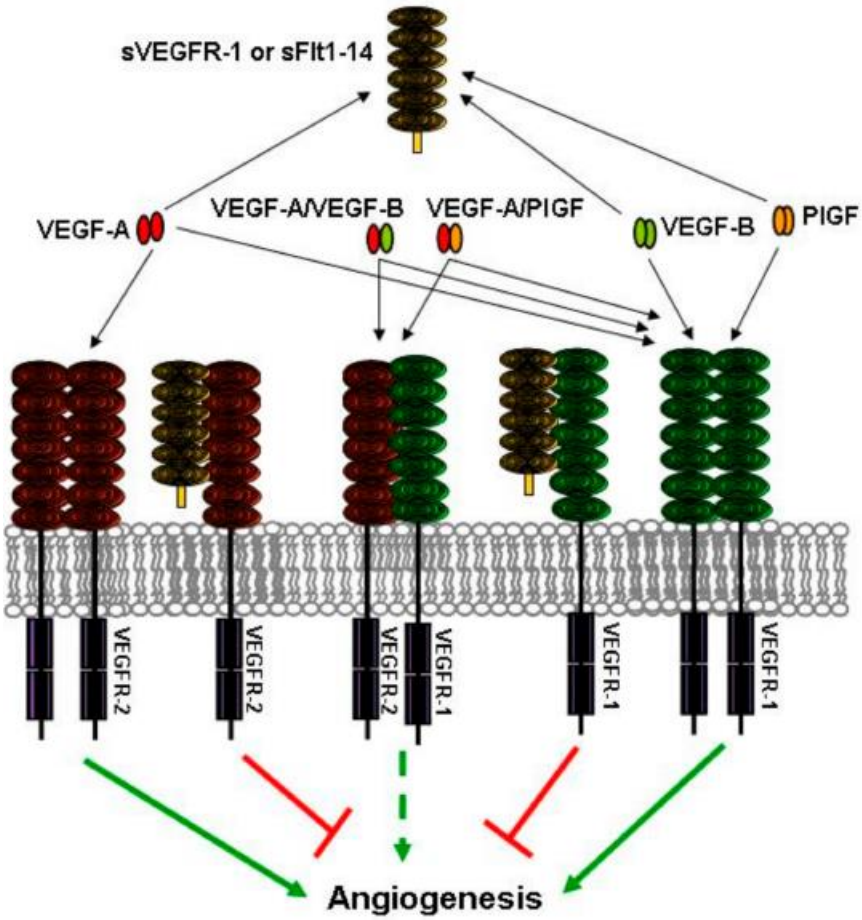

Figure 1.6. Schematic diagram illustrating VEGF family members binding to its receptors mediated by angiogenesis.

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The VEGF-A gene has five different isoforms as a result of alternative splicing, of which VEGF165 and VEGF121[5,41] were found to be predominant in melanoma[41]. Recently, Zhang *et al.* (2018)[59] found that F-box only protein 22 (FBXO22) enhanced melanoma cell migration and invasion and tumour angiogenesis by upregulating the VEGF-A pathway[41,59]. Moreover, the knockdown of FBXO22 suppressed melanoma cell migration, invasion and angiogenesis *in vitro* and *in vivo*, which may indicate a target for melanoma treatment[59].

1.3.3.2 Fibroblast growth factor 2

Fibroblast growth factor-2 is an angiogenic protein secreted by fibroblasts and ECs[57]. It is produced by the progression of melanoma from atypical melanocytes to the aggressive vertical growth phase. It can trigger the proliferation of ECs, fibroblast proliferation and migration[57].

It mediates by binding to its key receptor, fibroblast growth factor receptor-1 (FGFR-1), on the surface of its target cells to induce autophosphorylation, thereby activating different functional proteins and participating in most signal transduction pathways that control cell proliferation, differentiation, survival and angiogenesis[56]. The VEGF165 and VEGF121 along with FGF-2 were found to be the major factors of angiogenesis[5]. In human melanomas, the overexpression of FGF-2 was demonstrated to induce an increased release of matrix metalloproteinases (MMPs) and degradation of the extracellular matrix, which in turn stimulate EC proliferation and tubulogenesis in melanoma[5,55,57] (Figure 1.5).

A study using FGF-2-specific antibodies or antisense oligodeoxynucleotides targeted against human FGF-2 mRNA showed that they inhibited secondary melanoma cell growth to inhibit angiogenesis *in vitro*[41]. A study revealed fibroblast growth factor-2 expression in melanoma tissue in patients with nodal metastasis than patients without lymph node involvement[60]. However, the importance of this growth factor in melanoma is not clear as there is a shortage of data. Another factor, PIGF, has been shown in a number of studies to promote melanoma progression.

1.3.3.3 Placental growth factor

Placental growth factor is a member of the VEGF-A family which binds to VEGFR-1 expressed on the surfaces of ECs (Figure 1.5). Placental growth factor is involved in melanoma tumour progression[38,41,61]. It exists in several isoforms from mature alternative splicing of mRNA, binds to two coreceptors, NRP-1 and NRP-2, on ECs[55] to enhance melanoma angiogenesis.

Placental growth factor stimulates melanoma angiogenesis by mobilising and recruiting VEGFR-1-positive haematopoietic precursors from bone marrow. Additionally, PIGF binds to VEGFR-1-expressing smooth muscles/pericytes to stabilise and stimulate new blood vessel formation[41,55]. During pregnancy, PIGF is substantially exhibited by placental trophoblasts and contributes as a mitogen to ECs and an inhibiting factor that averts trophoblastic apoptosis[41]. The expression of PIGF-1 and PIGF-2 as well as their receptors NRP-1 and NRP-2, are constantly upregulated by tumour cells to augment their pro-angiogenic potential in melanoma[41]. Interestingly, the co-receptors NRP-1 and -2 are not the only receptors that stimulate the angiogenic process, independently PIGF binds to VEGFR-1 and interacts with VEGFR-2 via the genesis of heterodimers with VEGF-A (Figure 1.7) causing the same downstream effect influenced by VEGF-A independently[41,55].

In an experiment using transgenic mice expressing significant levels of PIGF, it was found that melanoma tumour growth and metastasis, as well as mobilisation and recruitment of ECs and haematopoietic precursors from bone marrow, further augment tumour angiogenesis[41,62]. Furthermore, Pagani *et al.* (2016) demonstrated that PIGF contributes to the intrinsic or inborn (acquired) immune response to anti-VEGF-A therapies in melanoma. In turn, NRP-1 and PIGF enhance melanoma progression and metastasis despite VEGFR-1 interaction with PIGF[63-64].

This may indicate a compensatory pro-angiogenic mechanism to elucidate the recurring resistance of melanoma to such anti-angiogenic therapies[41,61,63-64] (Figure 1.7). Both VEGF-A and PIGF promote angiogenesis in part by affecting integrins.

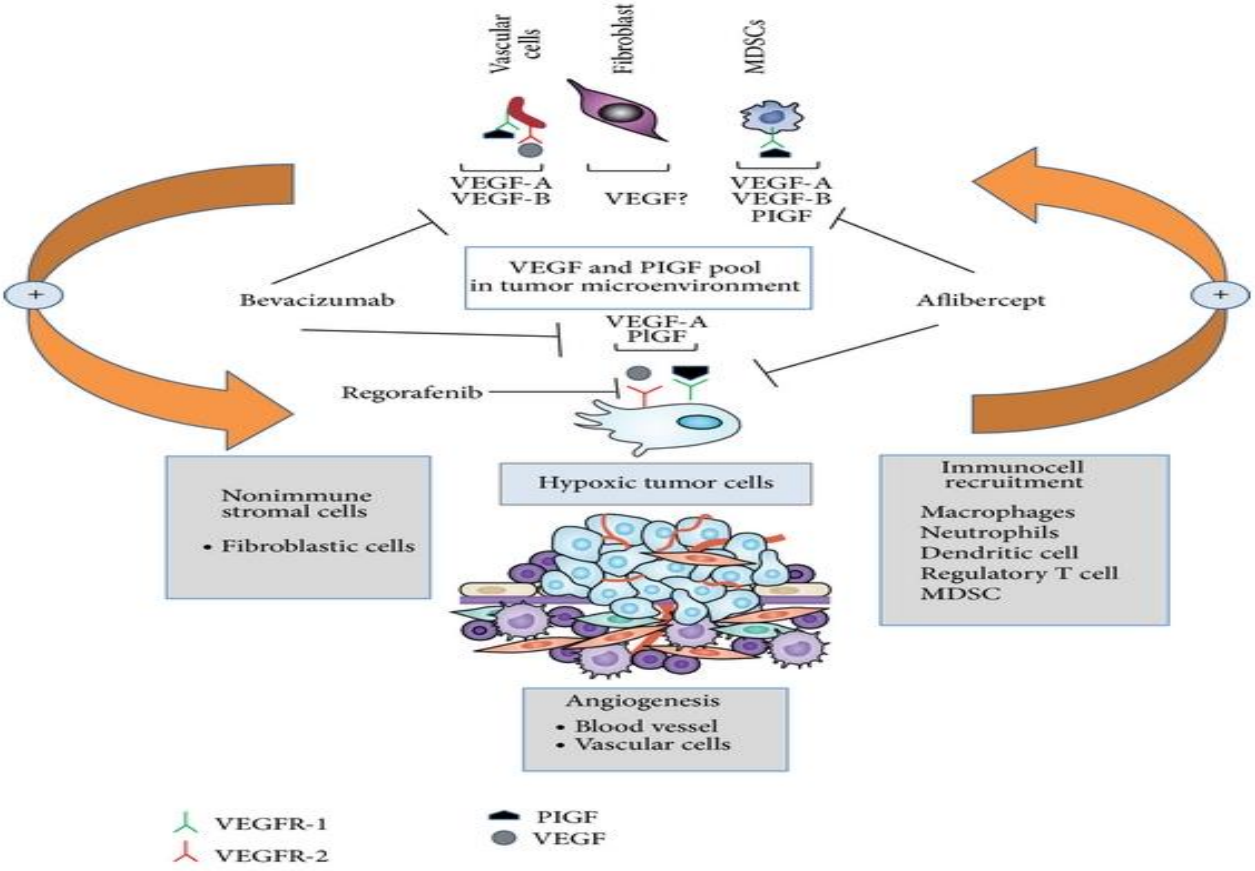


Figure 1.7. The role of PIGF in melanoma angiogenesis. Various factors are secreted due to an imbalance or disruption within the tumour microenvironment which results in malignancy.

Republished with permission from Giordano et al[1-2].

1.3.3.4 Integrins

Integrins are produced by various cell types such as ECs, pericytes, stromal fibroblasts, immune cells and numerous solid tumours, including melanoma[41]. The extracellular matrix generates degraded products such as vitronectin, fibronectin and collagen, enhancing their effects on tumour angiogenesis and invasiveness. To date, various integrin isoforms have been established and are strongly associated with tumour cell adhesion, migration, invasion, metastasis and angiogenesis[41]. Significantly, it has been established that integrin alpha V and integrin beta 3 (CD61) (αvβ3), (integrin alpha V and integrin beta 5 (sCD23 molecule) (αvβ5) and fibronectin receptor or integrin alpha 5 (CD49e) and integrin beta 1 (CD29) (α5β1) in human melanoma elucidate a vital role in neovascularisation and tumour progression via the VEGF-A/VEGF-2 and Ang/Tie signalling pathways[41].

The αvβ3 integrin isoform is the most plentiful and powerful pro-angiogenic of the integrin isoforms. It is involved in the progression of melanoma from the radial growth to the aggressive vertical growth phase[42-43]. It was also established that αvβ3 integrin decreases the size of the tumour spontaneously by causing apoptosis of proliferating angiogenic ECs[41]. In addition, several αvβ3 integrin ligands such as osteopontin, vitronectin and bone sialoprotein[65] modulate VEGF-A and FGF-2 tumour angiogenesis[5,41]. Osteopontin is mostly expressed in human cutaneous melanoma and corresponds with the aggressive, invasive phenotype of melanoma[41,55,66].

The αvβ5 integrin isoform is relative to the αvβ3 integrin isoform. It plays a role in angiogenesis of NRP-1-expressing melanoma cells; hindrance of the αvβ5 integrin isoform results in a remarkable decrease of extracellular matrix invasion, vascular mimicry, VEGF-A and MMP-9

expression by melanoma cells, supporting the pro-angiogenic nature of the αvβ5 integrin isoform[41,65].

The α5β1 integrin isoform is frequently expressed in melanoma. Fibronectin behaves as a ligand for α5β1 integrin that results from the degradation of the extracellular matrix from overexpressed MMPs. The upregulation of the α5β1 isoform was found to enhance MMP-2 and MMP-9 expression in melanoma mouse models to activate the respective ligands needed for malignant transformation of melanocytes[41]. Moreover, the α5β1 integrin isoform modulates the Ang/Tie signalling pathways in melanoma angiogenesis and is essential during the aggressive vertical growth phase of melanoma[5,41].

1.3.4 Current treatments of melanoma and their limitations

The therapeutic aim of melanoma angiogenesis is to enhance blood perfusion to hypoxic tissues or organs or to restore tissue functions and to suppress the uncontrolled angiogenesis that leads to disease conditions such as melanoma. Despite promising results demonstrated by animal experimental studies for pro-angiogenic factors, such results have failed to realise in clinical studies[11,67].

Current therapeutic approaches include surgical removal of the tumour (i.e. early stages), chemotherapy, photodynamic therapy, immunotherapy, biochemotherapy and targeted therapy[11,67-68]. However, the effectiveness of these therapeutic approaches may be determined by a single agent or combined therapies, restricted by the patient's health, stage and site of the tumour. Therefore, these treatments may fail due to resistance to therapy.

Table 1.1. Currently approved drugs in melanoma treatment.

Drug name	Limitations	Reference
Dacarbazine	Adverse effects: Nausea, leukopenia, some toxicities, vomiting, thrombocytopenia.	[69]
IL-2 (Aldesleukin)	Severe toxic effects include hypertension, fever, limited septic shock, nausea, vomiting and diarrhoea.	[70]
Ipilimumab	Adverse side effects of grades 3–4 in 15–56% of cases (pneumonitis, lethal skin rash, abdominal pain). Restricted usage because of high-rated toxicity.	[71]
Peginterferon alfa-2b	Substantial side effects and delay recurrences for some patients.	[72]
Recombinant interferon Alfa-2b	Substantial side effects (toxicities include fever, autoimmune response, chills) and lower lifespan for most patients. Efficiency is quite limited.	[73]
Vemurafenib	Side effects include skin problems, rashes, hair thinning, thick or dry skin, some sensitivity, joint pains, fatigue, nausea, fever and hair curling.	[74]
Dabrafenib	Fewer side effects such as hair thinning, thick or dry skin and some sensitivity. Offset refractoriness in melanoma patients.	[75]
Trametinib	Side effects include acne-like rash, itching, dry skin, inflammation and diarrhoea.	[76]
Pembrolizumab	Substantial side effects of grades 3–4 in 13–18% of patients (i.e. autoimmune response).	[77]
Nivolumab	Adverse side effects of grades 3–4 in 12–14% of patients (i.e. autoimmune response).	[78]
Taumogene Laherparepvec	Mainly flu-like symptoms.	[79]

Over the past years, ten out of 2415 clinical trials (www.clinicaltrials.gov) on melanoma medical therapies globally are currently being investigated in nanotechnology. To date, only ten melanoma medical therapies have been accepted by the United States Food and Drug Administration (US-FDA)[11,80-81] to treat melanoma in the advanced stage. The main available therapies are (1) Checkpoint inhibition immunotherapies: programmed cell death ligand inhibitors (nivolumab, pembrolizumab) as a single agent or combined therapies with anti-cytotoxic T-lymphocytes-associated-protein 4 antibodies (ipilimumab)[81]; (2) Molecular therapies in patients with driven mutations against the mitogen-activated protein kinase pathway, with BRAF (v-raf murine sarcoma viral oncogene homologue B1) and mitogen-activated protein kinase inhibitors (vemurafenib, dabrafenib, encorafenib, trametinib, cobimetinib) in patients harbouring a V600 mutation in the BRAF gene and tyrosine-protein kinase inhibitors (imatinib)[81]; (3) Tumour vaccines[81]; (4) Gene therapy: locoregional immunotherapy with oncolytic viruses (talimogene laherparepvec-T-VEC); (5) T-cell-directed therapies[81]; and (6) Additional current therapies: radiotherapy, cytotoxic therapy, photothermal therapy, photodynamic therapy, electrochemotherapy and others[81] (Table 1.1).

However, many challenges have emerged due to the adverse effects of these therapies, which have hindered their efficiency in clinical settings. Melanoma remains a highly metastatic tumour with high resistance to treatment[81]. These adverse effects include morbidities such as bleeding, leukopenia, hypertension and lymphopenia[11]. The current anti-angiogenic drugs are therefore not effective in these tumours due to their toxicity and the development of resistance. Furthermore, angiogenesis inhibitors have insufficient evidence and standardised biomarkers for demonstrating their efficiency, which is another therapeutic limitation[11,68].

Targeting pathological angiogenesis has been an advance in the use of nanotechnology in biological systems as it is developing rapidly. Nanoparticles are being applied in biomedicine in various ways[82] to enhance cancer diagnosis and therapeutic efficiency. Gold nanoparticles (AuNPs) are becoming optimistic contrast agents, photothermal agents, drug delivery systems and radiosensitisers[11,68].

1.4 Nanotechnology and medicine

The exact mechanism of action for melanoma has not been clearly established entirely, therefore treating this disease is often quite challenging due to frequent resistant to treatment. In order to overcome this situation, innovative therapies are constantly being applied in drug delivery systems using nanotechnology. Nanotechnology is one of the best-studied fields by researchers and most scientists with extreme promising treatments in tissue engineering and biomedical applications[83]. Nanostructures are potentially useful because of their unique inherent properties which include features such as a large surface area to volume ratio, fiber diameter, stability, hydrophilicity, permeability and porosity. In the past decades, researchers have developed various strategic therapeutics to target and eliminate tumour cells in clinical settings. Nanotechnology in medicine has been constantly utilised in imaging and drug delivery systems with the potential aim of diagnostic and prognosis of melanoma treatment[83-84].

Nanoparticles are being largely applied in medicine to potentiate drug delivery systems for tumour vasculature. These particles have the ability to bind the polymer in tumour cell membrane, to nuclear or cytoplasmic receptor sites allowing them to decrease the toxicity in normal tissue[83]. Moreover, this will enable an increase in the drug concentration focused on targeted cells[36,83].

In nanotechnology, enhanced permeability and retention is essential for drug delivery systems to the targeted areas in clinical settings. This works by suppressing the lymphatic filtration when molecules have invaded the interstitial tumour area making it feasible to keep the molecules in tumour cells. Numerous types of pore dimensions at the vascularity has the capability to interfere with the enhanced permeability and retention, tumour localisation as well as the size and shape of the tumour. The tumour vasculature is normally optimised by drug delivery, the extracellular matrix and lymphatic are accomplished small particle sizes at a nanoscale ranging at 20 nm in diameter[6,83]. Newly engineered biomaterials are essential to be established by scientists and researchers with the potential to develop novel agents in drug delivery systems with an understanding of melanoma skin cancer functions, biological information as well as drug kinetics[6,85-88].

1.4.1 Nanoparticles in melanoma cancer delivery

Nanoparticles can be defined as particle atoms ranging from 1-100 nm[83]. For drug delivery systems to be initiated at targeted areas in clinical settings, this system is accomplished at three sites namely, opened hair follicles, stratum corneum surface and lastly by the furrows[83]. These nanoparticles have the ability to interact with nanoparticles at the skin in an adjuvant fashion. They efficiently deliver therapeutic agents to the tumour site such as chemotherapeutics, immune and vaccine molecules as well as nucleic acids. Nanosystems are elucidated to overcome biological barriers and to target drug delivery to malignant site in clinical settings, thus allowing the usage of drugs in lower doses[68]. Additionally, this can enhance the efficiency of the drug to the tumour site by exhibiting any side effects.

Most nanotechnology and Nanosystems approaches are limited due to being in the developmental phase of research. However, a majority of these have been implemented in the clinical stage and are recently available in pharmaceutical markets. Currently, no commercial nanosystems are available for bioactive molecules to drug delivery systems for skin cancer[9]. Research is currently being elucidated to improve some nanoparticle applications and antitumour agents being investigated in clinical trials for stubborn solid tumours including melanomas as well as nonmelanoma skin cancer[9].

1.4.2 Cancer immunotherapy

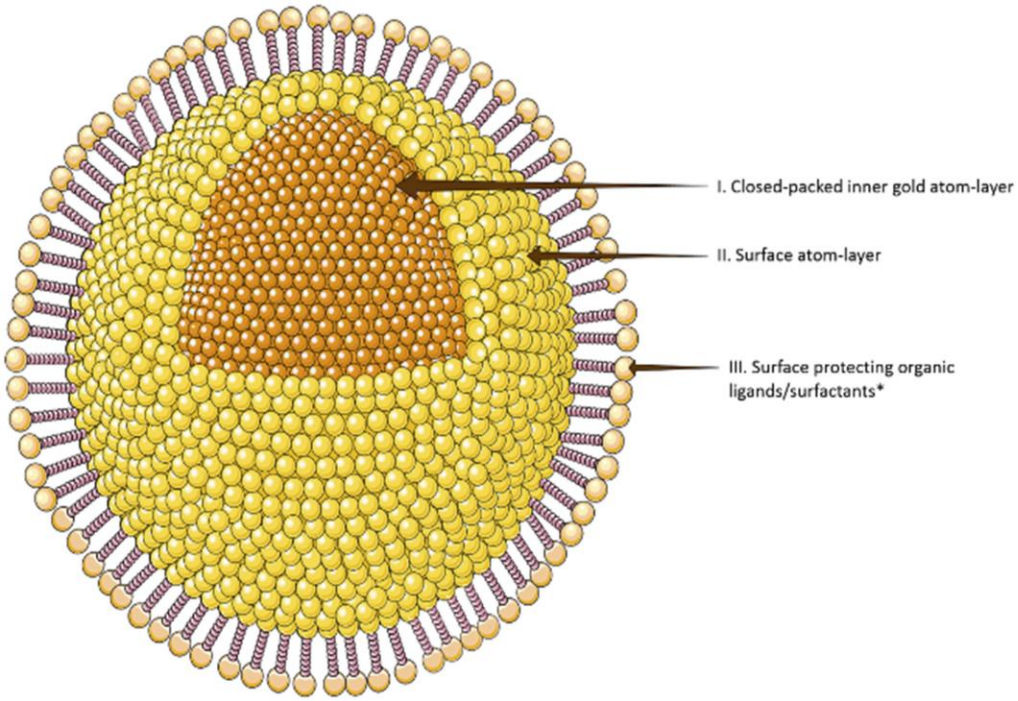
Nanoparticles are being initiated to various cells and tissues interrelated to an immune response involving antigen-presenting cells, lymphocytes and lymphoid tissues with an aim of overcoming restrictions to the direct targeting of tumour cells[9,81]. Moreover, this phenomenon allows nanoparticles to initiate several adjuvant therapies in treating various cancers, including melanomas[81].

Nanosystems and nanotechnology used in combination with physical techniques, nanocarriers can potentiate topical and transdermal drug delivery in melanoma treatment[9]. Clinical physical methods to enhance melanoma treatment includes laser irradiation such as thermotherapy, phototherapy and acoustic therapy. Moreover, additional methods include iontophoresis, ultrasound and microneedling systems[9]. Nanotechnology serves a promising strategy to overcome frequent resistant to drug treatment to help clinicians to better understand an efficient direct approach to melanoma treatment with extremely lower drug doses eliminating the possibility of toxic effects[9].

Nanocarriers in biomedical applications help potentiate the effect of various therapeutic agents synthesised from a combination of molecules aimed to act synergistically against various cancer types including melanomas that are resistant to treatment as well as unresponsive to conventional therapy[9]. Nanoparticles used in combination with high-cost therapies such as immunotherapy and gene therapy potentiates an effective treatment plan which can lead to a more cost-effective approach and enhanced clinical outcome[9]. Therefore, in this study gold nanoparticles will be implemented in the treatment of melanoma cells.

1.5 Gold nanoparticles in cancer

Gold nanoparticles (AuNPs) are colloidal suspensions consisting of gold particles with a diameter of 1–100 nm[89]. They have unique physiochemical properties, establishing their advancement in nanotechnology. Their size and shape allow them to form clusters and plasmonic crystals with different nanostructures[11] (Figure 1.8), which can be overcome by encapsulation with citrate coating[90]. Gold nanoparticles are aimed at cancer therapy to localise the primary tumour or metastases as well as to quantify the tumour size. The AuNPs have a capability to travel within the human body in search of all the cancerous cells while sparing healthy cells[91]. However, none of this imaging approaches have yet been validated for the purpose of clinical imaging of AuNPs. The structure of AuNP consists of a monolayer-protected cluster (MPC) with an inner core and an outer surface composed of gold atoms as well as a protective outer layer of surfactants[11] (Figure 1.8).



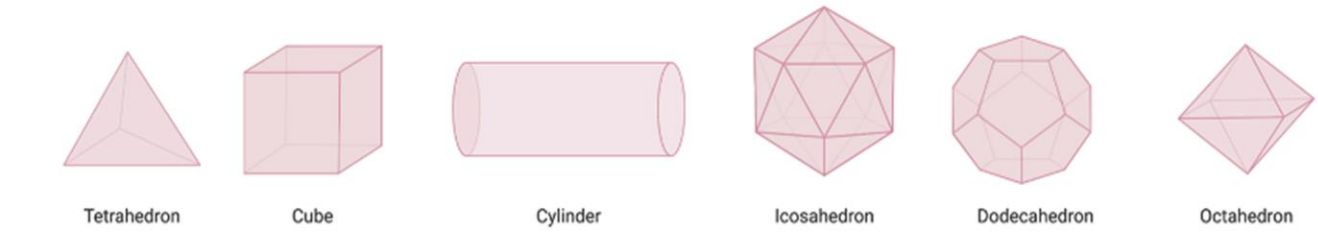


Figure 1.8. Schematic diagram of a single crystalline gold nanoparticle illustrating different nanostructures. The inner gold nanoparticle (AuNP) atom is stable and non-toxic. Depending on the composition of AuNPs, various ligands could functionalise the nanoparticle surface and facilitate their properties.

Republished with permission from Darweesh et al[11].

Gold nanoparticles can be synthesised by various methods such as citrate reduction by Turkevitch *et al*., uses tetrachloroauric acid ($HAuCl_4$) in combination with trisodium citrate as a reducing and capping agent for nanoparticles[92]. In contrast to Liu and Lu with slight changes, uses trisodium citrate and organic solvents in the aqueous phase [93], which enables their easy preparation and use. On a nanoscale, AuNPs have good biocompatibility and low cytotoxicity. These characteristics make them more desirable for application in biomedicine[17]. Applications such as gene and drug delivery, labelling, bioimaging for cancer treatment, and many others enhance AuNPs' capabilities.

1.5.1 Gold nanoparticles and angiogenesis

Several studies have shown the anti-angiogenic outcome of AuNPs with decreased viability, migration and tube formation of human umbilical vein endothelial cells (HUVECs)[11,82]. Moreover, the effects of VEGFR-2 inhibition in HUVECs were dose-dependent[11,81,94-95] and to downregulate Ang-1 and -2 expression in tumour cells[81]. Gold nanoparticles were found to inhibit VEGF-A-induced migration of human retinal microvascular ECs *in vivo*[11] .In the chick chorioallantoic membrane model, AuNPs inhibited the formation of new vasculature and reduced tube formation. Furthermore, in a study using a nude mouse ear model, the administration of AuNPs was found to suppress the angiogenic process[81].

1.6 Problem statement

Melanoma is a highly metastatic tumour and the angiogenic process is a key step in melanoma progression[96]. However, current anti-angiogenic drugs are not effective in these tumours due to their toxicity and the development of resistance. Indeed, resistance to anti-VEGF therapy seems to contribute to the activation of other stimulators of vascularisation such as FGF-2. Therefore,

compounds that target more than one angiogenic factor and selectively kill cancer cells are needed. If AuNPs are cytotoxic to melanoma cells and effective in inhibiting angiogenesis, they could have potential in melanoma treatment.

1.7 Aim and objectives

Aim

To investigate the effects of AuNPs on the growth of melanoma cells and angiogenesis (endothelial cell growth and migration) as well as angiogenesis promoting proteins.

Objectives:

- To determine the cytotoxic effects of AuNPs on melanoma cells using the crystal violet assay.
- To determine the effects of AuNPs on endothelial cell growth using the crystal violet assay.
- To determine the effects of AuNPs on endothelial cell morphology using light microscopy.
- To determine the effects of AuNPs on endothelial cell migration using the scratch assay.
- To determine the effects of AuNPs on the levels of angiogenesis promoting proteins, VEGF-A/PIGF using the enzyme-linked immunosorbent assay.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study design

This study was carried out using two cell lines; mouse endothelial (sEnd.2) cells and mouse melanoma (B16-F10) cells. The study design is outlined in the flow diagram (Figure 2.1).

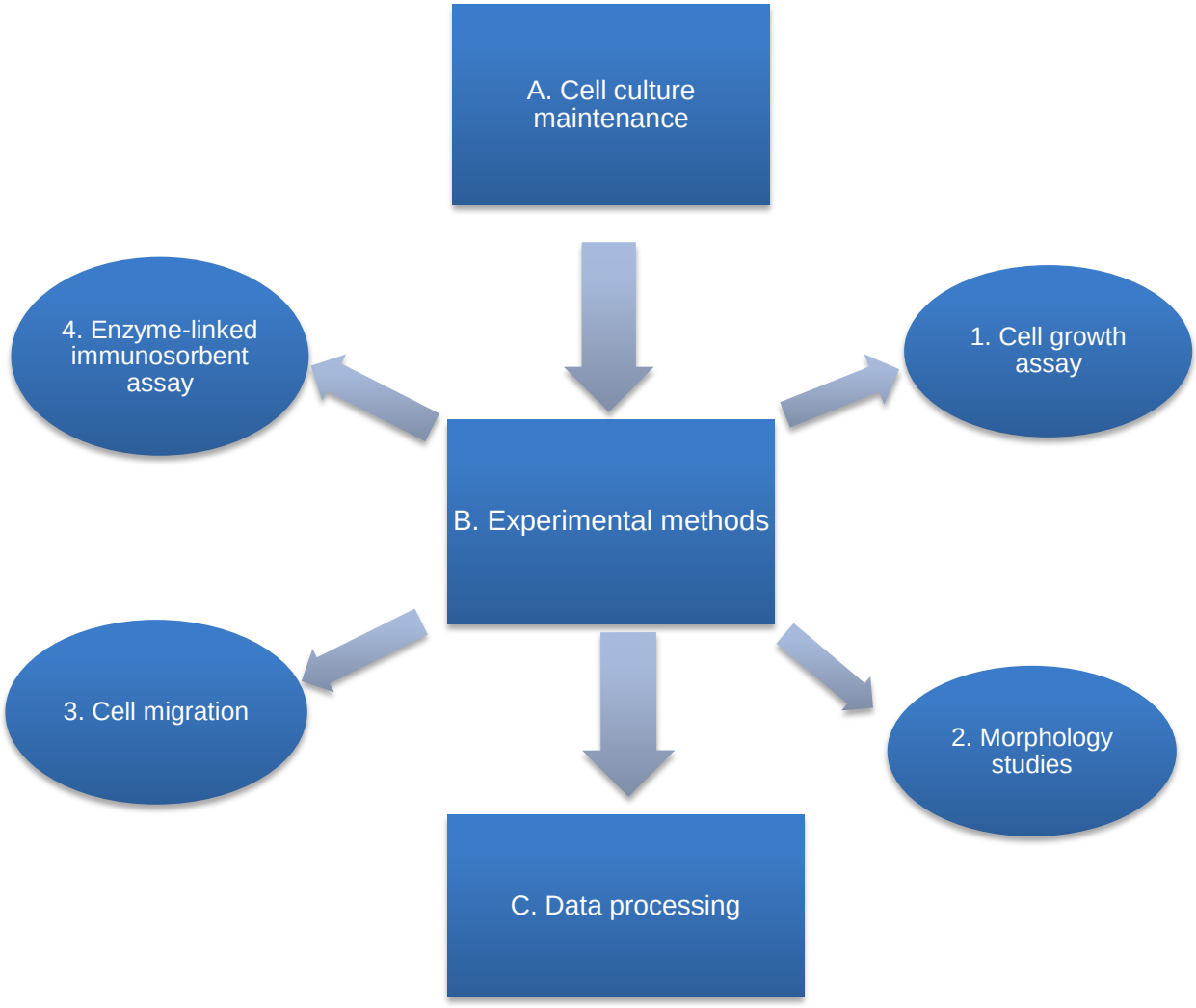


Figure 2.1. Flow diagram illustrating the study design.

2.2 Cell culture maintenance procedure

The sEnd.2 (obtained from Prof M.S. Pepper, University of Pretoria) and melanoma B16-F10 cells (purchased from the ATCC) were maintained in flasks (area 25 cm^2) in a humidified atmosphere containing 5% CO_2 at a temperature of 37°C. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, United States of America), supplemented with 10% foetal bovine serum (FBS) (Scientific Group, Midrand, South Africa), 2 mM L-glutamine and 1% penicillin-streptomycin (Whitehead Scientific, Johannesburg, South Africa).

2.3 Subculturing

All cell culture reagents (trypsin, PBS and medium) were prewarmed in a 37°C water bath before subculturing. When cells were confluent, they were split using a (1:1) trypsin dilution. After 30 minutes, PBS was discarded and 2 mL (1:1) trypsin was added and incubated while cells were monitored for detachment after 5 to 10 minutes. Gentle hitting of the flask on the sides was used for cells that did not detach easily. After cells had detached, the mixture was transferred into a 15 mL centrifuge tube and centrifuged (Thermo Scientific, Johannesburg, South Africa) for 10 minutes at 2000 rpm at room temperature. The supernatant was discarded, and the pellet was kept for cell counting.

2.4 Cell counting

Principal

Cell counting in cell culture is an essential application that uses cell suspensions necessary to determine the concentration of cells in a given sample. The cell suspensions are counted by

employing a coverslip designed for counting cell types and other microscopic particles[97]. The coverslip is loaded onto a hemocytometer and cells are then counted within the (4 × 4) gridlines.

Procedure

The pellet was gently resuspended in 1 mL DMEM, then 20 µL cell suspension was transferred into a 2 mL Eppendorf tube. Depending on the pellet size, the number of viable cells were counted by transferring the cell suspension and trypan-blue onto a clean coverslip. When the cell suspension was too concentrated, PBS was used for dilution. The cell density was calculated using the following equation:

$$\text{Cell density (cell/mL)} = \frac{\text{Total number of viable cells}}{\text{Number of squares (4)}} \times \text{Dilution factor} \times 10^4$$

A coverslip was put in place and a small amount of the trypan blue-cell suspension was pipetted to fill the chamber on a haemocytometer.

2.5 Cell growth assay

Principle

Crystal violet dye binds to proteins and DNA[98]. Crystal violet may be used to detect viable cells that maintain adherence in a cell culture plate[98]. This is a valid, easy and rapid procedure to access cell viability[99]. In this study, crystal violet was employed to determine the effects of AuNPs on melanoma and EC viability.

Procedure

In a 96-well plate (Lasec, Cape Town, South Africa), 5 000 cells were seeded in each well. After 24 hours the medium was removed and fresh medium containing 30 ng/mL VEGF-A and AuNPs (1.2-3.2 nM) was added and IC_{50} concentration that was determined (IC_{50} = 3,5135 nM). Control cells were treated with 0.9% saline instead of AuNPs. After 24 hours the medium with AuNPs or saline was removed and 100 µL of 1% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, United States of America) was added to each well. After 30 minutes 100 µL of 0.1% crystal violet solution was added for 15 minutes and thereafter the plate was rinsed in running tap water for 15 minutes. The plate was air-dried thoroughly and 100 µL of 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, United States of America) added. The absorbance was read at 570 nm on an ELx 800 Universal Microplate (BioTek instruments Inc, Weltevreden, South Africa).

2.6 Morphology studies

Principle

Polarisation-optical interference contrast (PlasDIC) is a new polarisation-optical transmitted light differential interference contrast technique. Unlike conventional DIC, linearly polarised light is only generated after the objective[100]. Polarisation-optical interference contrast provides high-quality imaging of cells to observe the morphological characteristics specific for types of cell death which can be easily recognised[100]. Endothelial cells morphological characteristics were observed using PlasDIC to determine EC morphology.

Procedure

Endothelial cells were seeded in a 6-well plate (Sigma-Aldrich, St. Louis, MO, United States of America) on heat-sterilised coverslips and cultured overnight. The cells were treated for 24 hours with AuNPs at IC_{50} concentration that was determined (IC_{50} = 3,5135 nM). The control was treated with 0.9% saline. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours, the cells were fixed in 3.7% paraformaldehyde and washed with phosphate-buffered saline (PBS). Images were then visualised using light microscopy and images were captured using a Zeiss AxioCam (Thermo Fisher Scientific, Johannesburg, South Africa). The cells were cultured in a 6-well plate and treated for 22 hours with 3.2 nM AuNPs. The cells were viewed using a Zeiss Axiovert CFL 40 microscope and PlasDIC images were captured using a Zeiss Axiovert MRm digital camera (Zeiss, Oberkochen, Germany) at 40× magnification.

2.7 Cell migration

Principle

The scratch assay is employed to study cell migration *in vitro*, which is the directional movement of cells from one area to another following a chemical signal in the microenvironment[101]. A scratch is created in a monolayer of cells, and the cells that migrate into the artificially created wound are counted as migrated cells[101].

Procedure

Endothelial cells were seeded into 100 mm × 20 mm culture dishes pre-treated with 1% gelatin in PBS at a density of 1,2 x 10^6 cells/dish for 24 hours. A monolayer was gently scratched using

a sterile 1 mL pipette tip across the centre of the dish, taking care that the long-axial part of the tip was perpendicular to the bottom of the well throughout. The straight line was scratched in one direction. Cells were washed twice with 1x PBS to remove cells that were detached while scratching. Culture dishes were replenished with fresh medium containing AuNPs (1.6 nM and 3.2 nM) or 0.9% saline, and the dishes were incubated at 37°C for 22 hours. Images were captured using a camera (Olympus Corporation, Shinjuku, Tokyo, Japan), attached to a light microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) at 0, 6 and 22 hours. The number of cells that migrated into the wound were calculated for control and treated cultures at the three intervals. Three dishes were analysed per drug concentration and the experiment was done in triplicate for a sample size of 9.

2.8 Enzyme-linked immunosorbent assay

Principle

The enzyme-linked immunosorbent (ELISA) assay, a plate-based method designed to detect and quantify antigen-antibody reactions[102]. An antigen is preserved to a solid surface which will be complexed with an antibody linked to an enzyme. In this study, the human vascular endothelial growth factor-A/placenta growth factor (VEGF-A/PIGF) heterodimer assay is a solid-phase ELISA employed to the levels of angiogenesis promoting proteins, VEGF-A and PIGF monomers[103] (Figure 2.2).

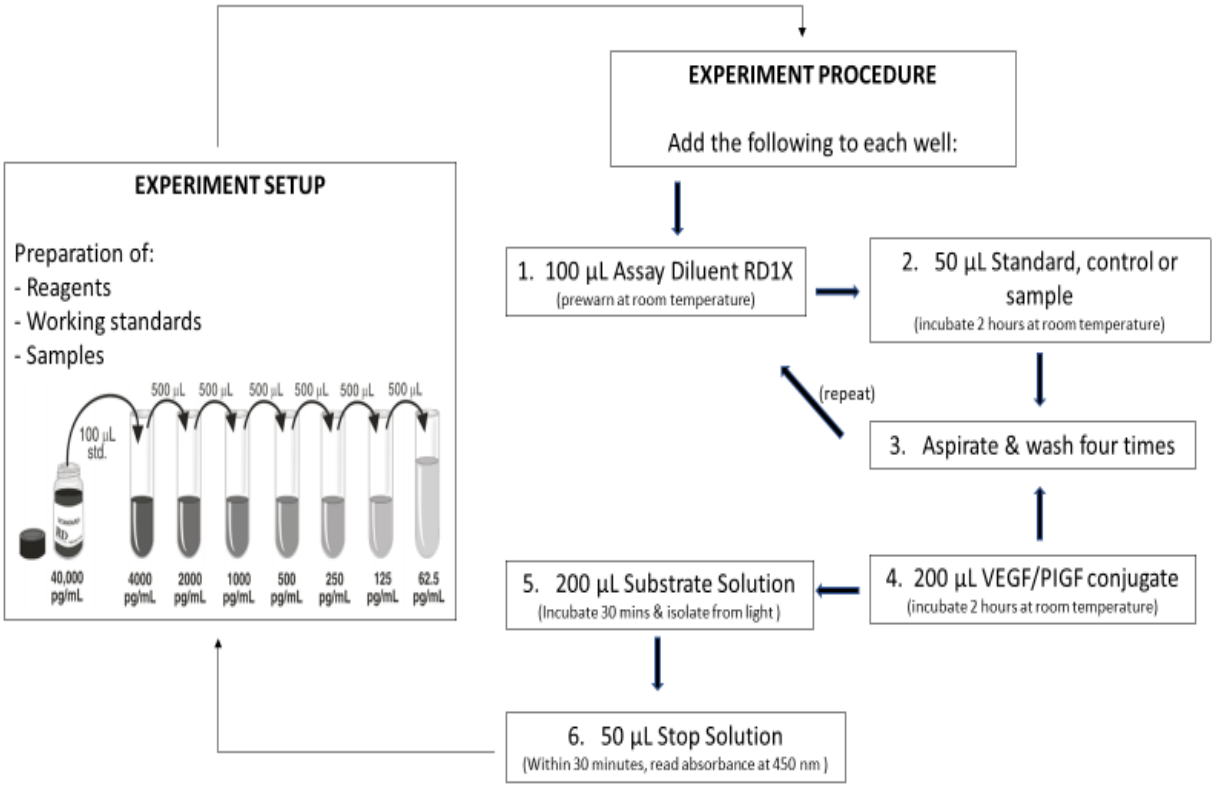


Figure 2.2. Flow diagram illustrating the assay procedure for quantification and determination on the levels of angiogenesis proteins. Figure drawn by LJ Matutule using Microsoft PowerPoint 2016.

Procedure

In a 96-well plate (Lasec, Cape Town, South Africa), 5 000 cells were seeded in each well. A sandwich enzyme immunoassay was performed using the Quantikine ELISA (R&D Systems, Inc., United States of America) according to the manufacturer's instructions (Figure 2.2).

Briefly, a monoclonal antibody specific for VEGF-A or PIGF and samples were added onto a 96-well microplate. The heterodimers were measured by crossmatching capture and detection

antibodies using the same ELISA kits. Excess microplate strips from the plate frame were returned to the foil pouch containing the desiccant pack and resealed.

An Assay Diluent RD1X was prewarmed at room temperature and 100 µL was added to each well and thoroughly mixed before and during use. This was followed by 50 µL standard, control or sample being added to each well (covered with adhesive strip) and incubated for 2 hours at room temperature. Each well was aspirated and washed three times with 400 µL Wash Buffer using a squirt bottle (total of four washes). After the last wash, the remaining Wash Buffer was aspirated off by inverting the plate and blotting it against a clean paper towel. Thereafter, 200 µL VEGF-A or PIGF conjugate was added to each well, covered with a new adhesive strip and incubated for 2 hours at room temperature (washing step was repeated). Substrate solution (200 µL) was added to each well and incubated for 30 minutes at room temperature without exposing this solution to light. Thereafter, 50 µL Stop solution was added to each well and the colour in the wells changed from blue to yellow. The optical density of each well was determined within 30 minutes by reading the absorbance at 570 nm on an ELx Microplate (BioTek instruments Inc, Weltevreden, South Africa).

2.9 Data analysis

Data analysis was conducted in consultation with Prof Becker, an expert in the field of Biostatistician of the Medical Research Council. This experiment with three replicates was conducted in a three-factor design, i.e. cancer at two levels, AuNPs at four concentrations and times in three different incubation periods. By convention, an adequate sample size will provide at least 30 residual degrees of freedom (df). For this 2×4×3 factorial design with three replicates, the residual df comes to exactly 54 if third-order interaction is excluded and 48 if included. The sample size for each concentration level was 9.

For the cell line, sEnd.2 and B16-F10 cells, viability and migration data were described using the mean, standard deviation and 95% confidence interval over concentration levels, over time points and by concentration-time combination.

The data was laid out in a three-factor design and was analysed using a three-factor analysis of variance (ANOVA) with replicates. For the factors AuNPs and time, there were four levels each and when a factor(s) in significant particular differences between levels were of interest. For the post hoc pair-wise comparison, use was made of the regression form of the analysis number, employing the margins command in Stata Release 15. Testing was done at the 0.05 level of significance. Furthermore, the angiogenic marker expression outcomes were dealt with descriptively.

CHAPTER 3: RESULTS

3.1 Nanoparticle characterisation using transmission electron microscope

The transmission electron microscope images of (14 nm, 20 nm and 40 nm) samples were spherical and consistent as expected for each AuNP (Figures 3.1(a)–(c)). The sizes of AuNPs described in this experiment were verified by a transmission electron microscope (TEM). The results (Figures 3.1(a)–(c)) revealed the uniform size of the well-formed spherical shape and in smooth surface of the sample with non-aggregated AuNPs. Results also demonstrated the spherically shaped and slightly larger cell sizes of the (Figure 3.1(b) samples with aggregated AuNPs. (Figure 3.1(c) revealed hybrid silica particles with slightly larger porous spheres and an increased surface area in the sample.

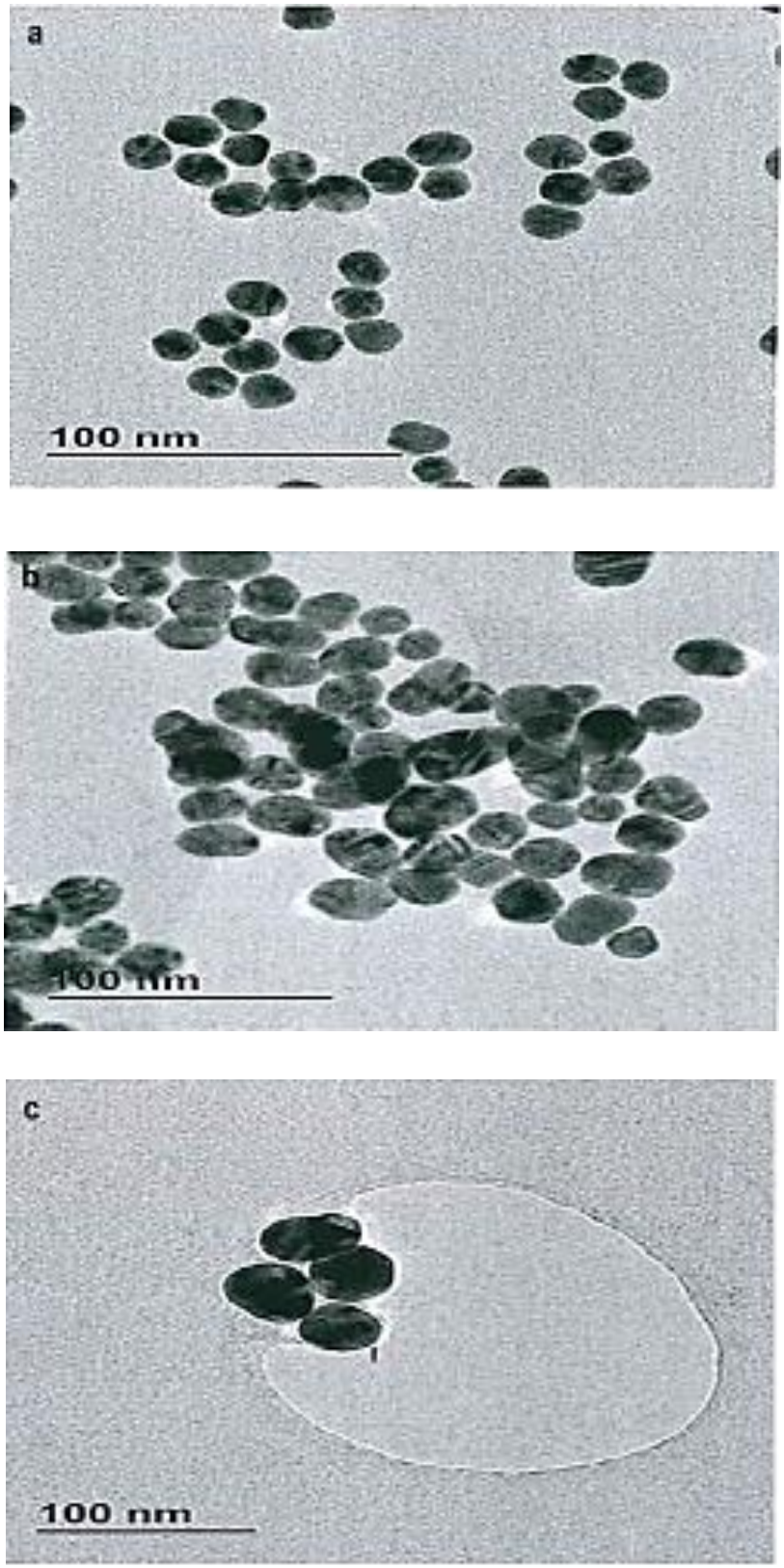


Figure 3.1. Transmission electron microscope images of nanoparticles at 100 nm scale. (a) 14 nm sample (b) 20 nm sample and (c) 40 nm sample.

The 14 nm gold nanoparticles were employed in this study because they did not aggregate in solution (PBS). The effects of the AuNPs were first tested at various concentrations using crystal violet assay and from the data, the IC_{50} concentration was calculated (IC_{50} = 3,5135 nM) and then employed in further investigations.

3.2 Cell viability assay

The tested concentrations of AuNPs were shown in both cell lines and cell viability indicated as a percentage of the control (100%). Minor cell growth inhibition was noted after 24 hours of exposure with AuNPs in the B16-F10 cell line, although the degree of inhibition is not statistically significant (Figure 3.2A); however, ECs cell viability decreased to (58%, $p < 0.05$) at 2.4 nM AuNPs and (46%, $p < 0.05$) at 3.2 nM AuNPs when compared to the vehicle control (Figure 3.3A). Following 48 hours of exposure, although AuNPs significantly decreased the percentage of viable cells, no significant change was observed in cell viability at $P < 0.05$ of B16-F10 cell line (Figure 3.2B). It was further noted that AuNPs induced a more significant reduction in the percentage of cell viability on EC growth to 70% at 1.2 nM AuNPs, 65% at 2.4 nM AuNPs and 40% at 3.2 nM AuNPs respectively (Figure 3.3B) when compared to the vehicle control (Figure 3.2B). This implies that increasing the concentration of AuNPs will further induce a decrease in cell viability.

Viability of B16-F10 cells

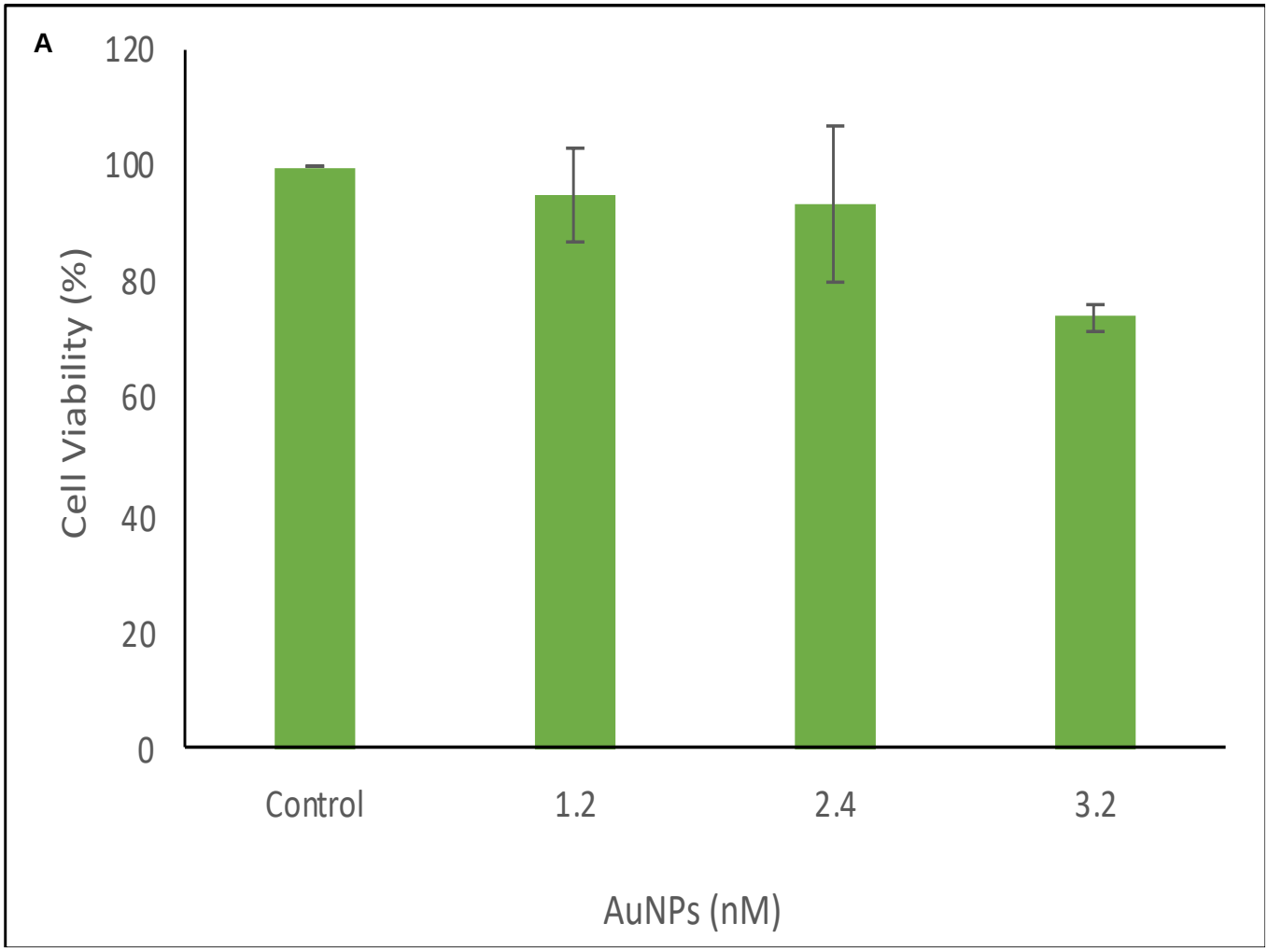


Figure 3.2A. Effects of AuNPs on B16-F10 cell viability. (A) 24 hours after exposure. There was no significant change in cell viability following AuNP treatment at the designated level of significance or $P<0.05$.

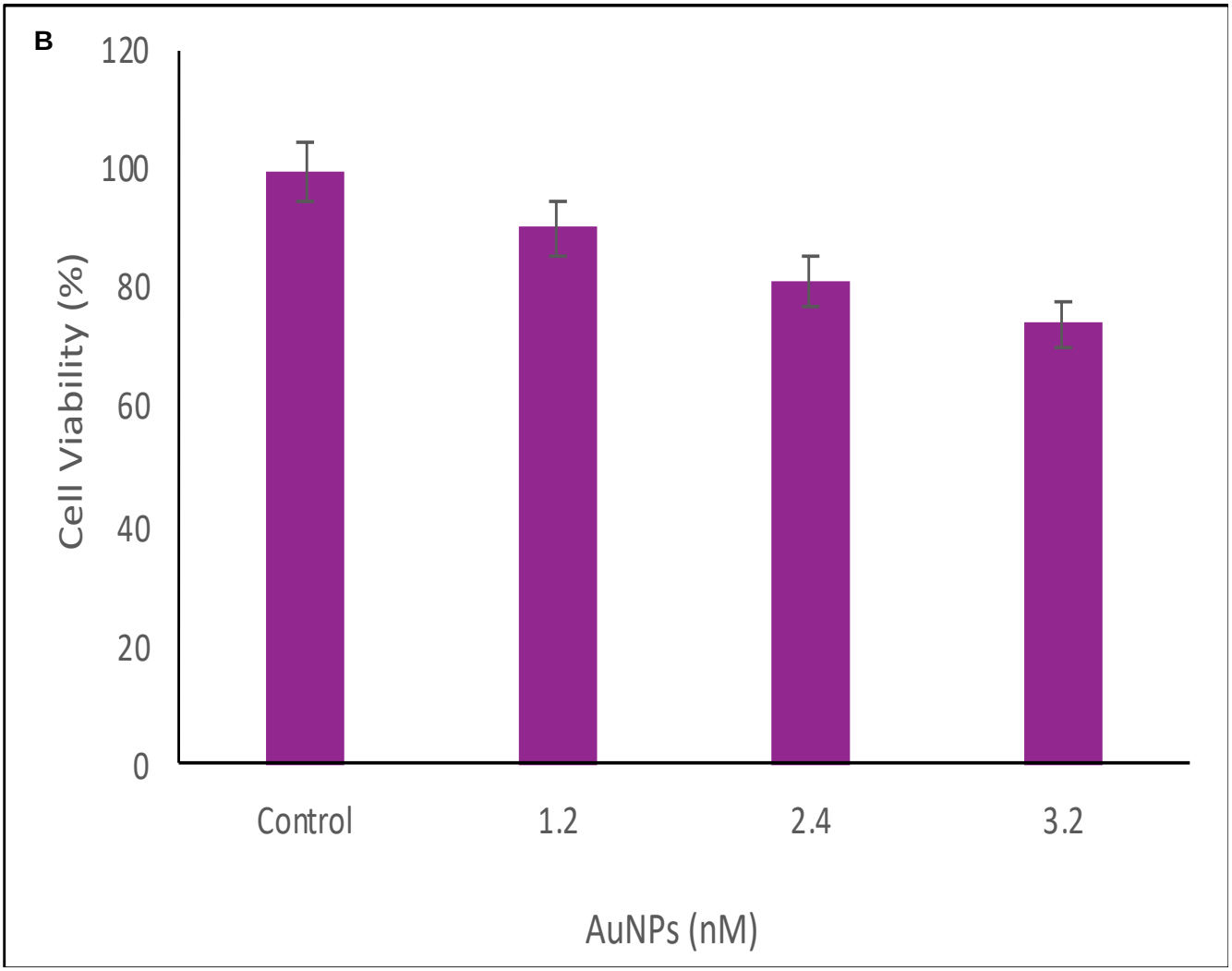


Figure 3.2B. Effects of AuNPs on B16-F10 cell viability. (B) 48 hours after exposure. Although AuNP treatment decreased the percentage of viable cells, no significant change was observed in cell viability at $P<0.05$.

Viability of endothelial cells

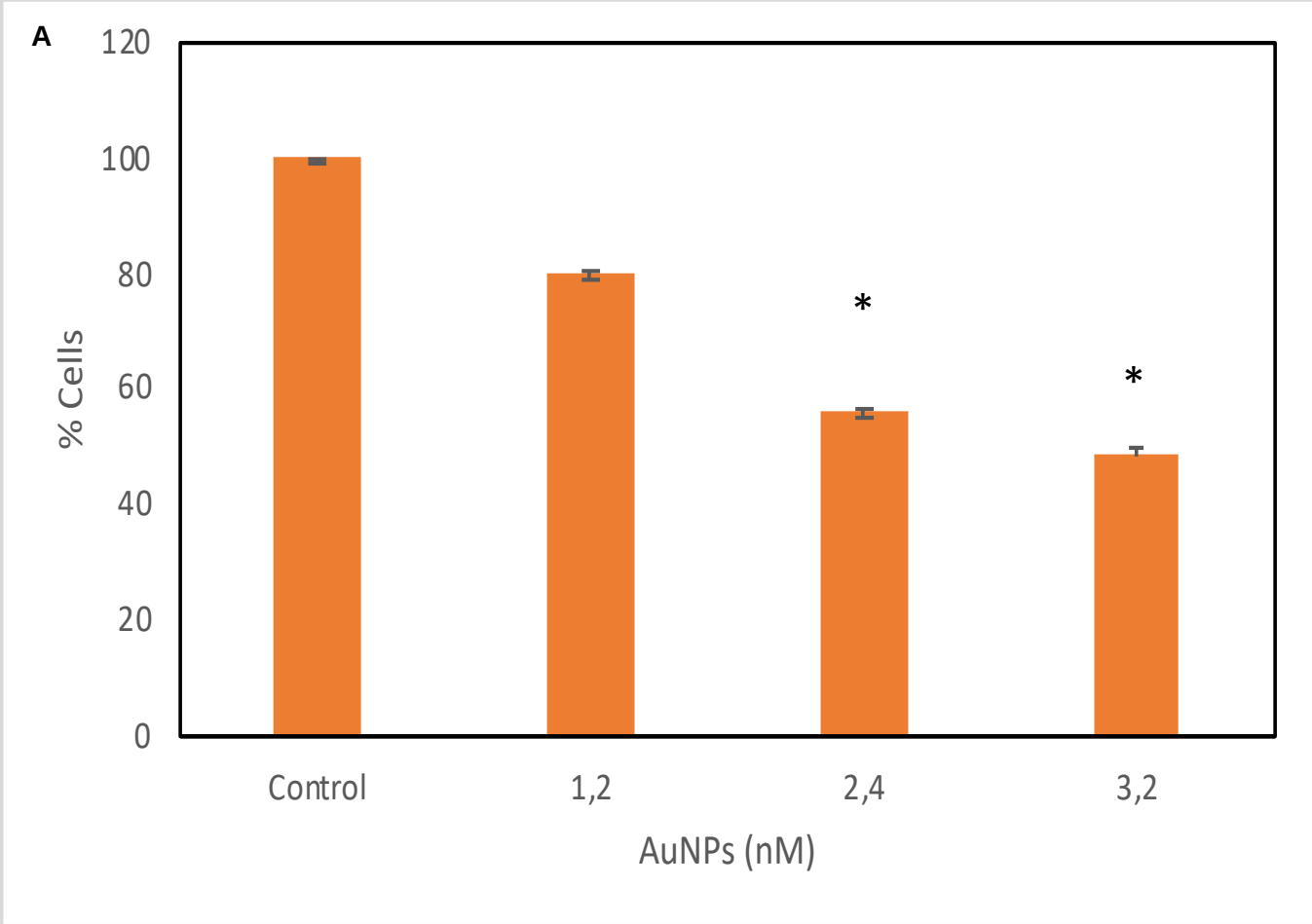


Figure 3.3A. Effects of AuNPs on endothelial cell viability. (A) 24 hours after exposure. Values are mean ± SD. * indicates significant difference ($p < 0.05$) compared to control.

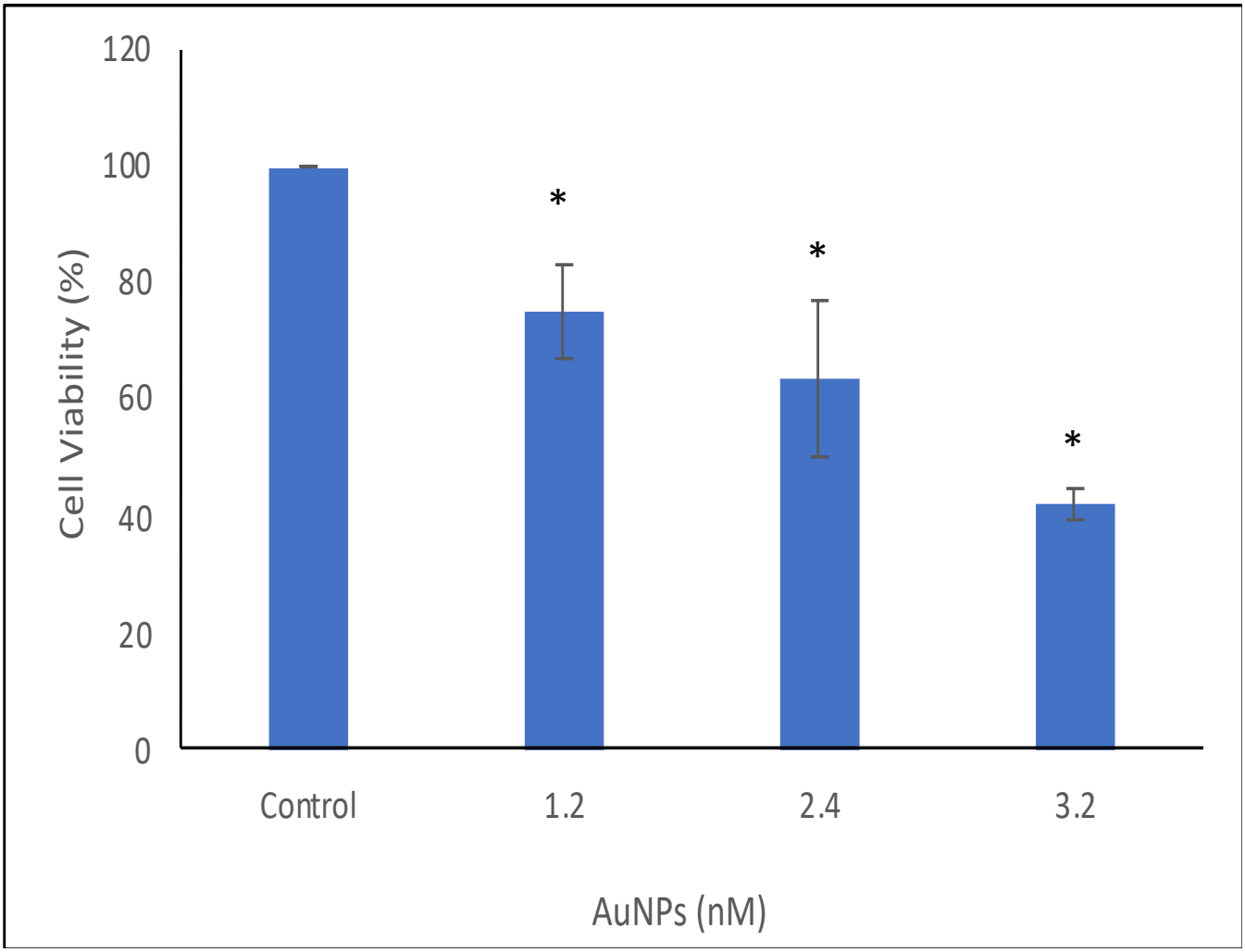


Figure 3.3B. Effects of AuNPs on endothelial cell viability. (B) 48 hours after exposure. Values are mean ± SD. * indicates significant difference ($p < 0.05$) compared to control.

3.3 Morphology studies

Polarisation-optical interference contrast was employed to determine the effects of AuNPs at a concentration of 3.2 nM in EC morphology after 0, 6 and 22 hours of exposure. Initial morphological studies at IC_{50} concentrations revealed a decrease in cell density of treated cells compared to control. The concentration was increased to 3.2 nM which was the highest concentration at which an effect on cell viability was observed.

At 0 hours when compared to control (Figure 3.4 A), cells were not displaying any characteristics of distress, they appeared confluent with a detectable nucleolus (40× magnification) (Figure 3.4 B) indicating normal nuclear morphology. In the control cultures, cells were observed in interphase and prophase (Figure 3.4 E). Moreover, cells had a higher density, indicating possible cell proliferation.

Following 6 hours of exposure to AuNPs, when treated cells were compared to the control (Figure 3.4 C), few noticeable changes were observed for both control and AuNPs-treated cells.Cells still had a confluent monolayer and displayed cells in interphase (Figure 3.4 D). However, after 22 hours of exposure to AuNPs, morphological alterations were observed in treated cells; the cell density was compromised (decreased cell density) with some cells appearing round with denser nuclear chromatin fragments characterising cells blocked in metaphase (Figure 3.4 F) when compared to the control (Figure 3.4 E). Decreased cell density revealed the ability of the concentration of 3.2 nM AuNPs to inhibit cell growth and the presence of structures that looked like apoptotic bodies, which may indicate the ability of the compound to induce cell death (Figure 3.4 F).

Control 3.2 nM AuNPs

0 hours

6 hours

22 hours

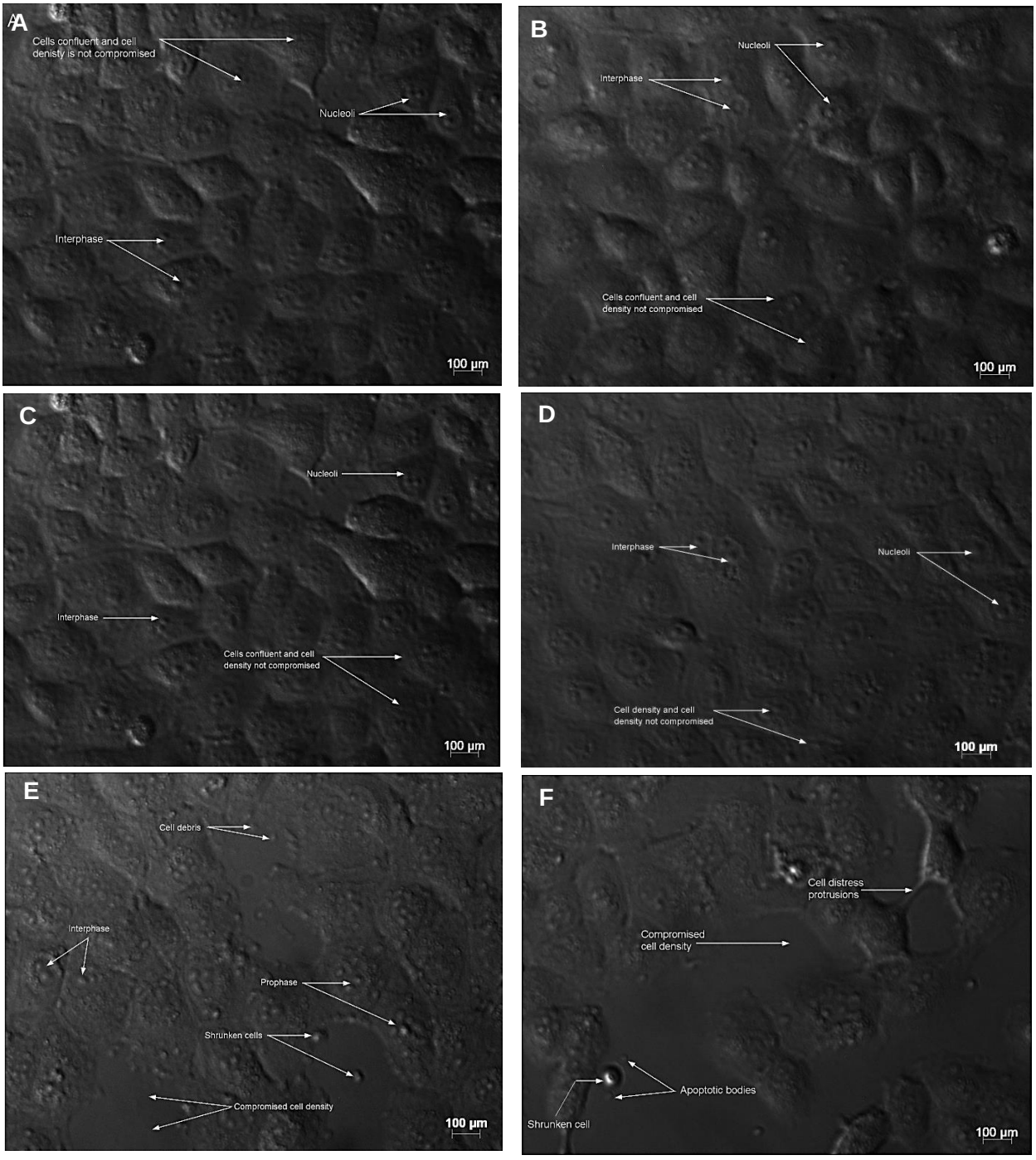


Figure 3.4. Morphology images of endothelial cells. A, C and E represent control cells while B, D and F represent 3.2 nM AuNPs after 0, 6 and 22 hours of exposure. Magnification = 40×. Arrow lines represents annotations of different morphological characteristics. Scale bar at 100 µm.

3.4 Cell migration

The effects of AuNPs on EC migration were evaluated in a two-dimensional assay by measuring the number of migrated cells to the artificially created wound/scratch in a confluent monolayer. ImageJ software was used to analyse data related to the migration process such as wound area, healing speed and the spreading rate (cells migrating to the scratch area).

At 0 hours the size of the scratch was similar in control, 1.6 nM AuNPs and 3.2 nM AuNPs-treated cultures (Figure 3.5A). Following 6 hours of exposure, there was a slight inhibition of migration in 1.6 nM AuNPs and 3.2 nM AuNPs-treated cultures compared to the control. After 22 hours, control cells had migrated to the 'wound space'. Very few cells migrated to the scratch area causing minimal inhibition to the artificially created wound area.

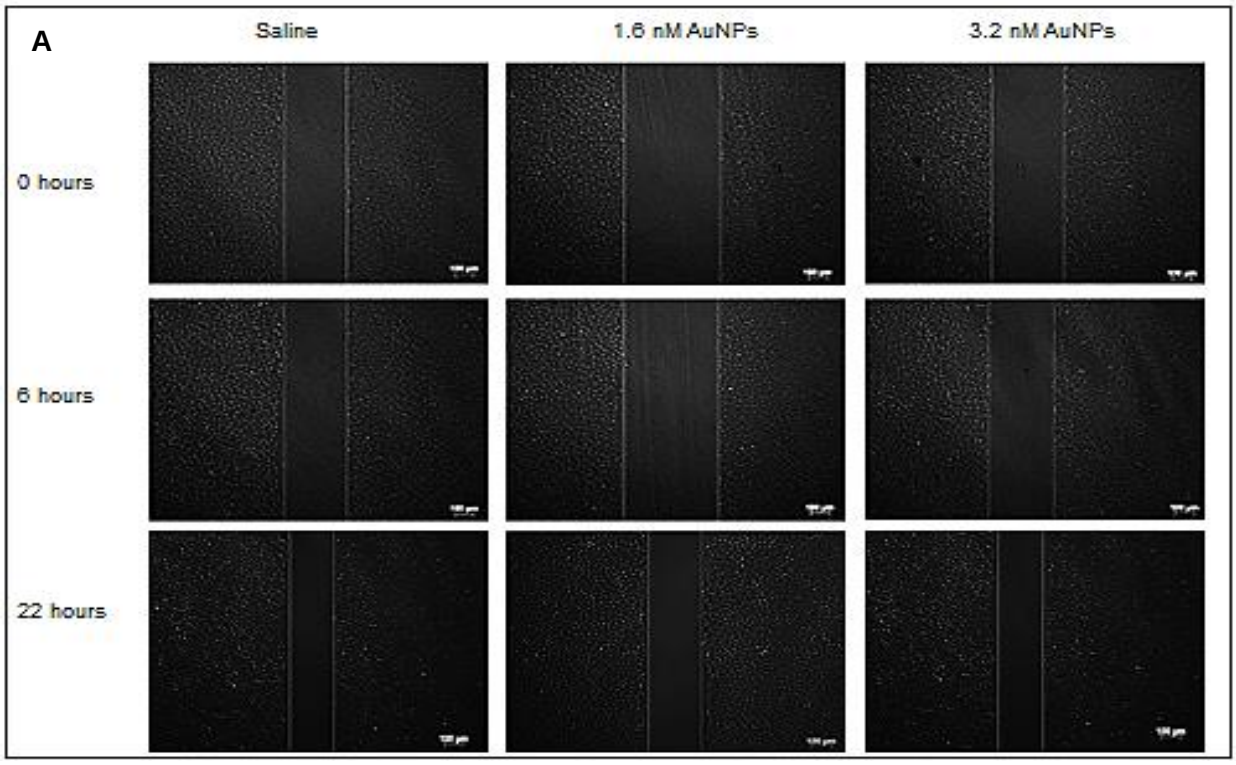


Figure 3.5A. Images from a scratch assay experiment taken at different time intervals. Endothelial (sEnd.2) cells were plated on gelatin-coated petri-dishes, wounded with a p20 pipette tip and then imaged at 0, 6 and 22 hours after exposure to AuNPs using a microscope. Straight line represents the area of the artificially created wound (scratch). Scale bar at 100 µm.

The wound width and data were analysed using ImageJ software. The data represented the number of migrated cells in micrometer (µm) and the rate of migration within the 22 hours' time lapse (Figure 3.5B). When these two AuNPs concentrations (1.6 nM AuNPs = 39,73 µm and 3.2 nM AuNPs = 37,16 µm) were compared to control cells, not much noticeable change was detected in regard to the scratch area at 0 hours (Figure 3.5B). Following 6 hours of exposure to AuNPs, the width of the 'wound' in control was 30,83 µm whereas 1.6 nM AuNPs = 38,21 µm and 3.2 nM AuNPs = 33,98 µm. This implies that cells were migrating in the direction of the wound area, but there was some inhibition due to AuNPs. After 22 hours, the wound width in the control was 18,12 µm; the 1.6 nM AuNPs = 24,45 µm and 3.2 nM AuNPs = 19,56 µm (Figure 3.5B) had a much slighter inhibition to the scratch area. Therefore, more ECs migrated to the artificial scratch area causing minimal inhibition to the 'wounded' area.

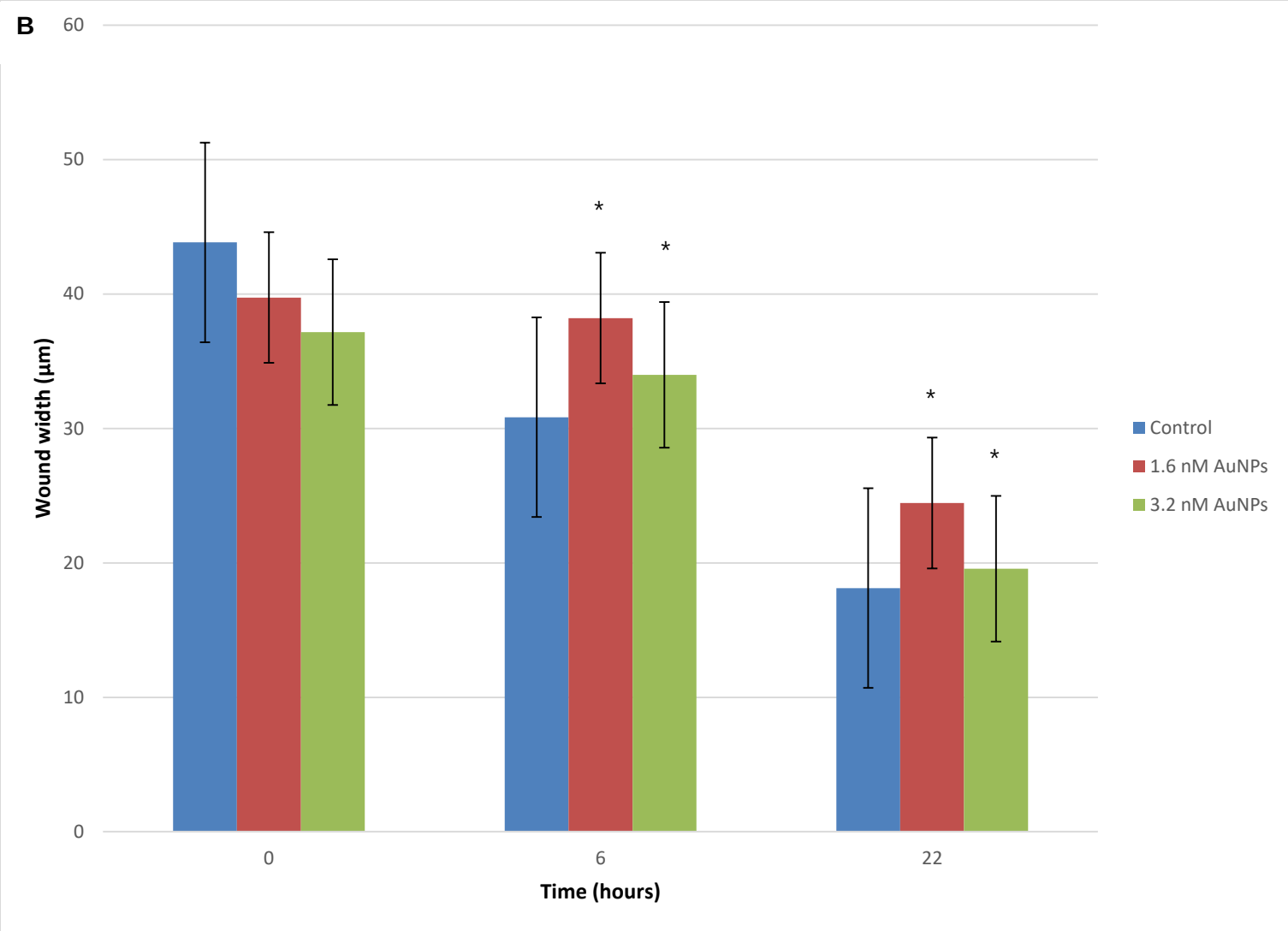


Figure 3.5B. Graph analysed using ImageJ software and Microsoft Excel, representing the number and rate of migrated cells withing the 22-hour time lapse. Data represent mean ± SD of experiments performed in triplicates. * indicates significant difference ($p < 0.05$).

3.5 Enzyme-linked immunosorbent assay

The human vascular endothelial growth factor/placenta growth factor (VEGF/PIGF) heterodimer immunoassay was used for the determination of the effect of AuNPs (0.1–3.2 nM) on VEGF-A and PIGF protein levels.

No statistical significance was observed for concentration 0.1 nM AuNPs at 390 pg/mL, thus no change was noted on the levels of VEGF-A/PIGF when compared to the control. At a concentration of 1.2 nM AuNPs the levels of VEGF-A/PlGF were decreased to 320 pg/mL but there was no statistcally significant difference whencompared to the control (Figure 3.6). After exposure of ECs to AuNPs at different concentrations, a statistically significant decrease in VEGF-A/PlGF was observed in cultures treated with 2.4 nM AuNPs (210 pg/mL; $p < 0.05$). The significant decrease in VEGF-A/PlGF occurred after cells were treated with 3.2 nM AuNPs (160 pg/mL; $p < 0.05$) (Figure 3.6). Therefore, VEGF-A and PIGF were reduced significantly following treatment with AuNPs at those two concentrations, meaning that the particles exhibited anti-angiogenic properties.

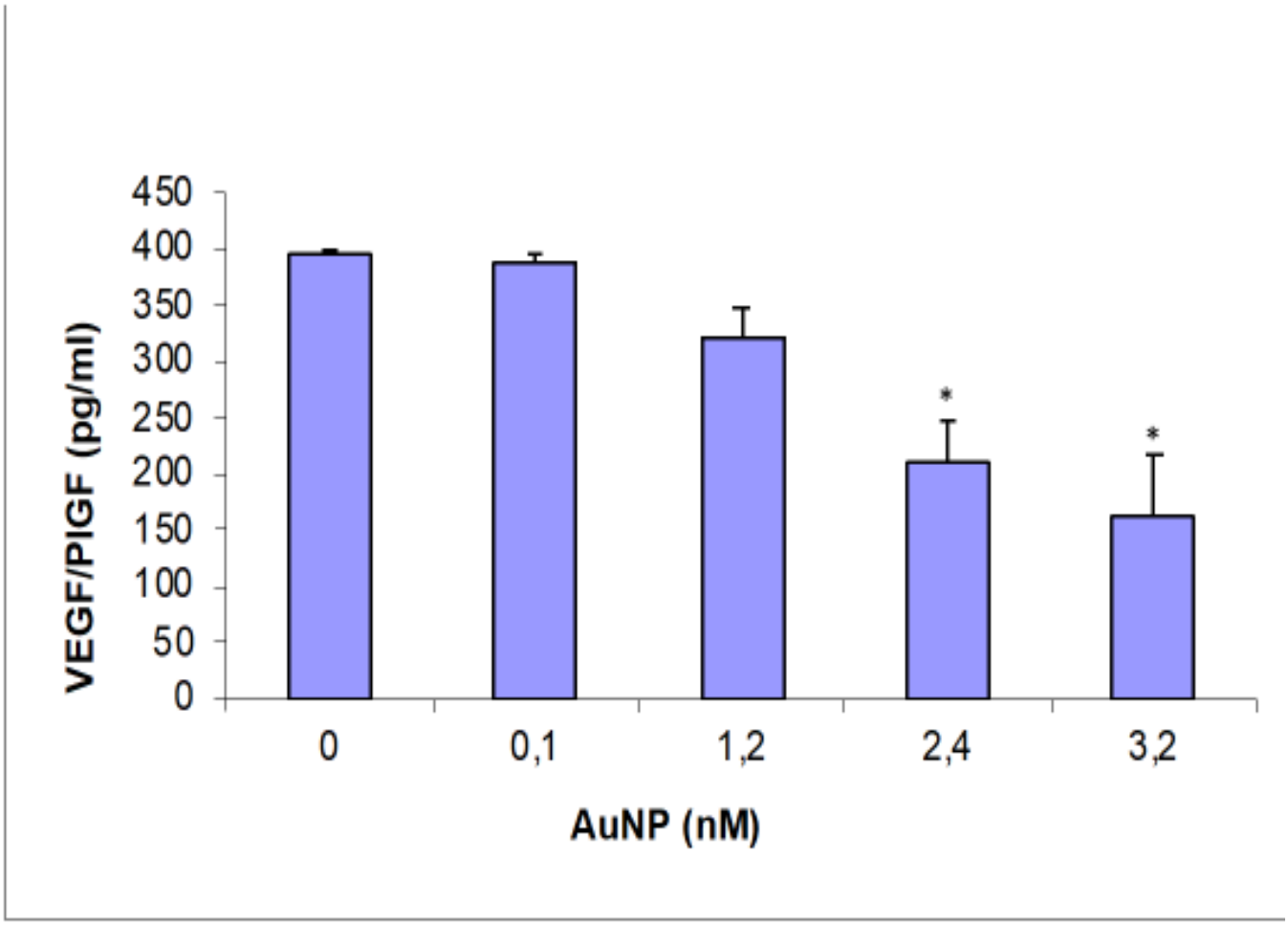


Figure 3.6. Angiogenesis protein levels, VEGF-A and PIGF after exposure to AuNPs at different concentrations. Data represent mean ± SD of experiments performed in triplicates. * indicates significant difference ($p < 0.05$).

CHAPTER 4: DISCUSSION

Compounds that target more than one angiogenic factor, cytotoxic to melanoma cells and effective in inhibiting angiogenesis could have a potent effect in melanoma treatment. Gold nanoparticles have been extensively studied and have been found to be essential in biomedical applications[81-82] with good biocompatibility and low cytotoxicity. In the present study, AuNPs were investigated as a possible novel agent in melanoma growth mouse melanoma (B16-F10) cells and angiogenesis parameters (EC growth, migration, and angiogenesis protein levels) using mouse endothelial (sEnd.2) .

Angiogenesis plays an essential part in numerous physiological and pathological processes which include wound healing, cell growth, tumour progression and metastasis[5,43,104]. Targeting angiogenesis can be a successful approach to inhibit or prevent new vascular growth for tumour cells leading to pathological angiogenesis. However, angiogenesis plays a limited role in normal physiological processes and may not be the best option for stubborn cancers such as melanoma. Currently, AuNPs are being investigated as potent anticancer drugs in melanoma, a stubborn cancer with angiogenesis as a crucial process. Previously, AuNPs were found to be concentration-dependent and to inhibit the process of angiogenesis[105]. Additionally, nanoparticles have an effective ability to target biological systems and to regulate various processes due to their unique physiochemical properties. In our study, the particle size of AuNPs were synthesised at Mintek at a diameter of 20 nm as confirmed by a transmission electron microscope. Moreover, the 14 nm AuNPs employed in this study did not aggregate in solution (PBS). The sizes of AuNPs plays an essential role in tumour uptake and tissue penetration[106]. This compound is more appealing because it is biocompatibility with low

cytotoxicity as well as physiochemical properties making it more desirable in biomedical applications[11].

The present study was aimed to characterise the particle size of AuNPs using a transmission electron microscope. These findings correlate with a study done by Pluchery *et al*. (2013) when synthesising AuNPs[107] using Turkevich's (1951) method[104]. The nanoparticle size of AuNPs have been extensively studied, AuNPs can simply cross natural barriers when comparing them to cellular component and proteins for biomedical applications[108]. Gold nanoparticles demonstrated remarkable physiochemical characteristics depending on the particle size. In biomedical applications, AuNPs (1–100 nm) can easily evade the immune system and blood-brain barrier at 12 nm making them easier to interact with biological systems[11,81,109-110].

Results demonstrated significant morphological changes in that the particle sizes were spherical and consistent as expected for each AuNP (Figure 3.1). Liu *et al.* (2019) demonstrated that AuNPs with sizes between 1–4 nm were found to be highly toxic for different cells, while AuNPs between sizes 14–100 nm were found to be relatively nontoxic[84,88] which is similar to a study done by Lu and co-workers (2017). A study by Zhang *et al.* (2019) synthesised AuNPs of 20 nm in diameter which were proven to exhibit the highest efficiency in inhibiting VEGF165 induced proliferation of HUVECs as well as to characterise their physiochemical properties[111]. Therefore, this correlates with this study as the sizes of AuNPs were 14 nm as verified by a transmission electron microscope, proving that they are nontoxic, and this size was therefore chosen for further evaluation in this study to ensure safety. Zhang *et al.* (2019) and Boyoglu *et al.* (2013) demonstrated that AuNPs (±1 nm in diameter) exhibited toxicity by entering both cellular and nuclear membranes by attaching themselves to deoxyribonucleic acid (DNA) molecules easily

than larger nanoparticles[111-112]. It is clear that the smaller nanoparticle sizes of AuNPs can efficiently penetrate the cells easily and enter the tumour vasculature to exert the effect locally[34].

In addition,.another recent study revealed that no sufficient evidence tested the concentrations of AuNPs contribution to melanoma B16-F10 cells being aggressive and malignant, which is crucial since AuNPs are already largely being used in biomedical applications[113]. Finally, our study is in agreement with Boyoglu *et al*. (2013) who reported that AuNPs sizes that are below 20 nm could be essential for DNA viruses while larger sizes could be essentially finer in RNA viruses[108].

Crystal violet staining was employed as a DNA stain after 24 and 48 hours of exposure using AuNPs (1.2–3.2 nM concentrations) in melanoma (B16-F10) and endothelial (sEnd.2) cell lines. Cell viability studies demonstrated significance in choosing the best optimal drug concentrations for biomedical applications. In our study, results on cell viability revealed that the AuNPs decreased the viability of melanoma and endothelial cells by inhibiting cell proliferation to varying degrees, depending on the concentration which was in correlation with previous studies[84,87]. Gold nanoparticles (14–100 nm) were reported to be not cytotoxic to mammalian cells, however, AuNPs (1–2 nm) were reported to be extremely cytotoxic to various cells including melanoma cells, connective tissue fibroblasts, epithelial cells and macrophages[114-115]. However, AuNPs larger than 15 nm were reported to be harmless[114]. In our study, AuNPs were synthesised at 20 nm to ensure safety.

From our findings, it was observed that AuNPs suppressed cell viability severely in ECs when compared to melanoma (B16-F10) cells, where they suppressed viability at slightly lower concentrations. Cell viability was reduced significantly after 48 hours of exposure to AuNPs at 1.2–3.2 nM concentrations in sEnd.2 cell line compared to control. This finding suggest that the

survival of melanoma cells could be reduced in a concentration-dependent manner. The concentrations of AuNPs used in this study (1.2–3.2 nM) suggested that, the smaller the nanoparticle size, the more effective the inhibition effect. Moreover, the sizes of AuNPs is known to impact their effect on the growth of various cell types.

A study by Lu *et al.* (2017) demonstrated various sizes of AuNPs, 1-3 nm and 3-5 nm were proven to be highly toxic whereas sizes 10-15 nm exerted effects in melanoma (B16-F10) cells in a concentration-dependent manner[114]. Finally, Liu *et al*. also reported that crystal violet staining is cost-effective method with convenient and highly reproducible results[116]. Therefore, our findings suggest that increasing the concentration of AuNPs will further induce cell viability and a significant toxic effect was seen against endothelial viability (Figure 3.3A and 3.3B).

In this study, microscopic analysis was conducted to display morphological characteristics that AuNPs caused during a 22-hour time-lapse exposure to ECs. Qualitative data was demonstrated by means of PlasDIC images of ECs after 22 hours of exposure with AuNPs (3.2 nM) compared to control (treated with saline). Our findings demonstrated a decrease in cell density; some cells were round and shrunken, some displayed evidence of cell debris and the result of a loss of membrane structure after 22 hours of exposure with AuNPs (Figure 3.4). Similarly, our findings are in line with Wang *et* al. (2019) who reported that a metastatic block is caused by accumulation of cells with condensed chromosomes[117]. A cell remaining in a metastatic block for a prolonged period of time resulted in the induction of apoptosis. However, after the initial 0 and 6 hours of exposure, treated and control cells appeared confluent and structurally intact without any morphological alterations. The data demonstrated morphological alterations as a result of apoptosis characteristics. Apoptosis is defined as a physiological homeostatic procedure where cells are eliminated from the body. These findings correlate with

what was found by Raobaikady *et al*. (2015) using a different compound compared to ours. Therefore, choosing the best concentration of AuNPs with maximum inhibitory effects on tumour cells but minimal effect on ECs can be a possible application as an important anti-tumour agent.

The effects of AuNPs on cytotoxicity are emphasised by most researchers, however, cellular behaviour of AuNPs are important. Numerous malignant tumours employ the capabilities of metastasis as a key of death cancer-related cases[84,114,118]. Previous studies have found that determining cell migration of tumour cells and understanding the mechanisms is crucial for novel clinical strategies in cancer diagnosis, prognosis, drug development and treatment[7,17,62,119]. Cell migration in tumour cells is a well demonstrated step in tumour progression and metastasis[114]. Many patients die from cancer as a result of tumour cells migrating to various parts of the body. Solid tumours metastasise to various parts of the body, constituting 90% cancer-related death cases as described by metastatic dissemination[119]. Previous studies have proven AuNPs to inhibit migration of cells and suppress metastasis.

In this study, cell migration assay was employed to evaluate the effects of AuNPs (1.6 and 3.2 nM concentrations) in EC migration. It was found that following 22 hours of exposure to AuNPs, AuNPs minimallyECs to the scratch area when compared to the control (treated with saline), Figure 3.5. Our results are similar to a study done by Liu *et* al. (2020) using a different compound named dioscin[120]. In contrast, a study by Lu *et al*. (2017) using 3-5 nm AuNPs demonstrated significant suppression of migrated melanoma cells demonstrating an essential role in melanoma metastasis[114] compared to our study using endothelial cells at 1.6 and 3.2 nM AuNPs. Murphy *et* al. (2013) have shown AuNPs with various sizes and surface charges can cause an impact on cell migration[121].

Therefore, this finding demonstrated that the concentrations of AuNPs when compared to the control revealed that cultures treated with AuNPs demonstrated more ECs migrating to the artificial created wound within the 22-hour time lapse.

This is the first study describing the effects of AuNPs on human VEGF-A and PIGF protein levels. Vascular endothelial growth factor-A and PIGF are good indications of melanoma angiogenesis and play an important part in the angiogenic process. These pro-angiogenic factors are postulated to impact angiogenesis parameters (induce angiogenesis, EC growth, enhance cell proliferation and migration). The expression of VEGF-A is dependent on PIGF while the VEGF-A/PIGF heterodimer induces pathological angiogenesis[122]. In our study, the enzyme-linked immunosorbent assay was used for the determination of the effect of AuNPs on VEGF-A/PIGF levels.

Findings revealed that at a higher concentration of AuNPs (2.4 and 3.2 nM AuNPs), data was found to be statistically significant following treatment with AuNPs. These findings demonstrated that the concentrations of VEGF-A and PIGF were reduced and the particles exhibited anti-angiogenic properties of AuNPs. Results revealed that at lower concentrations of AuNPs (0.1-1.2 nM), VEGF-A and PIGF displayed not much noticeable changes whereas the particles failed to exhibit anti-angiogenic properties of AuNPs when compared to the control (Figure 3.6). Pro-angiogenic factors such as VEGF-A and PIGF were found to affect endothelial cell functions involved in the process of angiogenesis[36]. These pro-angiogenic factors were reported to induce cell proliferation and migration of ECs. The placental growth factor revealed minimal activity in assays of vascular EC growth and permeability; however, it could improve the activity of VEGF-A *in vitro* and *in vivo*. Heterodimers of VEGF-A and PIGF that are naturally occurring have been

purified and characterised[123]. Moreover, these heterodimers were found to induce EC mitogenesis and PIGF showed minimal activity.

CHAPTER 5: CONCLUSION

The purpose of this study was to investigate possible cytotoxic effects of AuNPs on melanoma cells and angiogenesis parameters (EC growth, migration, and angiogenesis protein levels) *in vitro*.

The effects of AuNPs on melanoma and EC growth were determined using the crystal violet assay. Gold nanoparticles inhibited cell viability to varying degrees, with higher concentrations being more potent. Cell viability was decreased more in ECs when compared to melanoma cells. Moreover, it has been demonstrated that EC growth is central to the angiogenic process.

In this study, the effects of AuNPs on EC morphology using PlasDIC light microscopy demonstrated morphological alterations with apoptosis characteristics. The findings revealed that morphological changes were induced by AuNPs in ECs, indicating disruption of the membrane structure and presence of apoptosis when compared to the control. However, further testing is needed to support and confirm these results, using other assays or staining methods.

Gold nanoparticles were found to have anti-angiogenic activity. A scratch assay was employed to determine the effects of AuNPs on EC migration. The study found that more ECs migrated into the artificially created wound with AuNP treatment over a 22-hour period.

In this study, the effects of AuNPs on angiogenesis protein levels were assessed using the human VEGF/PIGF heterodimer immunoassay kit. The findings revealed that at higher concentrations of AuNPs, the levels of VEGF-A and PIGF were reduced significantly ($p < 0.05$) following treatment with AuNPs and the particles exhibited anti-angiogenic properties when compared to AuNPs at lower concentrations (Figure 3.6). This suggests that increasing the concentration of AuNPs will induce EC mitogenesis and exhibit anti-angiogenic properties of AuNPs although the PIGF levels revealed minimal activity.

In summary, this study has shown that AuNPs decreased the viability of melanoma cells. The anti-tumour effects were, however, not potent. The growth inhibitory effects of AuNPs were more potent in ECs. Additionally, studies have shown that AuNPs inhibited EC migration and angiogenesis proteins, VEGF-A and PlGF. Since angiogenesis is necessary for the continued growth of melanoma, the anti-angiogenic effects of AuNPs may limit melanoma progression.

Implications for future research

The following areas have been identified for further experimental research:

- Further testing is needed for the quantification and determination of angiogenesis factors.

- Studies have shown that oxidative stress is an important stimulus for vessel formation in many cancers, including melanoma. Current drugs that inhibit angiogenesis formation also promote ROS production. The formation of ROS is one of the main factors leading to drug resistance. Future studies should investigate the effects of AuNPs on oxidative stress. If AuNPs can inhibit properties of angiogenesis without leading to any significant increase in ROS, that will further make the nanoparticles good candidates for the treatment of melanoma.

- In ECs, AuNPs had a potent inhibitory effect on cell survival. This study did not determine the cell cycle phase distribution following treatment with these particles and the mode of cell death. Therefore, to further clarify the mechanism of action of AuNPs, cell cycle analysis and cell death studies need to be undertaken in future.

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APPENDICES

Appendix I: Declaration

Title of project:

Effects of gold nanoparticles on vascular endothelial growth factor-A-induced melanoma cell growth and angiogenesis

I, Lebogang Johanna Matutule (Student number: 19156759), declare that:

1. I understand what plagiarism is and am aware of the University's policy in this regard.
2. I declare that this dissertation is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.
3. I have not used work previously produced by another student or any other person to hand in as my own.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature

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Appendix II: Ethical clearance letter

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Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

28 February 2020

Approval Certificate
New Application

Ethics Reference No.: 816/2019
Title: Effects of gold nanoparticles on vascular endothelial growth factor-A-induced melanoma cell growth and angiogenesis

Dear Miss LJ Matutule

The **New Application** as supported by documents received between 2020-01-28 and 2020-02-26 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2020-02-26.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-02-28.
- Please remember to use your protocol number (816/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
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Appendix III: MSc committee letter

UNIVERSITEIT VAN PRETORIA
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MSc Committee
School of Medicine
Faculty of Health Sciences

MSc Committee
2 March 2020

Dr P Mabeta
Department of Physiology
Faculty of Health Sciences

Dear Dr,

Ms L Matutule, Student no 19156759

Please receive the following comments with reference to the MSc Committee submission of the abovementioned student:

Student name	Ms Lebogang Matutule	**Student number**	19156759
Name of study leader	Dr Peace Mabeta		
Department	Physiology		
Title of MSc	**New revised title: Effects of gold nanoparticles on vascular endothelial growth factor-A-induced melanoma cell growth and angiogenesis** Inhibition of VEGF-A-induced melanoma cell growth and angiogenesis using naked gold nanoparticles		
Date of first submission	**July 2019**		
October 2019	• Thank you for submitting the revised protocol and supporting documents.		
February 2020	• Thank you for submitting the ethics approval letter.		
Decision	This protocol has been approved. Ethics approval has been obtained. The internal and external examiners can be nominated and submitted to the MSc Committee six months prior to submission of the dissertation. Please ensure that the CV of the examiners includes: supervision, examination and publication records.		

Yours sincerely

Prof Marleen Kock
Chair: MSc Committee

MSc Committee, School of Medicine
Faculty of Health Sciences
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Appendix IV: Turnitin report

Effects of gold nanoparticles on vascular endothelial growth factor-A-induced melanoma cell growth and angiogenesis

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