The therapeutic/anti-carcinogenic effect of cord blood stem cells-derived exosomes in malignant melanoma

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Abstract

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Keywords: Extracellular vesicles, Exosomes, Melanoma, peripheral blood lymphocytes, apoptosis.

Malignant melanoma is an invasive type of skin cancer with high mortality rates, if not detected promptly. The mortality trends are generally linked to multiple dysplastic nevi, positive family history, genetic susceptibility and phenotypic features including fair skin, freckles, numerous atypical nevi, light coloured hair and eyes, inability to tan and prolonged exposure to ultraviolet radiation B (UVB). To date, the major anti-cancer therapeutics for melanoma include surgery, chemotherapy, radiotherapy, and immunotherapy. Recently, extracellular vesicles, especially exosomes, have been highlighted for their therapeutic benefits in numerous chronic diseases such as cancer. Exosomes display multifunctional properties, including inhibition of cancer cell proliferation and initiation of apoptosis. Hence, this study aimed to evaluate the genotoxicity and cytotoxicity of cord blood stem cell-derived (CBSC) exosomes on 6 samples of peripheral blood lymphocytes taken from healthy individuals and melanoma patients and on 3 samples of melanoma (CHL-1) cells. The limited number of samples was due to the time limitations and restrictions that were in place due to the COVID-19 pandemic. In this in *vitro* study, the optimal concentration of CBSC-derived exosomes (0, 100, 200, 300, 400 μg/ml protein at 24, 48 and 72h treatments) was confirmed by the CCK-8 assay.

CBSC exosomes (300 μg/ml) were used to treat lymphocytes and CHL-1 cells in the Comet assay and evaluated using the real-time polymerase chain reaction (qPCR) and Western blotting (WB). The data of the CCK-8 and Comet assays illustrated that exosomes exerted genotoxic effects on CHL-1 cells (CCK-8 assay, ****$p < 0.0001$), (Comet assay, *$p < 0.05$, **$p < 0.01$). However, the data portraying a reduction in the viability of lymphocytes needs further investigation as the number of samples was limited, therefore, further clarification is required. Importantly, no significant adverse effect was observed in healthy lymphocytes when treated with the same exosomes (p = ns). When further challenged with UVA+B radiation, the exosomes did not induce any genoprotective effect on ROS-induced CHL-1 cells, compared to the positive control (p = ns). Our data insinuates that the damage might be caused by inducing apoptosis. The anti-tumourigenic potential of exosomes was observed by activating the p53-mediated apoptotic pathway in CHL-1 cells, up-regulating p53, p21 and caspase 3 and down-regulating BCL-2 at mRNA (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) and protein levels (*$p < 0.05$, **$p < 0.01$). The potency of CBSC exosomes in inhibiting cancer progression in CHL-1 cells whilst causing no harm to the healthy lymphocytes makes it an ideal potential candidate for anti-cancer therapy. More samples are required to evaluate the therapeutic effect of exosomes on lymphocytes from cancer patients to fully understand their mechanism of action.

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8

8-oxo-7, 8-dihydroguanine (oxoG)

A

Acral lentiginous melanoma (ALM)
Alkali labile sites (ALS)
American joint committee on Cancer (AJCC)
Analysis of variance (ANOVA)

B

Basal cell carcinoma (BCC)
Base excision repair (BER)
BRCA-associated protein 1 (BAP1)
BCL-2 interacting domain agonist (BID)
BCL-2 interacting mediator of cell death (BIM)

C

Cord blood stem cell (CBSC)
Cyclin-dependent kinase (CDKs)
Cdkn1a (p21)
Cell counting kit 8 (CCK-8)
Complementary DNA (cDNA)
Cycle threshold (CT)

D

Dimethyl sulfoxide (DMSO)
DNA damage response (DDR)
Double strand breaks (DSBs)

E

Extracellular vesicles (EVs)
Endosomal sorting complex required for transport (ESCRT)
Ethidium bromide (EtBr)

F

Foetal bovine serum (FBS)

G

Gain of function (GOF)

H

Human melanoma black-45 (HMB-45)
Homologous recombination repair (HRR)
Hydrogen peroxide (H_2O_2)
Hydroperoxyl radical (HO_2)
Hydroxyl radical (•OH)

I

Inflammatory bowel disease (IBD)
Insulin-like growth factor (IGF)
Interleukin-2 (IL-2)
Intraluminal vesicles (ILVs)

L

Lactate dehydrogenase (LDH)
Lentiginous maligna melanoma (LMM)
Long-noncoding RNA (lncRNA)
Low melting point (LMP)

M

Malignant melanoma (MM)
Microgel electrophoresis (MGE)
Mismatch repair (MMR)
Mitogen-activated protein kinase (MAPK) pathway
Microgel electrophoresis (MGE)
Melanocortin 1 receptor (MC1R)
Mesenchymal stem cells (MSCs)
Microphthalmia-inducing transcription factor (MITF)
Microvesicles (MVs)
Microvesicular bodies (MVBs)
Mitochondrial RNA (mtRNA)
Morpheaform basal cell carcinoma (mBCC)
Mitochondrial outer membrane permeabilization (MOMP)

N

Neural cell adhesion molecule L1 (CHL-1)
Natural killer (NK) cells
Negative control (NC)
NADPH oxidase (NOX)
Not significant (ns)
Non-Homologous end joining (NHEJ)
Nucleotide-excision repair (NER)
Nicotinamide adenine dinucleotide (NADH)
Nodular basal cell carcinoma (nBCC)

O

Olive tail moment (OTM)

P

Papillomavirus (PV)
Platelet-derived growth factor (PDGF)
Positive control (PC)
P53- regulated modulated apoptosis (PUMA)
Polyvinylidene fluoride (PVDF)
Potassium chloride (KCl)

R

Reactive oxygen species (ROS)
Real-Time PCR (RT-PCR)
Retinoblastoma (RB)

S

Single-cell gel electrophoresis (SCGE)
Single strand breaks (SSBs)
Sodium dodecyl sulphate polyacrylamide gel (SDS- PAGE)
Standard error of the mean (S.E.M)
Single strand breaks (SSBs)
Superoxide ($O_2^{\bullet -}$)
Squamous cell carcinoma (SCC)
Superficial basal cell carcinoma (sBCC)

T

Tris-buffered saline containing Tween 20 (TBS-T)
Transmission electron microscope (TEM)
Transfer RNA (tRNA)
Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)
Tumour-node-metastasis (TNM)

U

Ultraviolet radiation A (UVA)
Ultraviolet radiation B (UVB)
Ultraviolet radiation C (UVC)

W

Western blot (WB)

Chapter one: Introduction

1 Introduction

1.1 Cancer

Cancer is a significant health concern and the second leading cause of death across the world (Hassanpour and Dehghani, 2017). In 2020, an estimated 19.3 million new cancer cases and approximately 10 million cancer deaths were reported, globally. Moreover, it is estimated that by the year 2040, the death rate will increase to 47 per cent reaching a total of 28.4 million (Sung et al., 2021). Neoplasia develops due to sustained abnormal cellular growth that possesses the ability to metastasise and invade the surrounding tissues (Sarkar et al., 2013). Numerous types of tumours exist that differ in aetiology and pathogenesis. The most common sex-associated cancers include prostate, and urinary bladder in men and breast, uterine and thyroid in women. Other carcinomas commonly occurring in both genders involve the lung, bronchus, colon, and rectum (Siegel et al., 2021).

Understanding the mechanisms underlying the induction of cancer is essential to lowering the death rates and occurrence of the disease. Studies indicate that carcinogenesis may consist of the deactivation of tumour suppressors or activation of oncogenes leading to a deregulated cell cycle that subsequently inhibits cellular death. Mechanisms triggering these events include mutation, chromosomal deletion or translocation, and activation or dysregulation of molecular signalling pathways (Sarkar et al., 2013).

The development of cancer is a complex multistep process. It is estimated that for a cell to become cancerous it must acquire several hallmarks (Figure 1). These include becoming independent of growth signals, inhibiting growth suppressors, suppressing apoptosis, genome instability, evading the immune system, tumour-promoting inflammation, deregulation of cellular energetics, exhibiting replicative immortal abilities, stimulating angiogenesis and inducing tissue invasion and metastasis and invasion of the surrounding tissues (Hanahan and Weinberg, 2011).

The three most common treatments for a variety of tumours comprise chemotherapy, surgery, and radiotherapy. However, current approaches may involve immunotherapy, angiogenesis inhibition therapy, vaccines, gene therapy, stem cell transplantation, hyperthermia-combined treatments, and targeted cancer therapies (Hanahan and Weinberg, 2011).

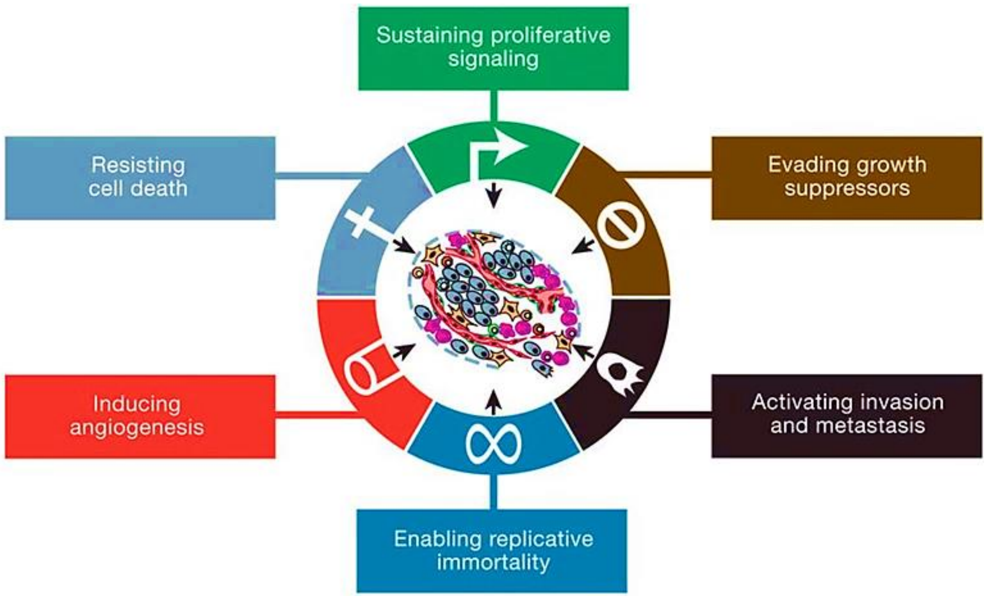


Figure 1 Six fundamental hallmarks of carcinogenesis (Hanahan and Weinberg, 2011), with copyright permission from the journal. It illustrates the mechanisms underlying the induction of cancer. These include inhibition of apoptosis, induction of angiogenesis, evading the immune system, deactivation of growth suppressors, continuous proliferative signalling and metastasis.

1.2 Tumour markers

A tumour marker is defined as the biological molecules produced by the presence of cancer in a patient. These are produced by either neoplastic or dysplastic cells (Tang et al., 2019). The widely used cancer biomarkers are identified from blood, plasma, serum, faeces, saliva, nails, urine, and various other tissues. These biomarkers possess multipotential properties that include the diagnosis and determination of disease status, establishing prognosis, and indicating treatment efficiency (Harismah et al., 2022).

1.3 Skin cancer and its types

Skin tumours evolve from uncontrolled, abnormal growth of cells in the skin that is left unrepaired by the mutations triggering DNA damage. The three most common types of skin cancer include squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and melanoma. Squamous and basal cell carcinomas are classified as non-melanoma skin cancers, whereas, melanoma particularly aggressive can present as a nevus (mole) (Leiter et al., 2014).

1.3.1 Basal cell carcinoma

Basal cell carcinoma (BCC) is the commonest type of skin tumour worldwide. Even though this carcinoma rarely spreads to secondary sites, it may, however, cause major morbidities if left untreated (Lai et al., 2018). Depending on the type of skin, this carcinoma can be classified as pigmented or non-pigmented. In addition, BCC can be further classified as nodular (nBCC), superficial (sBCC), and morphea form (mBCC) subtypes, respectively (Reiter et al., 2021).

It is well established that certain environmental and familial factors including genetics and ultraviolet radiation play fundamental roles in the development of this tumour (Niculet et al., 2022). Currently, novel treatment approaches are being considered in the treatment of BCC. These treatment strategies include dermoscopy combined with diagnostic imaging tools and the use of cellular inhibitors of the hedgehog signalling pathway (Niculet et al., 2022).

1.3.2 Squamous cell carcinoma

Squamous cell carcinoma (SCC) is the second most frequent type of skin cancer that can cause significant morbidity if not timely detected (Adalsteinsson et al., 2021). Although this carcinoma is mainly observed in the upper layers of the epidermis, nevertheless, studies suggest that the abnormal regulation of hair follicle stem cells is the major cause underlying the development of this tumour (Everts and Akuailou, 2021). Similar to BCC, chronic exposure to solar ultraviolet radiation is the primary extrinsic environmental factor responsible for the induction of this disease. In addition, papillomavirus (PV) is also considered a risk factor for SCC development.

The standard therapeutic approach for SCC is considered to be surgical excision complimented with radiotherapy. However, in high-risk patients, there is a greater recurrence rate due to a higher probability of metastasis, thus increasing the death rate by 70 per cent (Everts and Akuailou, 2021).

1.3.3 Malignant melanoma

Melanoma is an invasive type of skin neoplasia that originates in melanocytes, responsible for melanin production (Guy et al., 2015). During embryogenesis melanocytes or pigment-producing cells originate from neural crest cells. They are present as a minor population of cells within the stratum basale, the deepest epidermal layer of the skin. Structurally, melanocytes are highly differentiated cells that are dendritic in shape with a dark colour, owing to the presence of melanin. Research suggests that melanocytes migrate from the precursors of Schwann cells and their fate depends upon the regulation of IGF (insulin-like growth factor) and PDGF (platelet-derived growth factor), respectively (Adameyko et al., 2009). It is well established that melanin synthesis inside melanocytes depends upon the structural proteins present in melanosomes, proteins responsible for melanin synthesis, and proteins that are involved in melanosome transportation to the periphery of the cells (Uong and Zon, 2010). Moreover, Melanocyte inducing transcription factor (MITF) is also considered an important transcription factor in the regulation of melanin synthesis The synthesis of melanin in melanosomes is a multi-step process that involves the conversion of tyrosine to dopa by the enzyme tyrosinase (Lin and Fisher, 2007). In addition to being found profusely in the skin where they play a crucial role in protection against sunlight, melanocytes are also found in other organs like the inner ear, the middle layer of the eye, meninges, bones, heart, and the vaginal epithelium. In the epidermis, these cells come in contact with keratinocytes via their dendrites, enabling them to transfer melanin to keratinocytes and protecting them against the harmful effects of solar ultraviolet radiation (Williams et al., 2011).

Melanocytes produce two forms of melanin: eumelanin (black/brown pigment) and pheomelanin (red/yellow pigment). The colour of skin depends on the ratio of these pigments rather than the number of melanocytes (Davis et al., 2019). Malignant development of melanocytes into melanoma is a complex process and involves exogenous and endogenous triggers in addition to genetic alterations. Even though melanocytes rarely proliferate, in melanoma, however, this division index increases due to the accumulation and formation of melanocytic neoplasms, associated with point mutations (Schadendorf et al., 2018). Cutaneous melanoma can be a fatal disease causing 55000 deaths yearly. The prognosis and occurrence of this skin cancer depend upon early diagnosis and timely treatment (Schadendorf et al., 2018).

1.3.4 Risk Factors associated with malignant melanoma

The risk factors linked to malignant melanoma solely depend on certain endogenous and environmental factors. The most established primary risk factors include ultraviolet radiation A and B (UVA and UVB), sunburns, and indoor tanning beds (Palve et al., 2020). Host factors comprise multiple dysplastic nevi, positive family history, genetic susceptibility and phenotypic features including fair skin, freckles, numerous atypical nevi, light-coloured hair and eyes, and inability to tan (Rastrelli et al., 2014). Early detection of these factors in addition to the application of certain therapeutic strategies may be the key point to a better prognosis of the disease.

1.3.5 Ultraviolet radiation and melanoma

Solarisations produced by ultraviolet radiation lead to numerous skin diseases such as inflammation, sunburns and different types of skin tumours. Three types of ultraviolet radiation exist that display varying wavelengths. These are ultraviolet radiation A (UVA) (long wavelength 320-400 nm), ultraviolet radiation B (UVB) (middle wavelength 290-320 nm) and ultraviolet radiation C (UVC) (short-wavelength 100-290 nm) respectively (Baron and Suggs, 2014). It is suggested that only UVA and UVB penetrate the epidermal and dermal layers of the skin due to their long wavelengths, UVC on the other hand is completely absorbed by the ozone layer. Although, UVB is mainly linked to skin burns, nevertheless, research suggests that this type of UV radiation may also cause melanoma cells to metastasise to secondary sites via blood vessels, the induction of melanoma is therefore depicted to be initiated by the UVB radiations (Baron and Suggs, 2014). Prolonged exposure to the sun serves as an external insult that leads to irreversible genetic changes to the integumentary system, generating DNA damage by increasing the concentration of reactive oxygen species (ROS) and modification of base pairs (Battie et al., 2014).

1.4 The Tumour-node-metastasis (TNM) staging of melanoma

TNM staging is of utmost importance in clinical practice that involves the diagnosis, prognosis, and treatment of cancer. In research, it is crucial for data collection by cancer registries around the world. In melanoma, the TNM staging (T describes the size of the tumour, N describes the spread of cancer to nearby lymph nodes, and M describes metastasis) has been implemented by the American Joint Committee on Cancer (AJCC) in 2018 (Keung and Gershenwald, 2018). The TNM staging of melanoma is explained in the table below (Table 1)

Table 1 The TNM staging of Melanoma (Keung and Gershenwald, 2018).

T categories	**T0 No primary tumour** **T1 including T1a and T1b** **T1a: < 0.8 mm no ulceration** **T1b: < 0.8 mm with ulceration or 0.8–1.0 mm with or without ulceration** **T2 between 1-2 mm of melanoma thickness** **T3 between 2-3 mm of melanoma thickness** **T4 more than 4 mm of melanoma thickness**
N categories	**N0 no melanoma in lymph nodes** **N1: melanoma in one lymph node or Satellite or in-transit metastasis without regional nodal metastasis** **N2: satellite or in-transit metastasis in two or three regional lymph nodes** **N3: satellite or in-transit melanoma metastasis in four or more lymph nodes**
M categories	**M0 No spread** **M1 Cancer has metastasised**

1.5 Histopathological classification of melanoma

It is well documented that melanoma is classified into further major histogenetic subtypes. These include superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma and acral lentiginous melanoma. These subtypes are classified according to the growth pattern of the tumour.

1.5.1 Superficial spreading melanoma

Superficial spreading melanoma (SSM) manifests as a flat slowly growing, irregular, pigmented lesion (Figure 2), typically occurring in younger patients. The anatomical sites involved in this type of melanoma include the trunk, back and extremities. Histological analysis of SSM lesions generally indicates a large pleomorphic (cells occurring in various distinct forms in terms of shape and size or their nuclei) epithelioid melanocyte either in groups or as a single cell spreading superficially (Scolyer et al., 2011).

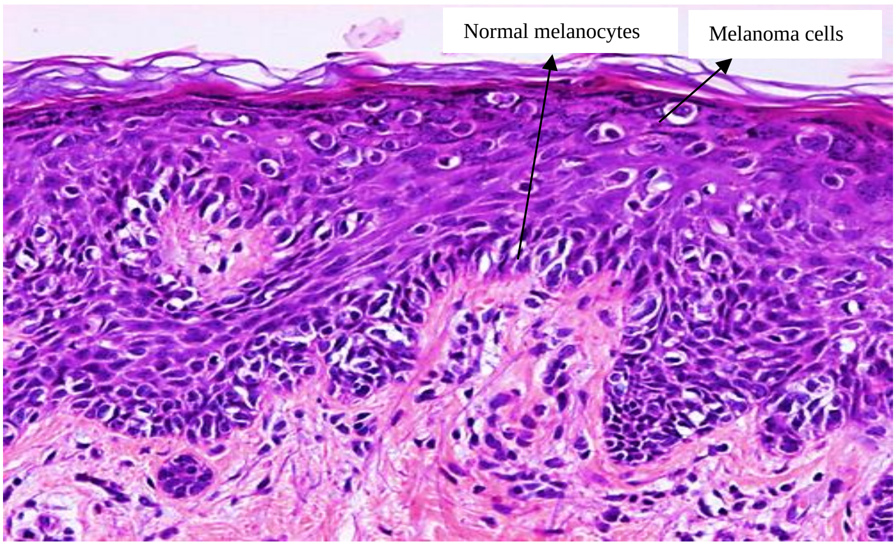


Figure 2 superficial spreading melanoma (Scolyer et al., 2011)

1.5.2 Nodular melanoma

Nodular melanoma (Figure 3) is mainly observed in slightly older patients as compared to SSM. They may be present at any site as a rapidly growing nodule, often associated with ulceration and haemorrhage (Scolyer et al., 2011).

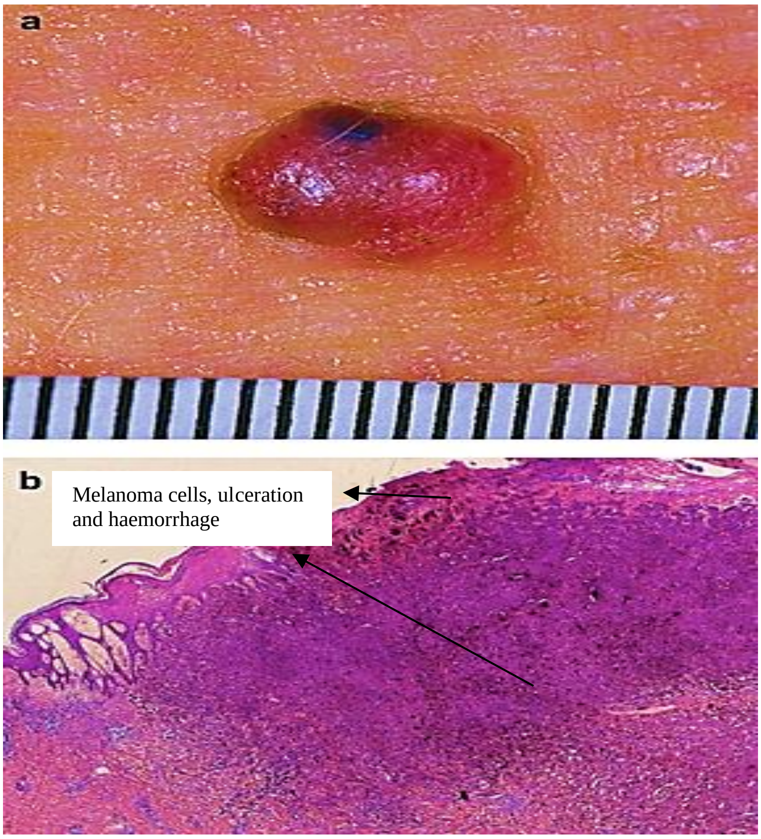


Figure 3 (a & b) nodular melanoma (Scolyer et al., 2011).

1.5.3 Lentigo maligna Melanoma

This type of melanoma typically presents with a large irregular pigmented lesion (Figure 4) involving the sun-exposed areas of the head, neck, and forearm. Lentigo maligna melanoma (LMM) is commonly observed in elderly patients with a part of their lesion raised that indicates dermal invasion.

Histologically LMM is observed by a lentiginous melanocytic proliferation in the skin severely damaged by solar radiation. In addition, epidermal thinning, epidermal atrophy and the absence of rete ridges are also seen in this subtype (Scolyer et al., 2011).

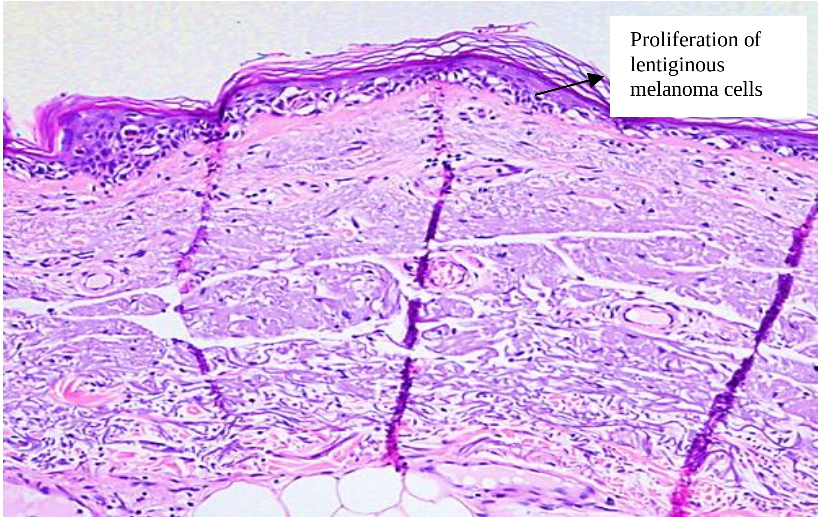


Figure 4 Lentigo maligna melanoma (Scolyer et al., 2011)

1.5.4 Acral lentiginous melanoma

The Acral (belonging to extremities of peripheral body parts) lentiginous melanoma (ALM) (Figure 5) is usually observed on the acral sites including the soles, palms, and subungual regions. They are slow in growth, characterized by atypical melanocytes scattered along with the junctional epidermal layer (Scolyer et al., 2011).

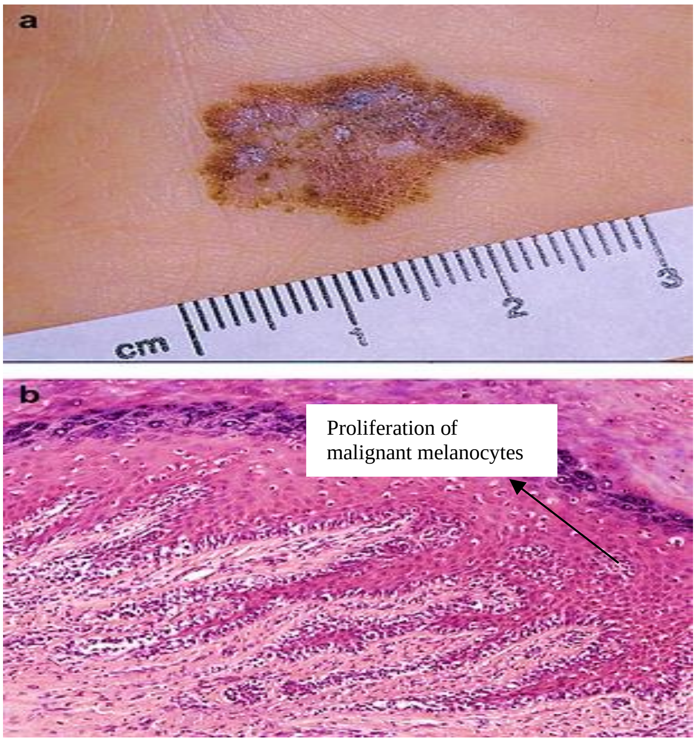


Figure 5 (a & b) Acral lentiginous melanoma (Scolyer et al., 2011).

1.6 Stages of melanoma

Staging of melanoma depends upon certain criteria (Table 2) including histological subtypes, Breslow depth, Clark level, presence of lymphocytic infiltration and ulceration, atypical hyperplasia, microsatellitosis, mitosis of cells and marginal evaluation respectively (Kauffmann and Chen, 2014) (Table 2).

Table 2 Staging of melanoma (Kauffmann and Chen, 2014).

Stage	Description	5-y Survival
Stage 0	In situ	99.9%
Stage I (A/B) T1a: 1.0 mm thick, no ulceration, mitosis <1/mm2 T1b: 1.0 mm thick, with ulceration or mitoses 1/mm2 T2a: 1.01–2.0 mm thick, no ulceration	Invasive	89%–95%
Stage II (A, B, C) T2b: 1.01–2.0 mm thick, with ulceration T3a: 2.01–4.0 mm thick, without ulceration T3b: 2.01–4.0 mm thick, with ulceration T4a: >4.0 mm thick, without ulceration T4b: >4.0 mm thick, with ulceration	High risk	45%–79%
Stage III (A, B, C) N1: Single positive lymph node N1a: Micrometastasis N1b: Macrometastasis N2: Two to 3 positive lymph nodes or regional in-transit metastases N2a: Micrometastasis N2b: Macrometastasis N2c: In-transit metastasis/satellites without metastatic nodes N3: Four positive lymph nodes, matted nodes, or in-transit metastases/satellites with metastatic nodes	Regional metastases	24%–70%
Stage IV M1a: Metastases to skin, subcutaneous or distant lymph nodes, normal LDH M1b: Lung metastases, normal LDH M1c: Other visceral metastases or any distant metastases with elevated LDH	Distant metastases	7%–19%

1.7 Diagnostic tumour markers in melanoma

The diagnosis of melanoma may occasionally be complicated due to the presence of various cytomorphological variants. Melanoma may bear resemblance to various carcinomas including neuroendocrine cancers, lymphomas, sarcomas and germ cell neoplasms (Weinstein et al., 2014). Hence, certain biomarkers including immunohistochemical staining for melanocytic markers are often used to diagnose this type of skin cancer. These include Human Melanoma Black-45 (HMB-45), Melan-A, tyrosinase, lactate dehydrogenase (LDH), microphthalmia inducing transcription factor (MITF), S100 (acidic-calcium binding protein), and SOX10 in addition to certain other diagnostic markers (Karagiannis et al., 2015).

1.8 Genetics of melanoma

Melanoma has often been associated with certain genetic mutations that fall under three major categories such as high, intermediate and low-risk germline alterations (Hill et al., 2013). Similarly, the somatic alterations are mainly composed of prolonged UV exposure such as cytosine-to-thymine alterations (Nikolaev et al., 2011). High-risk susceptibility variants frequently involve CDK inhibitor *CDKN2A*, Cyclin-Dependent Kinase *CDK4*, and BRCA1-associated protein 1 (BAP1) (Hill et al., 2013). Furthermore, the intermediate and low-risk melanoma susceptible genes are reported to involve Melanocortin 1 receptor *MC1R* and *MITF* (Rees, 2004, Law et al., 2012). In addition, in cutaneous melanoma, de-regulation in the mitogen-activated protein kinase (MAPK) pathway, results in a mutant BRAF oncogene that occurs in the majority of genetic melanomas (Tangella et al., 2021).

It is also reported that almost 10 per cent of invasive melanomas occur in people with positive family history (Read et al., 2016). Similarly, other factors such as environment, genetics, and host factors also contribute to the development of this type of carcinoma. The environmental factor associated with the occurrence of melanoma involves prolonged exposure to UV radiation. The host factors include the presence of melanocytic nevi mainly in light skin, hair and eye coloured individuals respectively (Carr et al., 2020).

1.9 Current therapeutic approaches in melanoma

1.9.1 Surgical

Surgical resection of the tumour is the main treatment for localized melanoma, and biopsy of sentinel lymph nodes is performed in patients with tumours greater than 0.8 mm in thickness or ulcerations.

If melanoma cells are present in sentinel nodes, then the additional lymph nodes might be surgically removed as well. In metastatic melanoma, surgical resection is normally carried out with other treatment options including chemotherapy (Davis et al., 2019).

1.9.2 Chemotherapy

For patients with invasive melanoma surgical excision alone will not be sufficient and drug therapies are often combined with their first line of treatment. So far, dacarbazine is known to be the chemotherapeutic drug of choice for the treatment of malignant melanoma with a one-year survival rate of 27 per cent (Davis et al., 2019).

1.9.3 Targeted therapies

Numerous targeted therapies have been introduced to prevent molecular defects in melanoma. These include the BRAF inhibitors namely vemurafenib and dabrafenib that were used for treating BRAF-mutated melanoma (Chapman et al., 2011, Rebecca et al., 2012). Although these drugs are suggested to be effective in most BRAF-mutated melanomas, nevertheless, a majority of patients build resistance to them over the treatment period (Davis et al., 2019).

1.9.4 Immunotherapy

Melanoma is considered to be an immunogenic tumour possessing a high level of infiltrating lymphocytes. Immunotherapy including cytokine interleukin-2 (IL-2) is considered to be the first immune-based therapy used to treat advanced melanoma (Byrne and Fisher, 2017).

Although, the response obtained from IL-2 is considered promising, however, it is also known to be highly toxic. Certain other immune inhibitors including nivolumab, pembrolizumab and ipilimumab have been approved for clinical use in melanoma, nonetheless, they possess severe complications and their long term use might not be suitable for all patients (Davis et al., 2019).

1.10 Nanotechnology

Nanotechnology has numerous advantages in biomedical applications. It is a systematic study of nanosized particles ranging from 1 nm to 100 nm, respectively (Ukkund et al., 2022). Nanomedicine comprises the production of nanoparticles including extracellular vesicles that possess ideal characteristics showing reduced toxicity and degradation time (Rasool et al., 2022). It also influences the prevention, diagnosis, prognosis, and treatment of a disease. In cancer therapeutics, certain extracellular vesicles (EVs) such as exosomes can be used either in conjunction with chemotherapeutic agents or as a separate treatment (Syn et al., 2017).

Besides being enriched with several biological components, these EVs are also considered bioinspired drug delivery vehicles in various fields of nanomedicine (Li et al., 2021).

1.10.1 Applications of nanomedicine

Nanomedicine is well known in research for its diverse applications in various fields including cancer diagnosis, drug delivery, vaccine synthesis, diagnostic and imaging devices, wearable instruments, implants *etc*., using different kinds of biological or non-biological materials. Nanoparticles are the basic components of nanomedicine that are being widely investigated (Zheng et al., 2020).

Recently research indicated that biological nanoparticles such as exosomes can be used in delivering various particles including drugs, peptides and enzymes as well as nucleic acids to the target tumour cells and are hence suggestive of serving as potential cancer biomarkers (Zheng et al., 2020).

1.11 Extracellular vesicles

Extracellular vesicles (EVs) are lipid-bound particles released by cells into the extracellular space (Zaborowski et al., 2015). Three subtypes of EVs exist, namely microvesicles (MVs), exosomes and apoptotic bodies that are categorized based on their biogenesis, physical properties, and release pathways (Yáñez-Mó et al., 2015). The composition of these vesicles comprises lipids, proteins, and nucleic acids respectively (Bebelman et al., 2018). Due to the differences in their biogenesis, so far, no distinguished protein marker has been identified to differentiate between their protein profiles (Jeppesen et al., 2019).

1.11.1 Exosomes and their biogenesis

Exosomes, also known as intraluminal vesicles (ILVs), are encompassed by a lipid membrane, secreted by most cell types and can be present in plasma, urine, serum, breast milk, semen, saliva, bronchial fluid, tears, amniotic fluid, serum, bile, lymph, cerebrospinal fluid and gastric acid (Doyle and Wang, 2019). These nanoparticles were considered a waste product when first identified in the 1980s (Johnstone et al., 1987). However, with advancements in research and technology, these nanoparticles signify a unique mode of intercellular communication that promotes biological processes including the treatment of numerous chronic diseases such as cancer (Kalluri and LeBleu, 2020).

Exosomes are characteristically 40-150 nm in diameter, synthesized from the endosomal pathway (Figure 6) that involves, the invagination of the cytoplasmic membrane forming early endosomes, followed by the formation of multi-vesicular bodies (MVBs) that result from endosomal maturation. These MVBs are either degraded by lysosomes or fused with the plasma membrane of the cell, ultimately releasing their cargo known as exosomes into the extracellular space (Ibrahim and Marbán, 2016). So far, there is not enough evidence to suggest the fate of MVBs. Nevertheless, some studies indicate that the fate of the MVBs relies on their cholesterol content, suggesting the content of a cholesterol-rich vesicle is secreted into the extracellular space while a cholesterol-deficient vesicle is degraded by lysosomes (Möbius et al., 2002). Although the exact mechanism is not fully elucidated, it is proposed that exosomes are released based on the cell's needs (Borges et al., 2013).

Since the transportation of MVBs and exosome formation are regulated by the endosomal sorting complexes required for the transport (ESCRT) pathway, certain proteins such as Alix, TSG101, HSC70, and HSP90β are found in the cargo of exosomes regardless of their origin (Tauro et al., 2012). This set of proteins is often referred to as exosomal marker proteins. Similarly, the tetraspanin set of proteins including CD63, CD9 and CD81 are transmembrane proteins that are frequently found in the membrane of exosomes and can be termed exosomal markers (Doyle and Wang, 2019).

Although exosomes were previously considered cellular waste products, research indicates that their main role is to participate in intercellular communication, cell maintenance and also the progression of tumour cells.

They are indicated to comprise bio-macromolecules such as lipids, proteins, mRNA, transfer RNA (tRNA), mitochondrial RNA (mtRNA) and long non-coding RNA (lncRNA) and micro RNAs (miRNA) (Keerthikumar et al., 2016). Moreover, these EVs are also reported to act as cell signalling molecules and based on their miRNA-rich cargo, they are also used as biomarkers for various diseases (Chung et al., 2020). In cancer therapeutics, exosomes have been highlighted for their multifunctional properties that include inhibition of cancer cell proliferation and stimulation of apoptosis in tumour cells (Zhao et al., 2020). Additionally, they are also considered a promising tool in tissue regeneration, however, this is still being investigated (Zhang et al., 2019).

Mesenchymal stem cells (MSCs) are considered naïve cells exhibiting self-renewal abilities. These cells possess the potential to differentiate into highly specialised cells under certain cues (Kim et al., 2017). Research suggests that exosomes isolated from MSCs could act as transferors of biomarkers that could facilitate diagnostic examinations (Muralikumar et al., 2021). MSCs have been highlighted to promote therapeutic interventions both in clinical and pre-clinical studies. The mechanism of action of these cells depends upon their origin, pluripotency, and ability to proliferate. A large body of evidence suggests that the trophic effects of MSCs play a vital role in regenerative medicine and their paracrine factors depend on the exosomes released from them (Muralikumar et al., 2021). Previous studies demonstrated that human cord blood-derived MSCs alleviated inflammatory diseases, and focal cerebral ischaemia (Li et al., 2016). Moreover, it was also reported that human embryonic stem cells were capable of reversing the tumourigenic microenvironment of melanoma cells to a non-malignant phenotype.

This study concluded that the inhibition of tumorigenicity in these cell lines was carried out by repressing cancer cell proliferation (Zhou et al., 2017). A similar study reported an inhibition in the growth of epithelial cancer cells by human embryonic stem cells, *in vitro*. This study revealed that the cargo of human embryonic stem cells possesses certain soluble factors that might prove beneficial as a new cancer strategy (Giuffrida et al., 2009).

Depending on the cargo of exosomes, a study has reported that exosomes released from menstrual stem cells lessened ROS and suppressed tumour angiogenesis in prostate cancer cells. This study concluded that exosomes isolated from menstrual blood could inhibit tumour-induced angiogenesis and prove beneficial in treating numerous carcinomas (Alcayaga-Miranda et al., 2016).

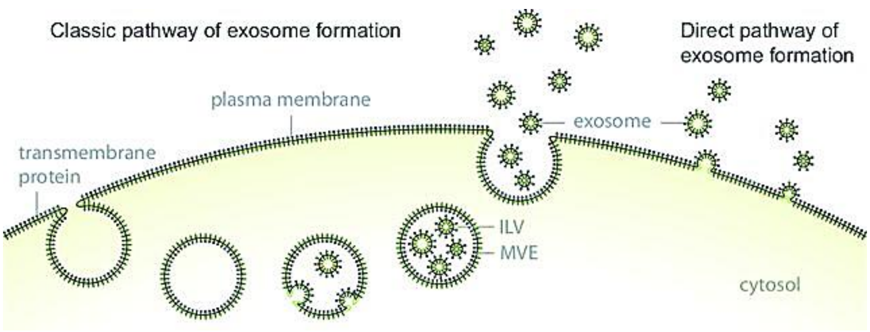


Figure 6 Biogenesis of exosomes from late endosomes formed by the inward budding of the microvesicle bodies (MVBs). These MVBs eventually degrade in the lysosomes or fuse with the plasma membrane and are released into the extracellular space as exosomes (van der Pol et al., 2012).

1.12 ROS induced oxidative stress

Damage to the DNA is termed genotoxicity and is caused by disruption of cellular homeostasis as a result of free radicals, primarily reactive oxygen species (ROS). This disproportionality of redox balance results in immense DNA damage including deformity in DNA structure, and segregation of genetic material that lacks repair mechanisms, ultimately leading to mutagenesis or carcinogenesis (Saikolappan et al., 2019). These radicals are derived from extrinsic and intrinsic factors. Intrinsic factors consist of enzymatic processes, inflammatory cells and mitochondrial pathways, while extrinsic factors involve environmental toxins such as drugs, chemical substances and radiation (Nourazarian et al., 2014). Under normal conditions, cells use their enzymatic and non-enzymatic defence mechanisms to combat harmful substances and ROS. The first line of defence against the accumulation of ROS in the presence of antioxidant molecules and enzymes that maintains their level, consistent with cell integrity (Chiaradia et al., 2021).

ROS comprise highly reactive molecules that cause oxidative stress. Examples include the superoxide anion ($O_2{\bullet}-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•HO) (Collin, 2019). Hydrogen peroxide is formed from the superoxide radical that causes immense damage to the DNA, proteins, and lipids of the cell (Zuo et al., 2015). However, an even more genotoxic form of oxygen radical is the hydroxyl radical (OH•) that is generated by the combination of H_2O_2 and $O_2{\bullet}-$ (Sakai et al., 2017).

Similarly, High levels of ROS can be detected in skin cells by photosensitizers followed by ultraviolet radiation (UV). Upon UVA radiation certain photosensitizer molecules like nicotinamide adenine dinucleotide (NADH) absorb the energy of UVA and intracellular ROS (Knak et al., 2014). Under normal circumstances, the damage caused to the genome is repaired by the base excision pathway (BER), in which the damaged nucleotide or nucleoside is removed and the gap in the DNA is re-synthesised to maintain the integrity of DNA. However, sometimes the BER pathway fails to repair the genome, due to the over-production of 8-oxoguanosine resulting in replication malfunctions (Seifermann and Epe, 2017).

During cellular metabolism or a similar biological process, ROS or oxygen exhibiting ions are produced as a cellular by-product. These free radicals have a key role in instigating apoptosis under certain biological conditions. Despite this, high levels of ROS are detrimental to cells by causing oxidative stress and disrupting cellular activities (Aggarwal et al., 2019).

DNA damage if not resolved, can lead to tumorigenesis. A varying degree of DNA damage exists among individuals depending on their ability to efficiently repair or detoxify the mutagenic agents. This results in dissimilarities in the occurrence of carcinoma among individuals (Srinivas et al., 2019).

1.13 DNA damage and repair mechanisms

DNA damage occurs in the presence of physical or chemical insults that may impact the interpretation or transmission of genetic material. These mutagens include chemicals, radiations, free radicals, cytotoxic compounds, etc. (Curtin, 2012). Our cells are at constant risk of exposure to these toxins that result in DNA damage leading to interrupted expression of genes, mutations and changes in the DNA structure. The mismatch repair system (MMR) plays a pivotal role in safeguarding the precision of replication. In case of a replication defect, the MMR repairs the DNA by recognising and excising the incorrect bases.

During replication, the most frequent damage that occurs is spontaneous deamination, when cytosine is converted to uracil resulting in a pairing with adenine instead of guanine (Niocel et al., 2019). During oxidative stress, abundant base lesions are produced, however, the most investigated oxidative lesion is 8-oxo-7,8-dihydroguanine (oxoG). If not repaired, this base lesion will result in mutations like A to T transversions (Whitaker et al., 2017). A high amount of oxoG has been reported to exist in multiple types of skin carcinomas including melanoma. Besides other mutagens, this point mutation could develop from UVA-induced oxidative stress that causes DNA strand breaks and base alterations (Khan et al., 2018).

To protect the integrity and stability of the DNA from mutagens, our cells possess a wide variety of repair mechanisms. These generally involve recognition and detection of the mutagen by activating certain pathways that include cellular death, cell-cycle arrest or DNA repair. Accumulation of unrepaired base lesions could lead to numerous chronic diseases including cancer (Srinivas et al., 2019).

1.13.1 Base excision repair (BER)

Base lesions are generally repaired by the 3'Rs i.e., Recognition, Removal and Replacement. During BER, an enzyme called glycosylase identifies the damaged base, excises it out of the DNA backbone, and fills in the gap. BER is a common method of repairing damaged lesions, usually observed in single-strand breaks (SSBs) (Caldecott, 2020).

1.13.2 Nucleotide excision repair (NER)

This type of repair mechanism only identifies the DNA damage that results in the distortion of the structure of the DNA helix. The damage is restored by using the intact complementary DNA strand (Vaughn and Sancar, 2021). The enzymes involved in this repair include XPC (xeroderma pigmentosum C), XPA, TFIIH, XPF, XPG and DNA ligase, respectively.

1.13.3 Homologous recombination repair (HRR)

Besides repairing double-strand breaks (DSBs) that may result from ionizing radiation, homologous recombinant entails a sister chromatid to be available in the G2 phase, as a template with an identical sequence of the damaged chromosome. In addition, this recombination mechanism is also considered to be important in meiosis. RAD51, CHEK2, BRCA1, BRCA2 and PALB2 are the proteins involved in this DNA repair pathway (Prado, 2018).

1.13.4 Non-Homologous end-joining (NHEJ)

Non-homologous end-joining (NHEJ) is an error-prone DNA DSB repair mechanism, possessed by the human cells to restore DSBs. The varying proteins present in this pathway exert substantial flexibility that permits NHEJ to generate various outcomes originating from the same ends. The broken ends of the DNA are joined together by DNA ligase IV and cofactor XRCC4, respectively (Chang et al., 2017).

1.14 The cell cycle and its checkpoints

Cellular growth and proliferation are significant characteristics of every living organism. The cell cycle initiates by the division of a parental cell into two daughter cells. The cycle comprises different stages including the M phase which is a short span phase that involves mitosis following cytoplasmic division.

The G1 phase is the longest stage where cells decide to divide and move on to the next stage, or to enter the resting phase G0. The S phase encompasses DNA synthesis, and replication and exhibits checkpoints that monitor DNA damage, followed by the G2 phase, the growth phase preceding the M phase (mitosis). The two main checkpoints of the cell cycle are the G1-S and G2-M interphases. The entire cycle is controlled by the cyclins and the cyclin-dependent kinases (CDKs), and CDK inhibitors through the induction of growth factors or anti-growth signals. Phosphorylation of a protein called retinoblastoma (RB), by the cyclin D-CDK4/6, leads to the release of E2F, a transcription factor directing the cell into the S phase. Carcinogenesis generally occurs due to a defect in the regulation of RB protein (Matthews et al., 2022).

Although the cell cycle is a highly regulated process, any disruption in it might lead to the formation of uncontrolled cellular growth giving rise to cancer. The tumour cells might either be benign (non-invading) or malignant (spreading to other tissue). Neoplastic cells originate from damaged DNA that is either caused by chronic exposure to chemical substances or chromosomal aberrations. When a cell is mutated, it eludes the cell cycle checkpoints prior to transforming into a tumour cell. The cancer genes are comprised of three major types namely, tumour suppressor genes, oncogenes, and DNA repair genes, respectively. The tumour suppressor genes are also termed anti-oncogenes and their main role is to regulate the proliferation of healthy cells. Mutations in these genes may lead to cancer formation and loss of function (Wang et al., 2018).

Examples of tumor suppressor genes include BRCA1, BRCA2, and p53 or TP53. Germline mutations in BRCA1 or BRCA2 genes increase the risk of developing breast or ovarian cancers in women and prostate or breast cancers in men. Oncogenes (cancer developing genes) play roles in the regulation of uncontrolled cellular proliferation and differentiation. The oncogene is the mutated form of the proto-oncogene. Proto-oncogenes are usually involved in cellular signalling cascades. Under normal conditions, the DNA repair genes rectify the errors that occur in replication prior to cell division or arise spontaneously. However, in the presence of a mutation, these genes fail to perform their function allowing the accumulation of mutated cells (Jeggo et al., 2016). Proto-oncogene is the HER2 gene. This gene codes for a transmembrane tyrosine kinase receptor called human epidermal growth factor receptor 2. This protein receptor is involved in the growth, repair and division of cells in the breast. More than 2 copies, leads to an excess production of the HER2 protein inducing breast cancer.

1.15 Role of p53 in cell cycle

Tumour protein TP53 (p53) is a type of phosphoprotein made of 393 amino acids. This protein plays a significant role as a transcription factor in regulating the cell cycle. In the presence of certain stimuli such as DNA damage, hypoxia, or oncogene activation the p53 gene activates certain metabolic responses that may lead to senescence, cellular death, cell-cycle arrest, and DNA repair. The G1-S checkpoint of the cell cycle plays a vital role in inhibiting the replication of damaged cells and p53 plays a major role in this transition. It is reported that the regulation of cell growth arrest is mediated by the regulation of p21, GADD45, CdC25C and 14-3-3s depending on P53, respectively.

Studies indicate that DNA repair is triggered by p53 via the stimulation of p21, a CDK inhibitor CDKs except on CDK4/6 that is important at the restriction point of the cell cycle, depending on p53 respectively (Williams and Schumacher, 2016). Bax is a pro-apoptotic protein that is upregulated by p53 during p53-induced apoptosis. On the contrary, BCL-2 is an anti-apoptotic gene that is down-regulated by p53 during apoptosis (Liu et al., 2016). Whether or not, the p53 induced cells enter apoptosis or cell-cycle arrest, wholly depends on the stimuli and cell type. In the presence of low DNA damage, p53 stimulates the genes responsible for cell cycle arrest, however, when the DNA damage exceeds the repair capacity apoptotic genes are activated (Chen, 2016). Apart from this, p53 also aids in preventing the cells with incomplete DNA in the S-phase checkpoint to enter mitosis (Senturk and Manfredi, 2013).

1.16 Mutant p53 and neoplasia

The wild-type form of p53 is a major tumour suppressor that protects the cells against cancer. A missense mutation is commonly caused when a single amino acid is replaced in p53 protein, disrupting the binding of the p53 to the DNA and leading to inhibition of tumour suppression (Mantovani et al., 2019). Structurally p53 is composed of a single negative domain, hence a single negative tetramer can damage the whole protein. Improper functioning leads to the absence of p53 expression or the formation of unstable mutated proteins. As the *p53* gene is a tumour suppressor gene, therefore it needs to be fully inactivated for cancer cells to grow and proliferate uncontrollably. This is only possible in the presence of two genetic defects in the gene, one in every allele of each cell.

The mutant p53 comprises various biological features, otherwise absent in its wild-type form that could result in cancer formation (Yue et al., 2017). These features have been commonly termed the gain of function (GOF), exhibiting dominant-negative influence on wild-type *p53*. An increase in genome instability is the key characteristic of tumour development that involves high levels of mutations and chromosomal aberrations. Mutated p53 affects genome instability by interrupting the checkpoint mechanism responsible for spindle formation that gives rise to cells with polyploidy genomes (Erenpreisa et al., 2014). A large body of evidence suggests that depletion of mutant p53 leads to suppressed tumorigenicity, chemotherapeutic resistance, and invasiveness both *in vitro* and *vivo* (Muller and Vousden, 2013, Kastenhuber and Lowe, 2017).

1.17 Mechanism of apoptosis and associated genes

Apoptosis or programmed cell death is considered a fundamental component in maintaining genome stability and cellular turnover. Impaired apoptosis could result in uncontrolled cellular growth and tumorigenesis. A study indicated a close association between the susceptibility of tumours to apoptosis and drug resistance. Based on this study, it was concluded that the initiation of apoptosis in neoplastic cells may prove to be a promising approach against drug resistance in various types of carcinomas (Hall et al., 2008). Apoptosis is stimulated by protein caspases and consists of two pathways extrinsic and intrinsic. The intrinsic (mitochondrial) pathway is regulated by the BCL-2 family of proteins that control the permeability of the mitochondrial membrane and the release of pro-apoptotic proteins including the effector caspases 3.

On the contrary, the extrinsic pathway is monitored in two ways, by the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) that involves Apo2L/TRAIL and related receptors, and by the Fas receptor and Fas ligand (Cavalcante et al., 2019). The equilibrium between the pro and anti-apoptotic genes monitored by p53 decides the fate of a cell. Thus the absence of p53 is associated with high levels of anti and reduced levels of pro-apoptotic genes, respectively (Kim et al., 2014).

1.18 Exosome isolation methods

Due to the recent advancements in nanomedicine, exosomes have been highlighted for their efficacy in the treatment of numerous chronic maladies including several types of carcinomas. Several methods have been proposed for the isolation of exosomes. These include ultracentrifugation, chromatography, filtration, polymer-based precipitation, and immunological separation. Choosing the right isolation method is of utmost importance to yield high purity exosomes without contamination (Brennan et al., 2020).

The most commonly used method is the ultracentrifugation which depends on the physical properties of the suspension including size, density, shape, centrifugal force and the viscosity of the solvent used. The ultracentrifugation is further divided into various other subtypes including differential ultracentrifugation, density gradient ultracentrifugation, and ultrafiltration. As the differential type of ultracentrifugation was used, hence in this method the suspension was subjected to low-speed centrifugation (*500* x *g*) 2 to 3 times to distinguish between cells, microvesicles, and extracellular matrix. The supernatant was re-centrifuged at 10,000 x *g* to remove apoptotic bodies and cellular debris.

The suspension was again spun at 100,000 to 200,000 x *g* (60- 120 min) to retrieve the exosomes, with subsequent washing of the pellets in PBS and stored at -80 °C (Nikfarjam et al., 2020) (Figure 7).

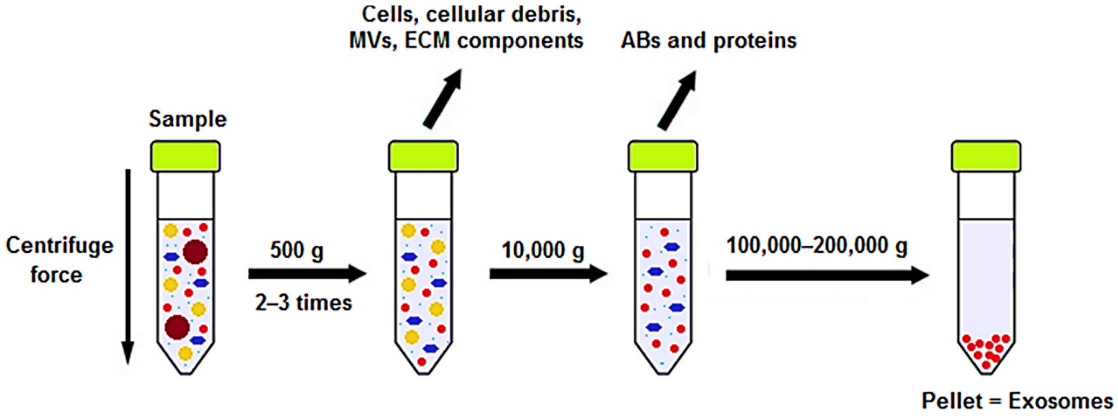


Figure 7 Illustration of a schematic presentation of exosome isolation by ultracentrifugation (Nikfarjam et al., 2020).

1.19 Methods to detect viability and genotoxicity

1.19.1 CCK- 8 assay

Cell viability refers to the number of live, healthy cells in a given sample. Determining the proliferation of cells is crucial in understanding the mechanism of action of certain genes or proteins involved in apoptosis or cell survival exposed to toxic insults. The proliferation/cytotoxicity assays are used for screening purposes to identify the cytotoxic effects of certain agents on the proliferation and viability of cells (Adan et al., 2016).

The cell counting kit-8 (CCK-8) is used to detect the proliferation or cytotoxicity of different agents. In this assay, a tetrazolium salt water-soluble tetrazolium salts (WST-8) is used which is highly water-soluble. This salt is reduced to a yellow-coloured formazan dye by dehydrogenases in cells.

The colour of the formazan dye is directly proportional to the number of viable cells (Yang et al., 2021).

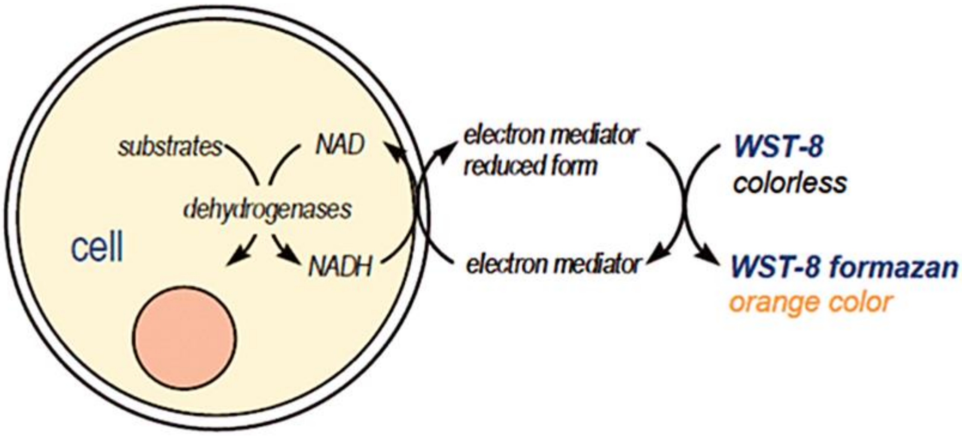


Figure 8 Formation of Formazan dye by CCK-8. The reduction of tetrazolium salt WST-8 to an orange colour Formazan dye is carried out by the presence of dehydrogenases in cells (Ishiyama et al., 1997).

1.20 Genotoxicity assay

1.20.1 The Comet assay

This assay is also referred to as the single-cell gel electrophoresis (SCGE) or microgel electrophoresis (MGE). This assay was established in 1984 for detecting the DNA damage produced by gamma radiation after electrophoresis in pH neutral conditions (Ostling and Johanson, 1984). This kind of Comet assay only assesses the double-strand DNA breaks (DSB) caused by radiation (Møller, 2005). However, a more common type was introduced that detected the DNA single-strand breaks (SSB), alkali labile sites (ALS) and DNA cross-linking in cells.

Some types of damage, such as base or phosphate alkylations, will decompose to form strand breaks in a process dependent on pH and time of alkaline treatment, known as the alkali labile sites. This version was alkaline with a pH >13 (Singh et al., 1988).

The alkaline Comet assay involves cell suspension using a pre-coated microscopic slide with agarose. spread on an agarose pre-coated microscopic slide. High salt concentration buffers are used to lyse the cells and release the DNA. The unwinding of the DNA is followed by electrophoresis under alkaline conditions, leading to the migration of damaged DNA towards the anode. After neutralizing, the DNA is stained with a fluorescent dye such as the Ethidium bromide (Kassie et al., 2000).

The extent of the DNA damage in the Comet assay is assessed by the morphology of the comet on a visual computer-automated scoring programme. The data parameter of this assay is presented as the tail length, % DNA in the tail and Olive tail moment (Tice et al., 2000).

Research has exemplified the Comet assay by its rapidity, sensitivity, and cost-effectiveness. This test can be used to detect environmental insults both *in vivo* and *in vitro* (Collins and Horváthová, 2001). Lymphocytes are suggested to be the most suitable surrogate cells for the Comet assay to detect human cancer that is caused by different agents (Faust et al., 2004). Nonetheless, this assay has been conducted on many other cells including buccal cells, nasal cells, optic cells, and surgical biopsy extracts (Collins, 2014). Similarly, the Comet assay has been used in several other studies to evaluate the genotoxicity caused by prolonged exposure to drugs, radiation and environmental risk factors (Valverde and Rojas, 2009).

In addition, research suggests that the Comet assay is likely to detect the presence of high levels of DNA damage in various types of cancer including breast cancer, oesophageal cancer, cervical cancer, and Hodgkin's disease (Collins, 2014).

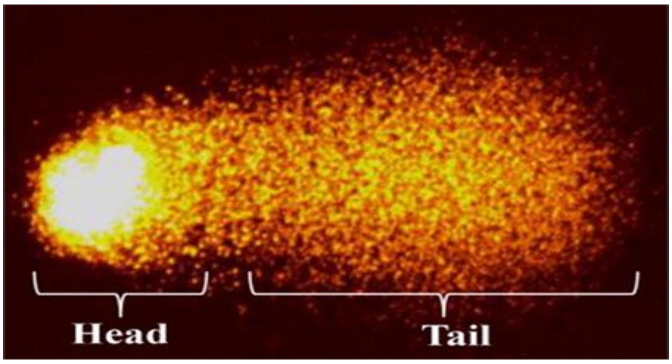


Figure 9 A typical image of a comet, illustrating the damaged part of the DNA (Tail) and the undamaged nucleoid part (Head) (Beedanagari et al., 2014).

1.21 Lymphocytes as potential surrogate cells in genotoxic assessments

Several determining factors predict the vulnerability to cancer. These include genome sensitivity, exposure to mutagens, and an efficient DNA repairing system (Collins, 2014). Peripheral lymphocytes are considered potential surrogates in determining genome sensitivity, due to their long lives and their ability to preserve genetic mutations for more than four decades (Anderson et al., 2014).

Since the haematopoietic system is at constant risk of oxidative stress due to exogenous and endogenous mutagens that might increase the chances of developing chronic diseases such as carcinoma, therefore, different biomarkers including imaging, biopsies and recent new blood tests with high specificity in detecting DNA damage exist to identify different types of carcinomas (Cohen et al., 2018).

In malignant melanoma, finding an efficient, affordable detecting technique is not readily available. Therefore, few recent studies indicated the importance of peripheral blood lymphocytes, as a biomarker in the detection of different types of cancers (Anderson et al., 2014). These studies observed an increase in DNA damage in peripheral lymphocytes belonging to a group of patients suffering from melanoma, COPD, suspected melanoma, lung carcinoma, and colon cancer as compared to the lymphocytes of healthy individuals. In yet another experiment, it was observed that the amount of DNA damage in peripheral blood lymphocytes of patients with malignant melanoma, was higher when irradiated with UVA as an external source of mutagen, as compared to that of normal volunteers (Najafzadeh et al., 2012).

1.22 Real-time PCR

Real-time PCR is a frequently used technique in biomedical research that permits the production of a large quantity of DNA from relatively small amounts. In this assay, a single or few copies of DNA is amplified into numerous other copies of a target DNA sequence. This technique was first introduced by Kary Mullis in the ’80s, who later received the Nobel Prize in 1993 for this innovation (Mullis, 1990). This is an easy, straightforward and economic assay with high sensitivity that replicates a specific segment of DNA into many other copies. The DNA polymerase requires a primer for the 3-OH group for the synthesis of DNA. This DNA polymerase then forms a complementary strand in a 5’ to 3’ direction by utilizing one strand as a template. By using two primers, both serve as templates and are copied.

This assay depends on the thermal cycling that comprises denaturation (denaturing the double-helical DNA into single-strand DNA), annealing (primers attach to single-strand DNA), and extension (synthesis of DNA from primers). The heating temperatures in all the above steps are as follows: 94°C for 30 s to 2 mins in denaturation, 45°C to 72°C for 30 s in annealing, and 72 or 78°C in extension. This technique (qPCR) utilises a DNA fluorescent dye (SYBR Green) which allows real-time observation of the amplified DNA. PCR is abundantly used in applications involving medical, diagnostic and research fields.

1.23 Western blotting

Western blotting or protein immunoblot is a commonly used assay in biomedical related disciples for the expression and quantification of the target protein (Mahmood and Yang, 2012). It facilitates the researchers to detect target proteins from a mixture of different types of proteins, isolated from cells. This technique is composed of three stages, the first involves gel electrophoresis, based on size and molecular weight distribution, next, blotting is done to produce a solid band for each protein and lastly, imaging of the target protein is done by using the right primary and secondary antibodies. The unbound bands are rinsed off and only one band represents the target protein on the membrane. In western blotting minute amounts of proteins are detected as low as 1ng due to its high specificity and sensitivity.

1.24 Aims

Exosomes are considered to play a crucial role in intercellular communication, cell maintenance and cell signalling molecules.

Besides their therapeutic potential in numerous chronic diseases, in cancer therapeutics, these nanoparticles have been highlighted for their multifunctional properties that include inhibition of cancer cell proliferation and stimulation of apoptosis in tumour cells. Although exosomes are well-researched nanovesicles exhibiting many health benefits in cancer and other chronic diseases, their effects on lymphocytes obtained from healthy individuals and melanoma patients have not been investigated. Therefore, this report aims to detect the DNA damage caused by cord blood-derived exosomes on the lymphocytes of melanoma patients compared to that of healthy individuals, as well as melanoma cancer cells (CHL-1).

To develop the hypothesis that cord-blood cells are an effective intervention in malignant melanoma, the following objectives were considered;

- To compare the genotoxic effects of cord blood stem cell (CBSC) exosomes on peripheral blood lymphocytes from melanoma patients and healthy individuals. To observe the same effects of exosomes in melanoma cancer cells (CHL-1). This will be done by performing the CCK-8 assay to detect the cytotoxicity of CBSC-exosomes in these cells. The Comet assay will be used due to its rapidity and sensitivity in detecting the genotoxicity of the DNA on the lymphocytes of healthy individuals and melanoma patients and CHL-1 cells.
- To investigate the effect of CBSC exosomes against ROS induced damage in CHL-1 cells by UVA+B radiation. This will be achieved by assessing the DNA damage induced by UVA+B radiation in the Comet assay.
- To determine the effect of CBSC-exosomes on the expression of p53, p21, BCL-2 and caspase 3 at mRNA and protein levels in melanoma CHL-1 cells.

Chapter two

2 Materials and Methods

2.1 Materials and Equipment

The list of reagents used in this study is listed in the table below along with their supplier's name and CAS/Cat number.

Table 3 List of chemicals and reagents used in the study along with their CAS/CAT numbers

Chemicals and reagents	Company Distributor	CAS/CAT Number
Acrylamide 30%	Sigma –Aldrich, UK	79-06-1
Black box	Sigma-Aldrich, UK	Z742411
Bradford Protein Assay Kit 1	Bio-Rad	5000201
Bovine Serum Albumin	Sigma –Aldrich, UK	9048-46-8
Caspase 3 (CASP3) Human qPCR Primer Pair	Origene	HP207674
CHL-1 Cells	ATCC	CRL-9446
Cell counting kit - 8 (CCK8)	Sigma –Aldrich, UK	96992
Cover glass	VWR	No.631
Distilled water(dH_2O)	n/a	n/a
Dimethyl sulfoxide (DMSO)	Sigma –Aldrich, UK	67-68-5
Donkey Anti Rabbit IgG H&L	Abcam, UK	ab6802
Dulbecco′s Modified Eagle′s Medium	Sigma –Aldrich, UK	56436C
Eppendorf ® tubes (1ml)	Sigma –Aldrich, UK	N/a
EDTA ($Na_2EDTA.2H_2O$)	Sigma –Aldrich, UK	10378-23-1
Ethidium bromide	Sigma –Aldrich, UK	1239-45-8

Exosomes (CBSC)	Taleghani hospital	N/a
Ethanol	Sigma –Aldrich, UK	64-17-5
Foetal bovine serum	Sigma-Aldrich, UK	F7524
Falcon tubes	BD, Swindon, UK	N/a
FAST SYBR, GREEN	Sigma –Aldrich, UK	163795-75-3
Anti-GAPDH antibody [EPR16891] - Loading Control	Abcam	ab181602
Hs_ACTB_1_SG QuantiTect	Qiagen	QT00095431
Hs_BCL-2_1_SG QuantiTect	Qiagen	QT00025011
Hs_CDKN1A-va. 1	Qiagen	QT00062090
Lithium heparin tube 9ml (LH)	Griener Bio-One, Austria	455084/A1803 3VS
iScript™ cDNA Synthesis Kit	Bio-Rad, UK	1708890
Lymphoprep	Axis-Shield-Norway	66720-17-0
Low melting point agarose	Invitrogen, UK	39346-81-1
2-Mercaptoethanol	Sigma-Aldrich, UK	60-24-2
Mini Gel Tank	Thermo Fisher Scientific	A25977
Normal melting point	Invitrogen, UK	9012-36-6
Pipette tips	VWR	N/a
Phosphate buffered	Sigma –Aldrich, UK	N/a
Protease inhibitor	Sigma –Aldrich, UK	66701-25-5
p53 primer	Qiagen	QT00050785
Recombinant Anti-p53	Abcam, UK	ab32049
Recombinant Anti-p21	Abcam, UK	ab109520
Recombinant Anti-BCL-2	Abcam, UK	ab32124
Recombinant Anti-caspase 3	Abcam, UK	ab32351

Sodium chloride (NaCl)	Sigma –Aldrich, UK	7647-14-5
Sodium hydroxide (NaOH)	Sigma –Aldrich, UK	1310-73-2
Superfrost microscope slides	Thermo Scientific	10504182
Triton X-100	Sigma –Aldrich, UK	9002-93-1
Trizma base	Sigma –Aldrich, UK	77-86-1
TWEEN 20	Sigma –Aldrich, UK	9005-64-5
RPMI-1640 medium	Sigma –Aldrich, UK	R8758
RIPA Lysis & Extraction	Thermo Fisher Scientific	88900
Penicillin-streptomycin	Sigma –Aldrich, UK	P4333
QIAamp® RNA Blood Mini	Qiagen	52304
Western ECL Substrate, 200	Bio-Rad	1705060
20X Bolt™ MES SDS	Thermo Fisher Scientific	B0002
4X Bolt™ LDS sample	Thermo Fisher Scientific	B0007
10X Bolt™sample	Thermo Fisher Scientific	B0009
96 well plates	VWR	10861-666

2.2 Equipment

The list of equipment pieces and materials used in this study are listed below

Table 4 Equipment list along with their company supplier.

Equipment list	Company / Distributor
Amersham™ Hybond™ Polyvinylidene difluoride (PVDF) Blotting Membrane (0.45 μM x 150	GE Healthcare Life Sciences, Germany
BioDrop™ Touch Duo Spectrophotometer	BioDrop Ltd, Cambridge, UK
Bolt™ Welcome Pack + iBlot™ 2 System	Thermo Fisher Scientific
BRAND® Filter Flask with lateral socket / Vacuum glass bottle	Sigma-Aldrich, UK
BRAND® Staining Trough/incubation box with tray	Sigma-Aldrich, UK
Centrifuge Mistral 3000	MSE, Albertville, USA

Centrifuge (biofuge 28 RS)	Heraeus, Sepatech, Germany
CCD camera	Hitachi KPMI/EK Monochrome,
Coplin jar	VWR, Lutterworth, UK
Corning® 15 mL centrifuge Tubes	Sigma-Aldrich, UK
Corning® cell culture flasks (25 cm2)	Sigma-Aldrich, UK
Culture flasks (25&75 Cm^3)	Corning Incorporated Costar®, NY, USA
Dry incubator (37º C) LKB BIOCHROM	Leech LTD, Nottingham, UK
Electrophoresis power supply	Consort (E861), Belgium
Electrophoresis tank (HU20)	Scie-Plas, Renfrewshire, UK
Falcon tubes	BD, Swindon, UK
Freezer -20º C	Sanyo, Ultra-low, Japan
Freezer -80º C	Sanyo, Ultra-low, Japan
Fluorescent microscope	Leica, Weztler, Germany
Fume cupboard	Milton, UK
Fume hood ray air	Maiche Aire, Bolton, UK
Ice maker (Scotsman AF 100)	Namur, Belgium
Incubator 37º C with 5% CO_2	Andor Technology Ltd, Belfast
Light microscope	Nikon, Japan
Komet 6 software	Kinetic Imaging, Nottingham, UK
Microcentrifuge MSE	GMI, Alberville, USA
Microplate reader	Dynex technology, Sussex, UK
Steponeplus Real-time PCR detector	Applied Biosystems, Warrington UK
Waldmann F15/T8-PUVA tubes	villingen-Scwennigen, Germany
Water bath	Grant instruments, Cambridge, UK

2.3 Methods

2.3.1 Ethical Approval

The ethical approval for the collection of blood samples was awarded by the Leeds East Research Ethics Committee (Reference No.:12/YH/0464), the University of Bradford Research Ethics Subcommittee on Research in Human Subjects (Ref: 0405/8) and the Research Support and Governance office, Bradford Teaching Hospitals, NHS Foundation (Ref: RE DA 1202).

The human cord-blood derived exosomes were a gift from our collaborators in Tehran's Taleghani Hospital. All the samples were collected from healthy mothers after obtaining consent. The cord blood samples were received from the Tissue Bank at the University of Bradford (Ref: ET-17-088).

2.3.2 Volunteer recruitment

Each volunteer was provided with a detailed information sheet regarding the study that contained two parts. The first part contained the background and purpose of the study and part two included the terms of participating in the study that involved points regarding the confidentiality of the study and the fact that the experiments conducted in the study are not predictive for the participants. The consent form included the following points;

- The willingness to participate in the study and complete the consent form
- The agreement to store the blood at the University of Bradford
- Previous medical history including inflammatory diseases, positive history of cancer, familial history
- History of present and past medication
- History of smoking, drinking, occupational risk exposure, and diet was also recorded.

2.4 Preparation of CBSC exosomes

The exosomes were diluted in RPMI medium when received. The concentration of exosomes was 3.4 x 10^9 particles/ml, stored at -80 ºC.

2.5 Transmission Electron Microscope (TEM) analysis of cord blood-derived exosomes

2.5.1 Visualisation and morphology of exosomes

Transmission electron microscope Tecnai 12, (FEI Company, Netherlands) was used to visualize the shape and size of the exosomes. The images were captured at different magnifications ranging from 100 to 250 K.

2.5.2 Method

A drop of the CBSC-exosome sample was placed on the filter paper on grids in proportionate amounts and left to dry for 10 min. The grids were then cleaned with distilled water and put on filter papers to dry. Next, parafilm was placed on a petri dish to avoid uracyl acetate spillage and the grids were then placed in uracyl acetate for 2 mins. The grids were again washed with DD-H_2O and dried on filter paper overnight, followed by visualization of the exosomes at different magnifications using a transmission electron microscope.

2.6 Cytotoxicity assay

2.6.1 Preparation of the cord blood stem cell exosomes and H_2O_2

The cord-blood derived exosomes were purchased as a stock solution dissolved in RPMI medium and stored at -80 ºC. The concentration of exosomes was 3.4 x10^9 particles/ml which were evaluated by the Nanoparticle Tracking Analysis (NTA), by the provider company.

Four concentrations were used in the CCK-8 assay namely 100, 200, 300, and 400 µg/ml protein of CBSC exosomes. For positive control (PC), 75 µM of H_2O_2 was used. This is in accordance with the previous studies that suggest 75 µM of H_2O_2 is the most damage-inducing concentration, without causing apoptosis (Park, 2018).

2.6.2 Cell counting kit-8 (CCK-8) (Cytotoxicity assay)

A colourimetric method was used to assess cell viability. The lymphocytes isolated from healthy individuals and melanoma patients in addition to the CHL-1 cells were added to a 96-well plate in triplicates at a concentration of 5000 cells per well. The cells were treated with different exosome concentrations (100, 200, 300, 400 µg/ml protein) at 24, 48 and 72 h in an incubator at 37 °C, with 5% CO_2. Ten microliters of the CCK8 reagent were added to each well and incubated at 37 °C for 4h. The absorbance was then evaluated at a wavelength of 450 nm using a microplate reader.

2.6.3 Statistical analysis

The statistical analysis was conducted on three melanoma patients and three healthy individuals (n=3). The assays on CHL-1 cells were also performed in three biological sets per experiment (n=3). The final result was assessed as mean values with an error bar. A one-way ANOVA was performed with multiple comparisons against the negative control to compare each sample to the negative control by the GraphPad prism 9. The significant p values were $p < 0.001$(****), respectively.

2.7 The Comet assay

2.7.1 Preparation of blood samples for the Comet assay

After consenting the volunteers, the blood samples were collected in lithium heparin tubes by trained phlebotomists. The samples were diluted 1:1 with RPMI-1640 medium in 15 ml falcon tubes, and 10% dimethyl sulfoxide (DMSO) were ultimately added to them. The prepared samples were divided and transferred to 1.5 ml Eppendorf tubes and labelled accordingly. These samples were then stored in -80^{o}C freezers.

2.7.2 Lymphocyte isolation from blood samples

Blood was mixed 1:1 ratio with 0.9% normal saline and slightly mixed. In a 15 ml falcon tube, 3 ml of lymphoprep was added and 6ml of the mixture of blood and saline was gradually added to it and centrifuged at room temperature (RT) for 20 min at 600 x *g*. Following centrifugation, the white coat present obtained from the plasma was extracted and added to a falcon tube containing 10ml saline and centrifuged for 375 x *g* for 15 min at RT. The pellet produced was resuspended in RPMI medium (RPMI 1640) and 10 % of DMSO and ultimately transferred to an Eppendorf tube and stored at -20 °C overnight, or at m-80 °C for prolonged storage purposes. The supernatant was added to 2% virkon and discarded.

2.7.3 Preparation of cell culture medium

The cell culture medium was practiced under sterile conditions, in a fume cabinet. The media comprised 500ml of Dulbecco's Modified Eagle's with 10 % foetal bovine serum (FBS), and 1% Penicillin-streptomycin, aliquoted in 75-cm^2 cell culture flasks.

2.7.4 Cell culturing of CHL-1 cells

The (CHL-1) cells were taken out of liquid nitrogen and thawed in a 37°C water bath for nearly two minutes and decontaminated with 70% ethanol. All the steps were conducted in a strictly aseptic condition. The contents of the vial were later transferred to a centrifuge tube containing 9.0 ml of the above-mentioned culture medium and centrifuged at *125 x g* for 5 minutes. The supernatant was discarded, and the cells were added to a T 75 cm^2 flask and incubated at 37°C and 5% CO_2. After the adhesion of the cells, the medium in the flask was removed and rinsed with PBS two times. Next, 1- 2 ml of 0.25 % Trypsin/EDTA was added and incubated for a few minutes until cells detached. Fresh medium was added with a sub-cultivation ratio of 1:10 in the culture flasks and incubated. When cells were confluent 80- 90%, they were passaged once and added to a new culture flask washed, trypsinised and the medium changed. The cells were then counted using a haemocytometer. After cells were adherent, they were again washed, trypsinised and 1.2 x 10^6 cells/ml were added to a 6 well plate. The first well plate was left untreated as a negative control, the second well plate was irradiated with UVA+B 1.2mW/cm^2 (15 min). The radiation dose was provided by using a lamp that consisted of two Waldmann F15/T8-PUVA tubes (Villingen-Schwennigen, Germany) in a box casing for 15 minutes as a positive control. The treatment groups comprised 300 µg/ml protein of CBSC exosomes (equivalent to 40 x10^7 particles/ml), and the second group comprised UVA+B treated cells co-treated with 300 µg/ml protein CBSC exosomes and incubated for 48h at 37°C and 5% CO_2. The cells were again washed, trypsinised and centrifuged *200 x g* for 5 minutes.

The supernatant was discarded, and the cells were additionally washed twice with cold PBS and centrifuged to re-suspend the pellet, cells were counted and finally placed in cryovials with 10% FBS and 5% DMSO and frozen gradually at 1°C/min or in an insulated box in a –70°C to –80°C freezer.

2.7.5 Cell treatment

The stored frozen blood samples were allowed to thaw at room temperature. Next, 100 µl of whole blood samples were incubated for 30 minutes at 37°C with varying drug concentrations, to achieve the final volume of 1000 µl with RPMI 1640 media (With sodium bicarbonate, without L-glutamine, liquid, sterile-filtered, suitable for cell culture). The test compounds used comprised a positive control of 75 µM H_2O_2, an exosome concentration of 300 µg/ml protein and negative untreated control. Following incubation, the tubes were centrifuged for 3 min at 160 x *g*, and later 900 µl of the supernatant was discarded. At this stage, the frozen cell pellets were also allowed to thaw at room temperature and the next steps, performed were similar to the whole blood. The remaining pellet was re-suspended by 100µl of 0.5% low melting point agarose (LMP) and added on pre-coated 1 % normal melting point agarose (NMP) labelled slides. The slides were finally placed on an ice tray for a few minutes to form a gel consistency and covered with coverslips.

2.7.6 Cell lysis

The coverslips were removed, and the slides were submerged in a cold lysis solution that was prepared earlier containing high salt and detergent content (100mM EDTA, 2.5M NaCl, 10mMTrizma base, 10% DMSO and 1% Triton X-100at pH 10). The high salt and detergent content of the solution caused the breakdown of the protein-DNA bond.

2.7.7 Electrophoresis

After cell lysis, the slides were placed horizontally on a gel electrophoresis tank and incubated in a fresh cold alkaline buffer of 2000 ml of which 70 ml consisted of (10M NaOH and200mM EDTA, at pH >13) and the remaining 1930 ml contained cold distilled water, for 30 minutes at 4°C, to initiate the unwinding of the DNA, respectively. After 30 minutes, electrophoresis was performed using 25V (0.78 V/cm) of constant voltage and 300 mA of current at 4°C.

2.7.8 Neutralisation

The slides were later removed from the electrophoresis tank and placed horizontally on a tray and covered with neutralising buffer (0.4M Trizma base-HCl, pH 7.5). This buffer was added three times for 5 min.

2.7.9 Staining of cells

The slides were stained with 60 µl of Ethidium bromide (20 µg/ml), on each slide and then covered by coverslips, in a dark room. To avoid the drying of cells, the cells were left in a damp black box and placed at 4°C, for later scoring.

2.7.10 Comet scoring

The cells were scored by randomly selecting 100 nuclei per slide using a fluorescence microscope outfitted with a CCD camera using Komet 6 software, Kinetic Imaging (Andor Technology Ltd, Belfast), attached to an image computer software. For the evaluation of each slide, a fluorescence microscope with a 20 X magnification was used. The data parameters namely % Tail DNA and Olive tail moment were used to express DNA damage.

2.7.11 Statistical analysis

The data from the Comet assay revealed the mean of 100 nuclei per slide with standard errors. The Comet assay comprised three healthy and three melanoma patients and three biological sets of CHL-1 cells ($n = 3$). The mean values were expressed as the final data with error bars. A one-way ANOVA was performed with multiple comparisons against the negative control to compare each sample to the negative control by using the GraphPad prism 9. The significant p values were $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) respectively.

2.8 Real-time quantitative PCR

Fresh blood was used for the real-time quantitative PCR from healthy volunteers as well as melanoma patients with appropriate consenting.

2.8.1 Cell culture

The frozen pellets were allowed to thaw at room temperature 37°C. The treated cells were in a suspension of 2×10^6 cells/ml prior to being cryopreserved.

2.8.2 RNA isolation from CHL-1 cells

Following the RNeasy Mini Kit (QIAGEN) manufacturer's protocol, the cells were placed in a sterile fume cabinet and centrifuged at 400 x *g* for 3 minutes. The supernatant was removed and 350 µl of lysis buffer (RLT) (10µl of 2-mercaptoethanol per 1 ml buffer RLT) was added to the pellet and centrifuged for 2 minutes at high speed. The lysate was then added to a QIAshredder spin column that was already placed in a 2 ml collection tube and spun at full speed for another 2 minutes.

Next, the lysate was kept and the QIAshredder was discarded. Later, 600 µl of 70% ethanol was added to the lysate and mixed well by careful pipetting. The pipetted samples along with any precipitates formed were then transferred into a new QIAamp spin column placed in a 2 ml collection tube and centrifuged for 15 s at 8000 x *g.* Moving on, the QIAamp spin column was rinsed by adding 700 µl of RW1 buffer (guanidine salt and ethanol) and centrifuging for 15 s at 800 x *g*. The QIAamp spin column was positioned in a new 2 ml collection tube. next, 500 µl of buffer RPE was pipetted into the QIAamp spin column and centrifuged for 8000 x *g* for 3 min to remove any ethanol present in the samples. Eventually, the QIAamp spin column was transferred into a 1.5 ml microcentrifuge tube and 50 µl of RNase-free water was added directly onto the QIAamp membrane to elute the RNA and spun at 8000 x *g* for 1 minute. The concentration and purity of RNA were measured using a NanoDrop ™ 1000 spectrophotometer device. The purity and concentration of RNA were circulated by a 260/280 ratio. The RNA samples were ultimately stored at -80ºC.

2.8.3 Complementary DNA synthesis

The iScript cDNA synthesis kit was used to synthesise complementary DNA (cDNA) from total RNA. This was done by mixing 4 µl of 5xiScript Reaction mix with 1 µl of iScript Reverse Transcriptase, 2 µl of Nuclease-free water and 1 µl of the thawed extracted RNA, in PCR tubes. The reactions were then transferred to a Bio-Rad PTC-200 Peltier Thermal cycler (Bio-Rad Laboratories, Inc. Hercules, CA, USA), and incubated for 5 min at 25°C, then at 20 min at 46°C, and finally for 1 min at 95°C, with the samples being additionally held for 10 min at 4°C. The samples were finally stored at - 20°C

2.8.4 The quantitative real-time PCR method

Following cDNA synthesis, the gene of interest was quantified by adding the real time-PCR reactions in a 96 well plate (MicroAmp TM), sealing with MicroAmp® Optical 8-Cap Strips. The assay was conducted in triplicates to minimize variation in the results. In each well, 10 µl of Fast SYBRTM Master Mix, 2 µl of target gene primer (TP53, p21, BCL-2, caspase 3 and β-actin), 4 µl of diluted cDNA and 4 µl of RNase-free water were mixed. For normalization of the reaction, β-Actin primer was used as a housekeeping gene. The house keeping gene is used as a reference point for the analysis of expression levels of other genes. The 96 well plates were sealed using MicroAmp™ optical 8 Cap Strips and spun in a plate spinner to remove any bubbles present. The plates were finally. The plates were then placed in StepOnePlus Real-time PCR Detection System (Applied Biosystems, Warrington, UK), with the following set up denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1minute. Ultimately, a melting curve of 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds.

2.8.5 Data analysis

The data was assessed using CFX Manager™ Software to evaluate the expression of the gene of interest against the housekeeping gene (β-actin). The 2-ΔΔCT was used to depict the differences in fold-change of genes expression between the control group and cells treated with UVA+B (1.2 mW/cm^2) and as a positive control, 300 µg/ml protein of CBSC exosomes alone and co-treated with UVA+B (1.2 mW/cm^2) (Schmittgen and Livak, 2008).

2.8.6 Statistical analysis

Each experiment was conducted three times in three biological samples and the results were illustrated as mean ± SEM. Data analysis was done using the GraphPad Prism® software, version 9, with two-way ANOVA and multiple comparison tests. The significant p values were $p < 0.01$ (**), and $p < 0.001$ (***), and $p < 0.0001$ (****), respectively.

2.9 Western blot assay

2.9.1 Cell culture

Three biological samples of CHL-1 cells with a concentration of 1×10^6 cells were cultured in six-well plates with Dulbecco's Modified Eagle's medium, 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin under aseptic conditions. The plates were incubated at 37°C and 5% CO_2 until confluent. The cells were then treated and labelled. The treatment group comprised 300 µg/ml protein of CBSC exosomes, and the second group comprised UVA+B treated cells co-treated with 300 µg/ml protein CBSC exosomes. The negative control group was left untreated.

2.9.2 Protein extraction

The cultured cells were scraped off in Eppendorf Tubes® and centrifuged at 400 x *g* for 3 minutes. The supernatant was removed, and the cell pellets were rinsed twice with cold PBS. The cells were then re-suspended in 100 µl RIPA buffer, and a 2 µl protease inhibitor cocktail and the cells were incubated on ice for 30 minutes. Following that, the cell suspension was quickly sonicated (~5 seconds) and centrifuged at 16 000 x g for 20 minutes at 4°C. Next, the supernatant was collected and 10 µl of the extracted protein was taken for protein quantification and the remaining lysate was stored at -80ºC.

2.9.3 Protein quantification

The quantification of total protein was done by the Bradford assay kit (Bio-Rad). The assay depends on the absorbance shift the in colour switch of Coomassie dye that changes from red to blue when a protein binds to it. The blue colour dye has a maximum absorbance rate of 595 nm. The absorbance rate is directly proportional to the amount of dye and the concentration of protein present. In this technique, the standard curve was represented by Bovine Serum Albumin (BSA) with varying concentrations of (0.125, 0.250, 0.5, 0.750, 1.00, 1.5 and 2 mg/ml). Ten microliters of each unknown sample and standard were added in triplicates in separate 96 well plates and 200 µl of diluted Biorad dye reagent. The absorbance of Bovine serum albumin (BSA) standards and unknown samples were read at 595 nm.

2.9.4 Sodium dodecyl sulphate polyacrylamide gel (SDS- PAGE)

For resolving and stacking gels Tris buffer was prepared (pH 6.8 & pH 8.8). Catalyst APS and TEMED were also added with a final concentration of 10.4% for the polymerization of polyacrylamide gel.

A resolving gel with a pH of 8.8 was poured at the bottom of the tank, to a level of 1 cm below the teeth of the comb and 0.1% (w/v) SDS solution was poured on top of it. Next, a 4% (w/v) stacking gel solution with a pH of 6.8 was poured after the gel had set in and solidified. The protein samples were prepared by mixing each protein sample with 2x sample buffer (1:1) and then heated in boiling water for 5 minutes. Laemmli buffer 2x used contained 2-mercaptoethanol that reduced the disulfide bonds. Due to the SDS detergent used, the proteins were separated depending on their size and not their charge.

Bromophenol dye was also used due to its blue colour to make the samples distinguishable. Next, the samples were briefly centrifuged and loaded into separate lanes. The pre-stained ladder was loaded in the first lane for reference purposes. Power Pack™ Basic (Bio-Rad, UK) was used to run the gels.

The gels were run at 50 V initially, giving sharp bands and then increased to 100 V (constant voltage). By visualizing the pre-stained ladder and the position of the dye in the tank, the end of the electrophoresis was determined. The gels were then submerged in the electrophoresis buffer (25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS). Next, 30 μg of proteins were loaded in each well carefully to prevent sample leaking. In each gel, the first well contained 10 μl of the pre-stained ladder (Precision Plus Protein™ Dual Color Standards). The electrophoresis buffer tank ran for ~ 2 hours at a power supply of 100V (constant). The SDS gel was used to separate the proteins based on their size i.e., low percentage gels are used to distinguish larger proteins and high percentage gels are used to separate smaller sized protein molecules. The SDS also neutralized the charge differences of the proteins.

2.9.5 Membrane transfer

Following electrophoresis, proteins were transferred to a blotting nitrocellulose membrane using the iBlot® Gel Transfer Device (Invitrogen) at a constant voltage of 25 V for 7 minutes.

2.9.6 Blocking

Following the transfer, the nitrocellulose membranes were incubated in 5% (w/v) BSA in Tris-buffered saline containing Tween 20 (TBS-T) (150 mM NaCl, 20 mM Tris base and 0.1% (v/v) Tween 20, pH 7.4) for one hour at room temperature on a gentle shaker. The membranes were washed thrice with TBS-T for 10 minutes.

2.9.7 Primary antibody

After blocking, a primary antibody was added to the mixture that could bind to the target protein. The membranes were incubated overnight at 4ºC with primary antibody over a gentle shaker. GAPDH monoclonal antibody was used as the housekeeping control protein. A monoclonal antibody was used for its higher specificity and a better signal. The dilutions of the primary antibodies were as follows GAPDH (1:10,000 dilution), p53 (1:1,000 dilution); P21(1:1,000 dilution); BCL-2 (1:1,000 dilution); Caspase 3 (1:1,000). These solutions were diluted with TBS-T containing 5% (w/v) BSA. Membranes were then washed thrice with TBST for 10 minutes.

2.9.8 Secondary antibody

A secondary antibody was added to determine where the primary antibody was located. A secondary antibody Donkey Anti-Rabbit IgG H&L (HRP) was used and diluted with 1:3,000 with 5% (w/v) BSA in a TBS-T solution. The membrane was incubated for one hour on a gentle shaker at room temperature. The membrane was finally washed with TBS-T thrice for 10 minutes.

2.9.9 Detection

The membrane was stained with a chemiluminescence (ECL) kit, by incubating the membrane witan h equal proportion of ECL membrane for a few minutes at room temperature. The membrane was placed on the G box (Gene flow, UK) and the excess detergent was removed. Images were finally captured.

2.9.10 Data analysis

The expression of the protein was assessed by image j software. GAPDH was served as the loading control. The data were presented as mean ± SEM of three biological samples. The data were analysed using a one–way ANOVA followed by Dunnet‘s post hoc test to determine the significance between the treatment groups by GraphPad Prism® 9.

Chapter three: The Comet assay in peripheral lymphocytes and CHL-1 cells after treatment with cord blood-derived exosomes.

3 Comet assay

3.1 Introduction

Chronic exposure to UV triggers the formation of reactive oxygen species that lead to DNA damage, modifications in protein synthesis and lipid peroxidation (Wu et al., 2021). Evidence shows that in the presence of redox homeostasis with increased ROS, exosomes are capable of transferring their bioactive cargo to the recipient cells (Zhou et al., 2017). Previous studies have demonstrated the positive effects of human umbilical cord exosomes on wound healing, hepatic fibrosis, and cardiac ischaemia (Zhang et al., 2015, Yan et al., 2017, Zhao et al., 2015). Furthermore, in malignant melanoma exosomes have been shown to reprogram the malignant phenotype into a benign phenotype. This was considered to be due to the cargo of exosomes that comprised certain factors that exhibited antitumorigenic properties (Zhou et al., 2017). In breast cancer, it was observed that exosomes not only inhibited the progression of cancer but also changed the morphology of cancer cells (Li et al., 2014). Moreover, it is also suggested that human cord blood exosomes are able to exert antitumorigenic effects on cancer cells by reprogramming them to a pluripotent level and re-establishing their normal pathways (Kim and Zaret, 2015).

Since UV radiation is considered a significant risk factor in the induction of melanoma, and one of the major genotoxic agents in photochemical modification of the DNA (Godic et al., 2014). As H_2O_2 and UV are chemical and physical stressors, hence, the results are more consistent with UV in causing DNA distortions. Therefore, in this project, UV was used as a co-treatment with CBSC exosomes in melanoma CHL-1 cells, to induce ROS and to evaluate whether exosomes exert protective or damaging effects in target cells under oxidative stress.

The Comet assay or single cell gel electrophoresis is the commonly used method to detect DNA damage in cells both in cells and lymphocytes *(*Wu and Jones, 2012*)*. Research has exemplified the Comet assay by its rapidity, sensitivity, and cost-effectiveness in assessing DNA damage (Cortés-Gutiérrez et al., 2011). The OTM and % DNA in the tail are the two parameters of the Comet assay that evaluate the damaged DNA (Intranuovo et al., 2018). The OTM is calculated as a product of two factors: the percentage of DNA in the tail (%Tail DNA) and the distance between the intensity centroids (centres of gravity) of the head and the tail along the x-axis of the comet.

In this study, the effect of CBSC exosomes on lymphocytes obtained from healthy individuals and melanoma patients as well as on CHL-1 cells was assessed by using the Comet assay. Melanoma CHL-1 cells were used in this study, due to the fact that these cells possess either mutated or deleted *TP53* gene, therefore in order to evaluate the expression of apoptotic mRNA and proteins through p53-mediated pathways exerted by CBSC exosomes, this particular cell line was chosen.

Previous studies have indicated that H_2O_2 is considered a genotoxic agent that induced nuclear abnormalities (Camargo et al., 2014). Hydrogen peroxide increases the reactive oxygen species by diffusing in the cell membrane through the aquaporins producing hydroxyl-free radicals (•OH) that in return lead to single-strand DNA breaks (Olson, 1988). Hence, due to its genotoxicity (75 μM) of hydrogen peroxide was used as the positive control in the Comet assay. Similarly, UVA and UVB are also considered genotoxic by yielding high levels of reactive oxygen species in the skin that results in DNA damage (Baczynska et al., 2013). Therefore, in CHL-1 cells, 1.2mW/cm^2 (UVA+B) (15 min) was used as the positive control. The dose-response experiments were conducted to determine the optimal dose of cord blood exosomes. An optimum dose of 300 μg/ml protein was used throughout the study. Melanoma CHL-1 cells were additionally co-treated with UVA+B.

3.2 Materials and Methods

The reagents used in the CCK-8 and the Comet assays are all listed in Tables 3 & 4. The Comet and the CCK-8 assays are described in Chapter 2 under sections 2.6 & 2.7 respectively.

3.3 Results

3.3.1 Particle size determination of CBSC-exosomes

The CBSC exosomes were characterized by a TEM scan, due to its simplicity, specificity, practicability, and ability to distinguish exosomes from the other microvesicles present in the biological matrix. CBSC derived exosomes showed various diameters in the given sample (Figure 10).

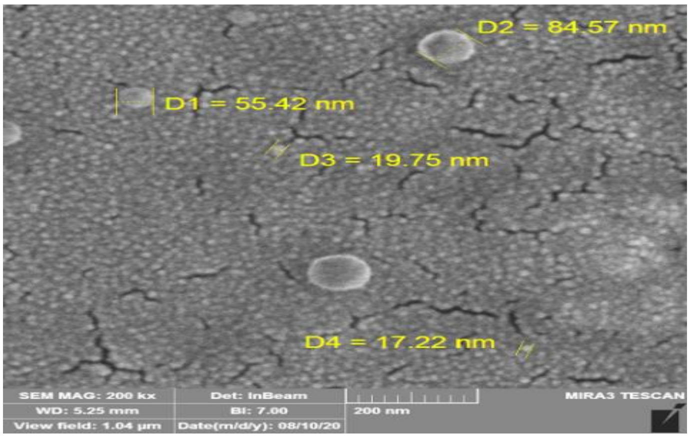


Figure 10 The different diameters of exosomes were detected by MIRA 3 Tescan at 200 kV magnification.

The surface morphology of exosomes was evaluated by scanning electron microscopy (SEM), with CBSC exosomes showing a uniform morphology, spherical bioparticles, with diameters ranging from 30 to 120 nm (50, 150 and 250 kV magnification) (Figure 11).

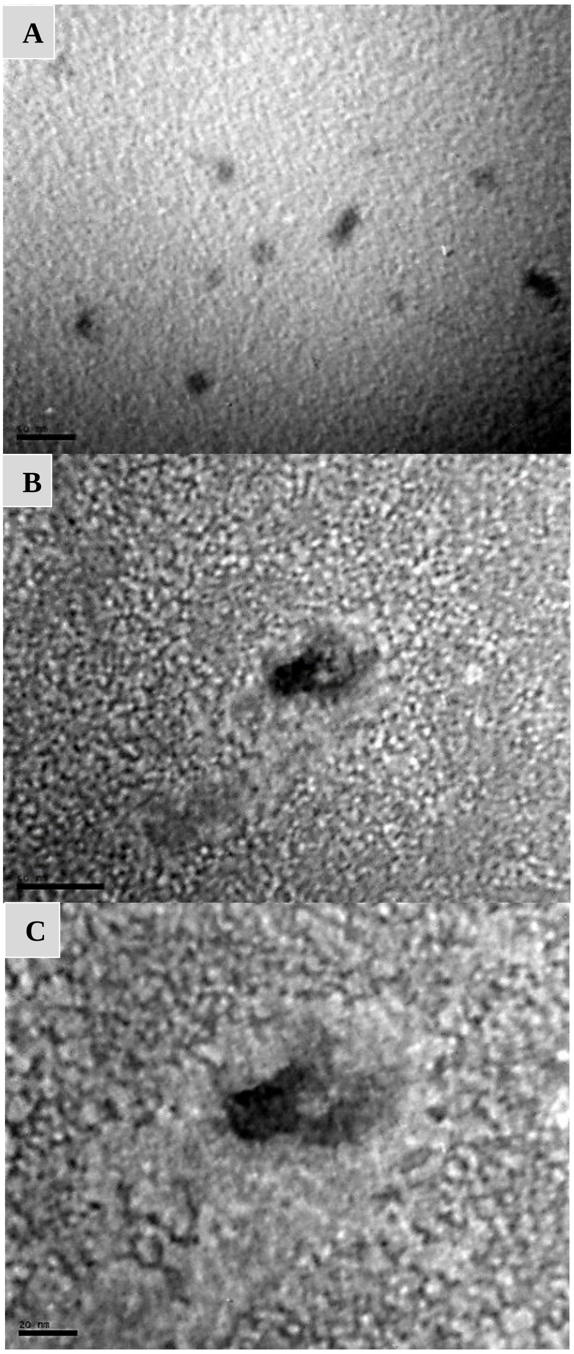


Figure 11 SEM representation of morphology of cord blood-derived exosomes with 50,100 and 250 kV magnification.

3.3.2 CCK-8 results

The viability of lymphocytes obtained from 3 healthy individuals and 3 melanoma patients, together with CHL-1 cells (three biological samples) were detected by the CCK-8 kit.

3.3.3 CBSC exosomes caused a significant time and dose-dependent decrease in the cell survival rate of CHL-1 cells and lymphocytes obtained from melanoma patients compared to healthy individuals.

The viability of lymphocytes obtained from melanoma patients and healthy individuals, as well as CHL-1 cells, were assessed by the CCK-8 assay after treatment with varying concentrations of exosomes (0, 100, 200, 300, 400 μg/ml protein) over 24, 48 and 72 hours, respectively (Figure 12A, 12B & 12C). The results showed a significant decrease in the viability of lymphocytes obtained from melanoma patients and CHL-1 cells treated with exosomes of 100, 200, 300 and 400 μg/ml protein at 24, 48 and 72 h (****$p < 0.0001$) ($n = 3$). The overall results contrasted with the high survival rate observed in healthy lymphocytes treated with exosomes as the data showed no significant cytotoxicity of the CBSC exosomes against the healthy lymphocytes throughout the different incubation periods of 24, 48 or 72 h (p = ns) ($n = 3$). By comparing the significant reduction in percentage survival rate at 24, 48 and 72 h; 300 μg/ml protein (equivalent to 40×10^7 particles/ml) at 48 h was chosen as the optimal dose with 51 per cent cell viability.

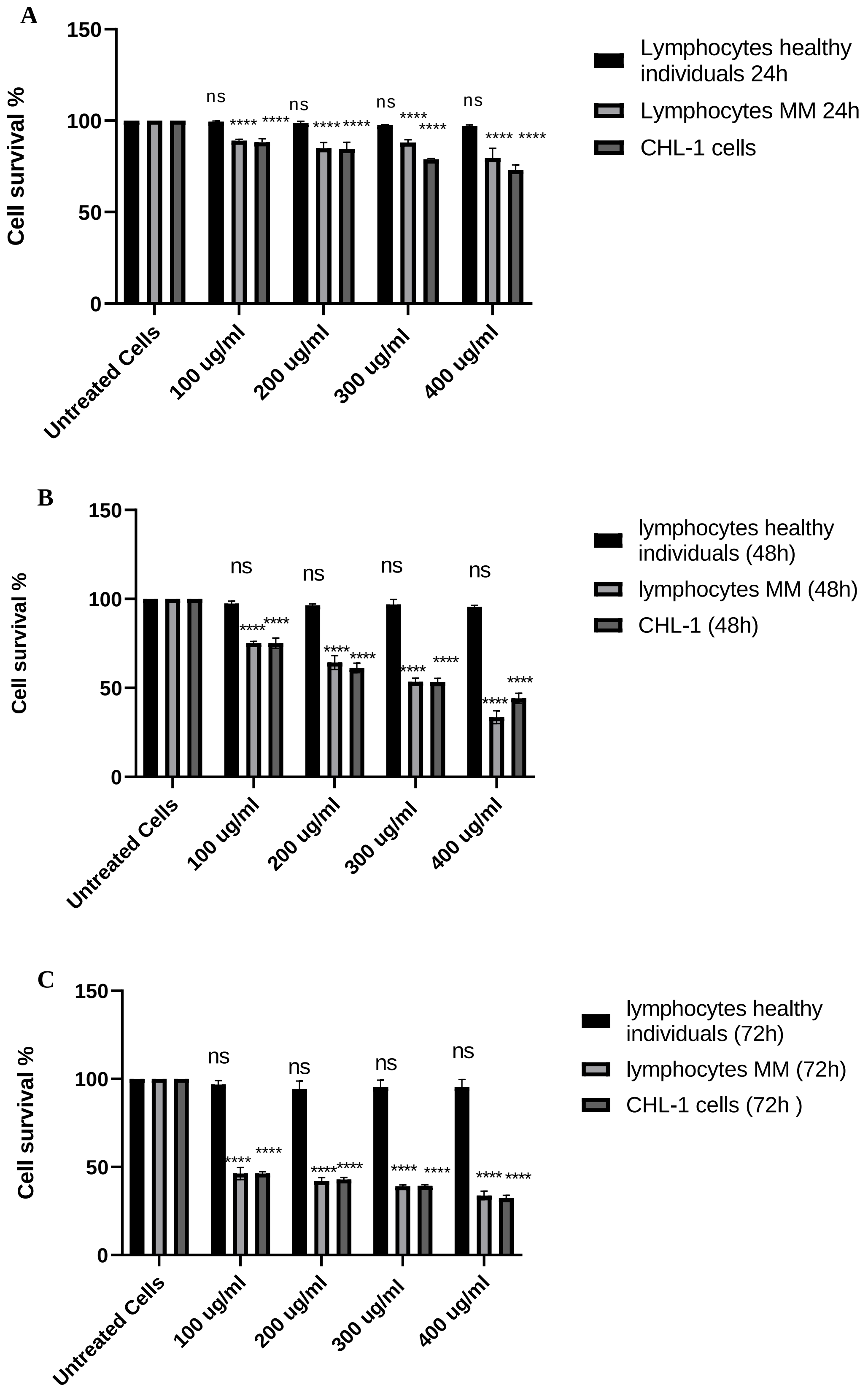


Figure 12 (A, B, C) Viability in 10 x 10^4 cells from lymphocytes of healthy individuals, lymphocytes of malignant melanoma patients (MM) and melanoma (CHL-1) cells, incubated

separately were treated with various concentrations of 0, 100, 200, 300 and 400 µg/ml protein of CBSC exosomes for 24, 48 and 72 h, (A, B and C respectively (n= 3)). Cell proliferation analysis was performed by CCK-8 assay. Errors bars represent SEM. (p = ns (not significant), ****$p < 0.0001$), data was compared to the untreated cells and analysed by one way ANOVA.

3.3.4 The Comet assay results

3.3.5 Determination of genotoxicity of CBSC exosomes in the lymphocytes of healthy individuals

The genotoxicity of CBSC exosomes at 300 µg/ml protein was evaluated on lymphocytes obtained from healthy volunteers and melanoma patients. As a positive control 75 µM H_2O_2 was used on the lymphocytes. The parameters %Tail DNA and OTM (Olive Tail Moment) characterise the DNA damage.

In the healthy volunteer group, results showed no significant DNA damage in the healthy lymphocytes after treatment with CBSC exosomes (Figure 13), compared to their untreated control (p = ns) ($n = 3$). This was observed for both parameters. The positive control (PC) 75µM H_2O_2 showed high DNA damage (***$p \leq 0.001$) compared to the untreated negative control group (NC) (Figures 13 A & B).

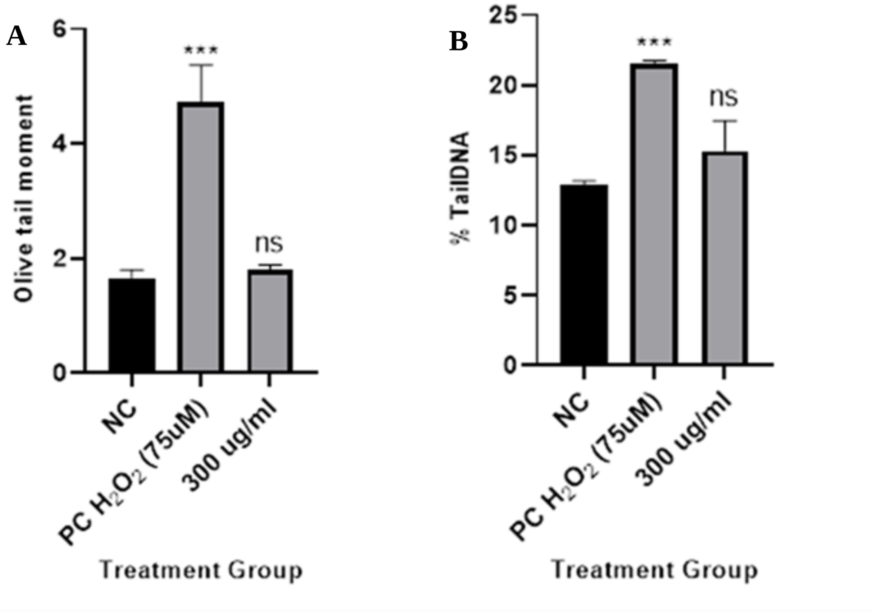


Figure 13 (A & B) The concentration-response of 300 µg/ml protein of CBSC exosomes in lymphocytes from healthy individuals using OTM and % Tail DNA. 100 random cells were counted for each treatment, including negative control (NC), positive control (PC), H_2O_2 75 µM and 300 µg/mL protein of exosomes. All the treatment groups were compared to the NC (***P < 0.001; ns means not significant evaluated by one-way ANOVA) (n = 3).

3.3.6 Determination of genotoxicity of cord blood-derived exosomes in the lymphocytes of melanoma patients

While the main aim of this study was to assess the anti-tumourigenicity of CBSC exosomes in cancer cells. The genotoxic potential of exosomes was also determined in lymphocytes of melanoma patients.

The lymphocytes obtained from melanoma patients revealed a significant increase (%Tail DNA) of 21.2% and 3.95 (OTM) compared to their respective untreated negative group (NC) of 12.1% (%Tail DNA) and 2.19 (OTM) (*p ≤ 0.05) (n= 3) (Figure 14). Highly significant DNA damage (***P < 0.001) was observed for the positive control at H_2O_2 75 µM when compared to the untreated control (NC).

Unlike the healthy lymphocytes, the % Tail DNA in the lymphocytes of melanoma patients illustrated significant DNA damage in the exosome treated group, compared to their untreated control (*p < 0.05) (n = 3) (Figure 14 A & B).

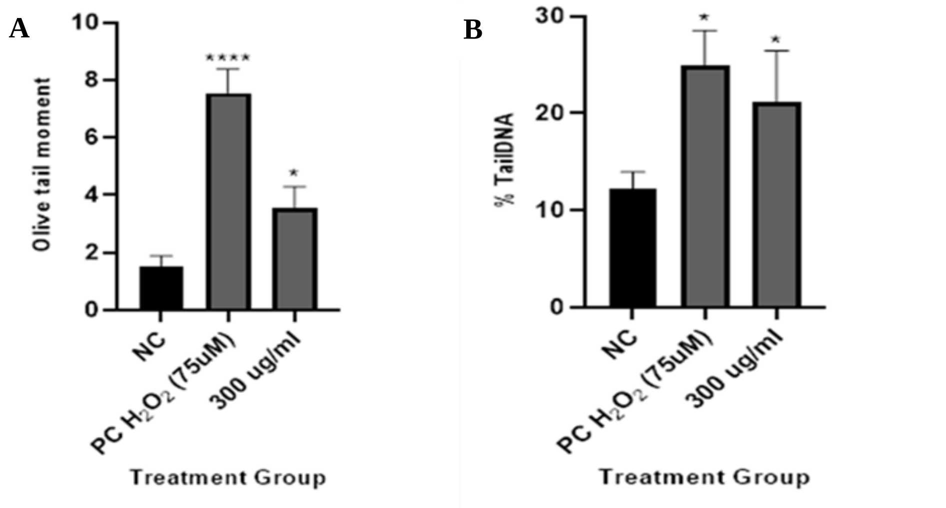


Figure 14 (A & B) The concentration-response of 300 µg/ml protein of CBSC exosomes in lymphocytes from melanoma patients using OTM and % Tail DNA. 100 random cells were counted for each treatment, including negative control (NC), positive control (PC), H_2O_2 75 µM and 300 µg/ml protein exosomes. All the treatment groups were compared to the NC (*p <0.05; ****p < 0.0001; evaluated by one-way ANOVA) (n = 3).

3.3.7 CBSC exosomes concentration responses on melanoma CHL-1 cells, both alone and in combination with UVA+B radiation

The genotoxic effects of CBSC exosomes on CHL-1 cells (3 biological samples) were analysed, both in isolation and co-treated with UVA+B radiation (1.2 mW/cm^2), using the Comet assay. The CHL-1 treated cells with 300 µg/ml protein of exosomes showed a significant increase of 19.3 % (% Tail DNA) and 3.54 (OTM) compared to their untreated control of 12.1 % (%Tail DNA) and 1.77 (OTM), respectively (*p ≤ 0.5, **p ≤ 0.01). Moreover, CHL-1 cells treated with 300 µg/ml protein of exosomes co-treated with 1.2 mW/cm^2 UVA+B revealed 19.04% (% Tail DNA) and 3.6 (OTM) that was not significant compared to the PC (1.2 mW/cm^2 UVA+B) (p = not significant) (Figure 15 A & B).

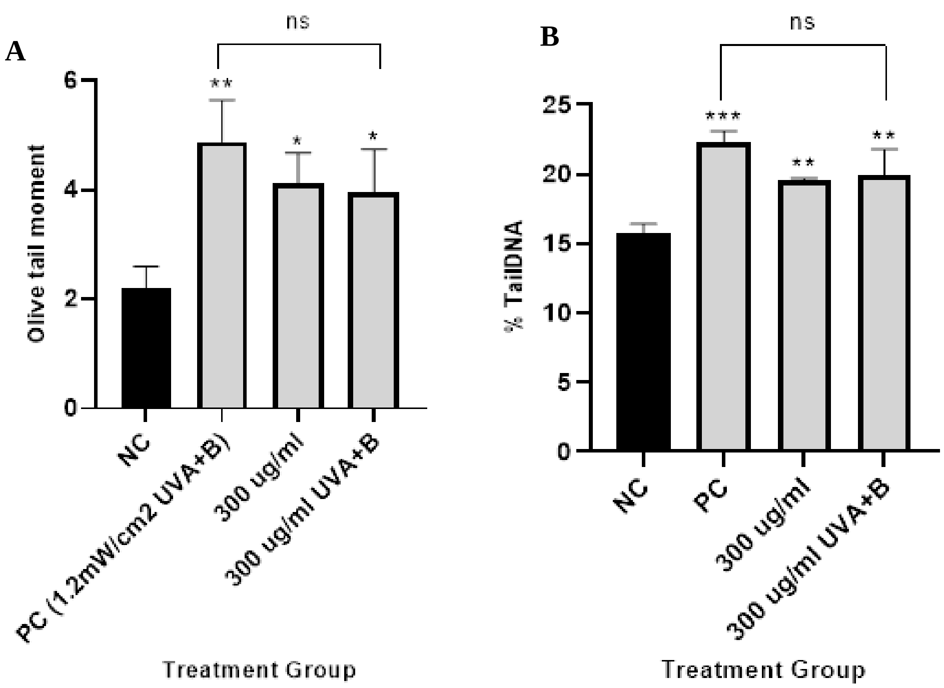


Figure 15 OTM and % tail DNA (**A** and **B**, respectively) data show the effect of CBSC on melanoma CHL-1 cells (3 biological samples) after treatment with an exosome concentration of 300 µg/ml protein alone and in combination with UVA+B 1.2 mW/cm^2.

100 random cells were counted for each treatment, including negative control (NC), positive control (PC), UVA+B 1.2 mW/cm^2 µM and 300 µg/ml protein exosomes alone and in combination with UVA+B 1.2 mW/cm^2. All the treatment groups were compared to the NC (p = not significant, *$p < 0.05$; ** $p < 0.01$, ***$p < 0.001$; evaluated by one-way ANOVA). The treatment group co-treated with UVA+B was compared to the PC (p = not significant).

3.4 Discussion

The present study investigated the viability (CCK-8 assay) and DNA damage (Comet assay) of CBSC exosomes in lymphocytes from healthy individuals and melanoma patients but also melanoma (CHL-1) cells. Previous investigations have illustrated that exosomes are involved in intercellular communication, cell maintenance and cell signalling. To date, numerous studies have been published both *in vitro* and *in vivo* that indicated promising effects of exosomes in tissue repair, regeneration, and treatment of multiple chronic diseases. Furthermore, the antitumorigenic effect of exosomes from different origins in the treatment of several carcinomas including tumour growth suppression in multiple myeloma and bladder cancer is well established (Karaoz et al., 2019).

Research has revealed the potency of exosomes derived from the human umbilical cord in mitigating bladder tumour growth both *in vitro* and *in vivo*. It was observed that these EVs possessed anticarcinogenic abilities that impeded the proliferation of cancer cells by inducing programmed cell death (Wu et al., 2013). Although, research has highlighted the antitumorigenic effects of exosomes in different cancer types.

Nevertheless, the genotoxic effects of cord blood-derived exosomes on the lymphocytes of melanoma patients and melanoma cancer cells by using the Comet assay, have not yet been investigated. Hence, this study aimed to evaluate the DNA damage caused by CBSC exosomes on lymphocytes from melanoma patients and healthy individuals as well as melanoma cancer cells (CHL-1). The Comet assay was used in this investigation for evaluating DNA damage in cells (Gopalan et al., 2011).

Since the cells in the circulatory system are at constant risk of oxidative stress due to exogenous and endogenous mutagens that could increase the chances of carcinoma, hence, a few studies have stated the importance of peripheral blood lymphocytes, as a biomarker in the detection of different types of cancers (Anderson et al., 2014). In addition, it has also been suggested that lymphocytes, due to their availability in the blood, could serve as ideal surrogates, in evaluating the different types of DNA damage that exist in various stages and types of carcinomas (Najafzadeh et al., 2012). A similar study was conducted to determine the DNA damage in lymphocytes caused by food mutagens in patients with inflammatory bowel disease (IBD), and high concentrations of ROS were observed in lymphocytes of IBD patients compared to that of healthy volunteers (Najafzadeh et al., 2009).

The major traits of cancer progression include continuous proliferation and resistance to apoptosis which is caused by recurrent changes in the genome. Most cancer cells proliferate more than the others, hence targeting their proliferation is of utmost importance. The aim of targeting their cellular division is to induce apoptosis by using cytotoxic drugs (Swift and Golsteyn, 2014).

Most of the cytotoxic drugs frequently used to treat cancer induce high levels of DNA damage on tumour cells that leads to apoptosis or cell cycle arrest (Helleday et al., 2008).

Evidence suggests that exosomes isolated from natural killer (NK) cells are capable of exerting cytotoxic effects and inhibiting the MAPK pathway in mouse melanoma cells (B16f10) (Zhu et al., 2017). Similarly, the anti-oncogenic effect of mesenchymal stem cell exosomes was observed to reprogram cancer cells to benign phenotypes by transferring their cargo to the target cells. The re-activation of suppressed differentiation pathways and suppression of tumourigenicity was indicated to be the reason leading to the differentiation into a benign phenotype (Zhou et al., 2017). Despite mounting evidence for the antitumorigenic role of exosomes in multiple types of cancer, to date, no investigation has been performed to determine the effect of exosomes on lymphocytes from cancer patients.

The role of exosomes in maintaining cellular homeostasis and cell survival is still unclear and needs further investigation. However, the role of intercellular communication of exosomes in contributing to the normal physiology of the nervous system mediated by calcium influx has been established (De Toro et al., 2015). The cardiovascular system is also highly dependent on exosomes for their normal function. During hypoxic conditions, the release of these nanoparticles is enhanced to induce an inflammatory response in stressed cells (Isola and Chen, 2017). Controversial studies have been published that have discussed the role of exosomes in cellular stress.

Exosomes are considered to play a protective role in H_2O_2 induced oxidative stress in mouse mast cells, compared to the cells under normal physiological cues (Eldh et al., 2010). On the contrary, in cisplatin incorporated exosomes, an increase in ROS levels in the surrounding cells has been observed in acute renal injury (Zhou et al., 2013). It is suggested that the protective or harmful effects of these nanoparticles in redox homeostasis depend on their cargo and origin.

In this project, the effect of exosomes on lymphocytes from healthy individuals and melanoma patients was evaluated. In lymphocytes obtained from melanoma patients, a significant decrease in cell survival rate was noticed which was confirmed by the DNA damage observed in the OTM and % TailDNA of the Comet assay. This could potentially be due to the limited number of available samples; therefore, more investigation is required to confirm the effect of exosomes on the viability and genotoxicity of lymphocytes. Conversely, no such effects were observed in the lymphocytes of healthy individuals. This could possibly be due to their cell-to-cell communication ability to transfer their bioactive cargo that depends on the needs of the recipient cells. Their cargo comprises lipids, proteins, and DNA (both mitochondrial and genomic) such as mRNA, microRNAs (miRNA), and long noncoding RNAs (lncRNAs) (Von Schulze and Deng, 2020). The genotoxic effect of CBSC exosomes on the melanoma CHL-1 cells could be due to their ability to inhibit the pathways responsible for cancer progression.

For instance, in bladder carcinoma, exosomes isolated from Wharton jelly (the gelatinous substance within the umbilical cord, made up of mucopolysaccharides such as hyaluronic acid and chondroitin sulfate. It also contains some fibroblasts and macrophages) are indicated to suppress the Akt pathway to facilitate apoptosis (Wu et al., 2013). Taken together, our data illustrated a significant decrease in the survival rate of lymphocytes obtained from melanoma patients and melanoma CHL-1 cells treated with CBSC exosomes in a time and dose-dependent manner. Similarly, significant DNA damage was observed in both of these groups, in addition to the ROS-induced UV co-treated with exosomes, by the Comet assay. This was in contrast to the results obtained from lymphocytes of healthy individuals that showed no significant reduction in the cell survival percentage and no DNA damage was observed. Our results agree with the previously published papers that suggest exosomes unload their cargo to the recipient cells depending on their need, by inhibiting the pathways responsible for cancer growth and progression (Wu et al., 2013). Whether the exosomes exhibit anticancer properties by disrupting the redox homeostasis that increases oxidative stress in cancer cells by eventually directing them toward apoptosis, is still a matter of debate and needs further elucidation.

Chapter four: Effect of CBSC-exosomes on major signal transduction pathways in melanoma cell lines

4. Determination of *p53*, *CDKN1A*, *BCL-2* and *Caspase 3* genes and protein expression in melanoma cell lines

4.1 Introduction

To date, there are no published papers that investigated the effect of CBSC exosomes on the expression of p53-mediated apoptotic pathways in melanoma cells. Moreover, while studies have investigated the therapeutic and apoptotic effects of exosomes in multiple cancer cells, no one has investigated their effects alone and in combination with UVA+B on the expression of mRNA and proteins associated with apoptosis in melanoma CHL-1 cells. Following the results obtained from the CCK-8 assay and the Comet assay, it was observed that CBSC-derived exosomes induced cytotoxicity and genotoxicity in the lymphocytes obtained from melanoma patients and CHL-1 cells. This was in contrast to the results obtained from the lymphocytes of healthy individuals that showed no significant cytotoxicity and DNA damage after treatment with CBSC-derived exosomes. Furthermore, the CBSC-derived exosomes co-treated with UVA+B radiation also showed a significant increase in UVA+B induced DNA damage in melanoma CHL-1 cells by the Comet assay. Due to the consistency of data observed in CCK-8 and the Comet assay in the lymphocytes obtained from melanoma patients and CHL-1 cells, therefore further investigations were conducted on melanoma CHL-1 cells.

P53 is a tumour-suppressor protein that plays a major role in monitoring the homeostasis of biological processes. This protein is activated in response to certain cellular stresses and monitors gene expressions in controlling proliferation, DNA repair, senescence, and apoptosis (Lacroix et al., 2020). The *p53* possesses a complex signalling mechanism in the presence of cellular stress that leads to aberrations in oncogene expression involving DNA damage and metabolic dysregulation. *P53* signalling impacts a series of biological events, these include induction of apoptosis, cell-cycle arrest, DNA repair, and metabolic homeostasis (Li et al., 2012). Another interesting characteristic of p53 is its ability to instigate apoptosis, as a result of diverse stress stimuli. Depending on the severity of the DNA damage, p53 is activated following a series of biological pathways. If the DNA damage is beyond repair, then the cell is directed towards programmed cell death or apoptosis (Aubrey et al., 2018).

P21 often termed Cdkn1a (p21), is a cyclin-dependent-kinase inhibitor and the first identified *p53* target gene. The main role of this gene is to regulate the cell cycle. During the activation of p53, $p21^{WAF1/CIP1}$ attaches to Cyclin-dependent kinase 2 (CDK2) leading to the suppression of the activities of CDK2 and CDK1 respectively. In addition, *p21* also plays a fundamental role in inhibiting tumour progression by regulating DNA replication (Aubrey et al., 2018).

Apoptosis is one of the fundamental mechanisms that is stimulated in response to cell death signals. The BCL-2 family of proteins play a significant role in controlling and monitoring the apoptotic pathway (Suvarna et al., 2019). It is well established that the BCL-2 family is regulated by the activation of pro-apoptotic effectors (BH3, BIM and BID) of Bax and Bak proteins.

Therefore, any aberration in the regulation of the BCL-2 family could be of high importance in cancer management (Martinou and Youle, 2011). During apoptosis, the BH3-activator proteins including Noxa, PUMA (p53-upregulated modulator of apoptosis), BIM (BCL-2 interacting mediator of cell death) and BID (BCL-2-interacting domain death agonist) often called the activators, inhibit the activation of pro-survival proteins and directly induce BAK and BAX allowing mitochondrial outer membrane permeabilization (MOMP) that subsequently leads to the release of cytochrome c activating caspase proteins such as caspase 9 that in return activates the effector caspase 3 leading to apoptosis (Luna-Vargas and Chipuk, 2016, Avrutsky and Troy, 2021). Apart from this, in the presence of stimuli such as mild heat, hydrogen peroxide or unbalanced pH, p53 is shown to directly stimulate the BCL-2 family of proteins including BAX and BAK facilitating the initiation of apoptosis through MOMP (Mihara et al., 2003).

4.1.1 Quantitative Polymerase Chain Reaction (qPCR)

Several methods exist to detect DNA amplification, one of which is called the real-time Polymerase Chain Reaction RT-PCR or qPCR (Maddocks and Jenkins, 2017). This technique mainly focuses on determining the fluorescence signal intensity that increases in every qPCR cycle. The PCR product amplification is evaluated in the amplified DNA quantity at a specific time (López-Sanmartín et al., 2019). Although at first, the fluorescence dye is vaguely detected; it increases after every cycle. The quantification cycle refers to the point at which the target DNA is assessed in a sample (Kralik and Ricchi, 2017). The cycle threshold (CT) is described as the number of cycles the fluorescent signal needs to cross the threshold (Gunay et al., 2016).

As real-time PCR is a rapid and very sensitive technique, therefore it is used in a wide range of applications. These include genomic expression, gene cloning, diagnostics and gene sequencing (Valones et al., 2009). A striking feature of qPCR is to quantify and amplify a gene of interest (Narrandes and Xu, 2018). In addition, qPCR can be used to target any specified DNA present in a single cell, this could also involve proteins and mRNA (Ståhlberg and Kubista, 2014).

To conduct PCR a few components should be considered such as the availability of DNA polymerase enzyme, primers, and nucleotides. The DNA sequence can be amplified using given primers. The primers are DNA fragments that match the sequence of the target DNA that is subsequently targeted and amplified. There are forward and reverse primers. The forward primers pair with the antisense strand of the DNA, whereas the reverse primers pair with the sense strand of the DNA. (Garibyan and Avashia, 2013). Thermal cycling is one of the major steps in the PCR technique that includes cooling and heating cycles in addition to denaturation, annealing and extension. In the denaturation of the DNA, the temperature is initially increased to 95°C for 30 seconds to 2 minutes, enabling the unwinding of the DNA. Annealing follows denaturation at a temperature range of 60°C for 30 seconds. Denaturation created 2 single strands. Annealing allows the primers to attach to the template strands. During extension, the primers get extended, re-synthesising the complementary strand.

4.1.2 Western blotting (WB)

Western blot, also known as immunoblot is an efficient technique for quantifying and evaluating specific proteins in a wide range of biological applications (Ghosh et al., 2014). In this assay, the protein of interest that needs quantification must be present in every given sample. Results appear in the form of bands. The thickness and shape of each band are compared to the control. This depicts whether the protein has been overexpressed or repressed (Barresi, 2011).

In addition to biological samples, WB is also used as a diagnostic method in clinical settings (Manole et al., 2018). Due to its high specificity and sensitivity, WB can detect picogram amounts of proteins in a given sample (Gogia et al., 2017).

As indicated, WB is highly effective in distinguishing the target protein from a sample containing different types of proteins. The proteins are initially separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS_PAGE gel electrophoresis). This is then followed by molecular weight separation of the samples (Bendickson and Nilsen-Hamilton, 2013). The proteins are transferred onto a polyvinylidene fluoride (PVDF) or nitrocellulose membrane instead of the electrophoresis gel, due to the high fragility of the latter. Electric current is required for the transfer of proteins onto the membrane to occur (Mahmood and Yang, 2012). Using a detection kit (Fluorophore, an enzyme, and a radioisotope) proteins are visualized when specific primary antibodies bind to the target protein. Secondary antibodies conjugated with an enzyme or fluorochrome then bind the primary antibodies.

Visualization of proteins is possible by either using an imaging system or via chemiluminescence on X-ray film (Manole et al., 2018).

This study aims to focus on the effect of CBSC-exosomes on the expression of *p53*, *p21*, *BCL-2* and *Caspase 3* genes and proteins in melanoma CHL-1 cells, using the quantitative PCR and western blotting methods.

4.2 Materials and methods

The equipment and chemicals used in these assays (qPCR and WB) have been described in chapter 2 materials and methods sections 2.8 & 2.9 for qPCR and WB and enlisted in Tables 3 & 4.

4.3 Results

4.3.1 Real-time PCR (RT-PCR)

4.3.1.1 Assessing the expression of *p53*, *p21*, *BCL-2* and *Caspase 3* genes in CHL-1 cells with and without UVA+B co-treatment.

The molecular processes involved in this study were evaluated by determining the effect of CBSC derived exosomes on the expression of p*53*, *p21*, *BCL-2* and *caspase 3* at mRNA levels. Real-time PCR was performed to determine the cell cycle regulatory genes *p53* and *p21*, in addition to the apoptosis-related genes i.e., *BCL-2* and *Caspase 3* (Figure 16). The results shown in figure 16 portrayed that CBSC exosomes (300 µg/ml protein alone and co-treated with 1.2 mW/cm^2 UVA+B radiation) significantly upregulated the cell cycle genes such as *p53* and *p21*, compared to the untreated cells (****$p \leq 0.0001$). In contrast, a significant downregulation in anti-apoptotic *BCL-2* was observed in both treatment groups (300 µg/ml protein alone and co-treated with 1.2 mW/cm^2 UVA+B radiation) compared to the untreated control (**$p \leq 0.01$, ***$p \leq 0.001$).

A significant upregulation in pro-apoptotic *caspase 3* was also noticed in both treatment groups (300 µg/ml protein alone and co-treated with 1.2 mW/cm^2 UVA+B radiation) compared to their untreated counterpart (**p ≤ 0.01, ***p ≤ 0.001). In addition, the UVA+B radiation (1.2 mW/cm^2) (PC) also showed a significant upregulation in *p53*, *p21*, *caspase 3* and repression of the *BCL-2* gene, respectively (****p ≤ 0.0001).

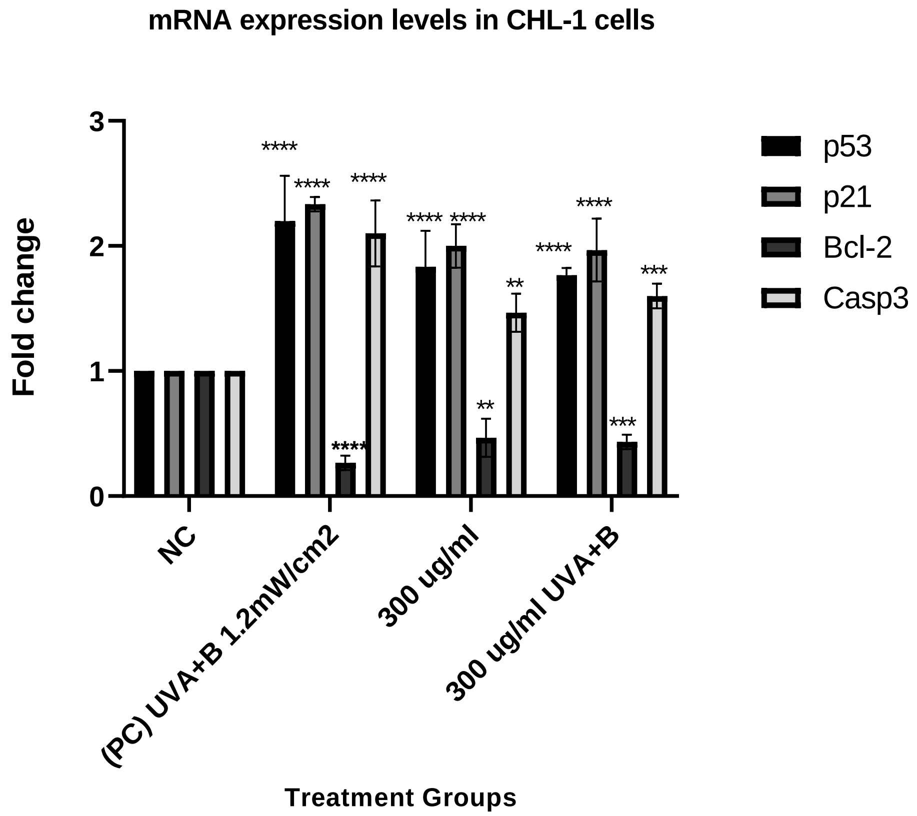


Figure 16 The effect of CBSC-derived exosomes in 300 µg/ml protein alone and co-treated with 1.2mW/cm^2 UVA+B radiation, on the gene expression of P53, P21, BCL-2 and Caspase 3 in melanoma CHL-1 cells. The treatment group data were compared against the control group and normalized against the housekeeping protein β-actin. The experiment was conducted thrice in three biological samples. The treatment groups comprised untreated cells, 300 µg/ml protein of CBSC-exosomes alone and co-treated with 1.2mW/cm^2 UVA+B radiation. The cord blood exosomes significantly upregulated the expression of p53, p21, and caspase 3 mRNA levels. However, the expression of BCL-2 was significantly repressed.

The data denotes the mean ± SEM of three experiments. (ns = not significant (**p < 0.01, ***p <0.001, ****p <0.0001).

4.3.2 Western Blotting (WB)

4.3.2.1 The effect of CBSC-exosomes on the expression of p53, p21, BCL-2, and Caspase 3 proteins in CHL-1 cells with and without UVA+B radiation.

In this study, a series of WB experiments were conducted to evaluate the protein expression of *p53*, *p21*, *BCL-2* and *caspase-3* in CHL-1 cells treated with CBSC-exosomes (Figure 17 A & B). The results were then validated with the data obtained from real-time PCR.

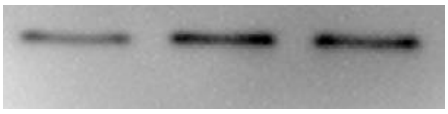
WB was used to detect the effect of CBSC exosome treatment (300 μg/ml protein equivalent to 4 x 10^7 particles/ml) on protein expression of p53, p21, BCL-2 and caspase 3 in melanoma CHL-1 cells. In this report, the p53 protein was used to confirm its role in the induction of apoptosis in CBSC exosome-treated CHL-1 cells with 300 μg/ml protein alone and in combination with UVA+B radiation.

Analysis of the data revealed a significant upregulation in the expression of p53, p21 and caspase 3 proteins. In contrast, protein expression of BCL-2 was significantly repressed which was also indicative of apoptosis induction. As shown in figure 17 the expression of p53 has increased by 1.8-fold in exosome-treated cells and by 2-fold in exosome and UV-treated cells when compared to the control group. Similarly, the expression of p21 protein was increased by 1.7 and 2-fold in both treatment groups, and an increase of 1.9 and 2.1-fold was observed by caspase 3 in both treatment groups.

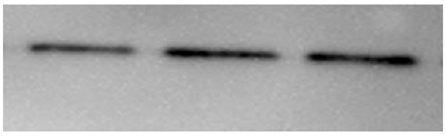
NC CBSC-exosomes CBSC-exosomes with UVA+B

P53 (53 kDa)

A

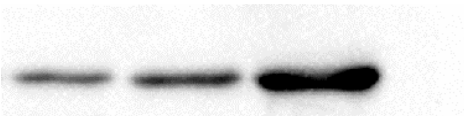


P21 (18 kDa)

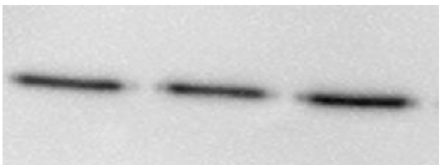


BCL-2 (26 kDa)

Caspase 3 (35 kDa)



GAPDH (37 kDa)



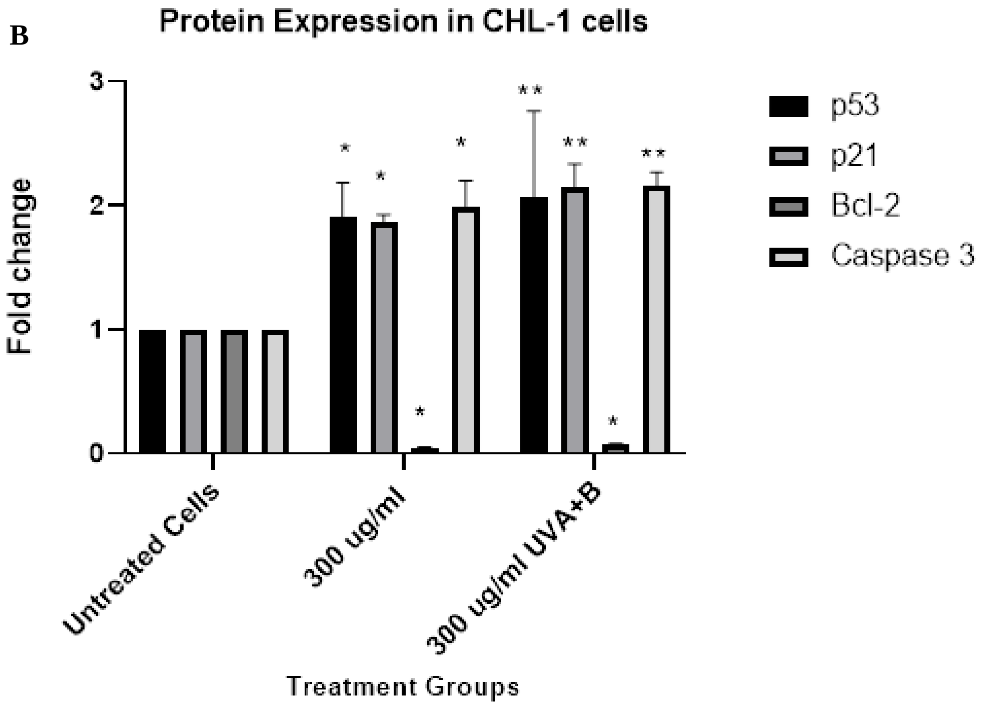


Figure 17 A & B The effect of CBSC-derived exosomes in 300 µg/ml protein of CBSC-exosomes alone and co-treated with 1.2mW/cm^2 UVA+B radiation on the protein expression of P53, P21, BCL-2 and Caspase 3 in melanoma CHL-1 cells. The treatment group data were compared against the control group and normalized against the housekeeping protein GAPDH. The experiment was conducted thrice in three biological samples. The treatment groups comprised untreated cells, 300 µg/ml protein of CBSC-exosomes alone and co-treated with 1.2mW/cm^2 UVA+B radiation. The cord blood exosomes significantly upregulated the expression of p53, p21, and caspase 3 proteins. However, the expression of BCL-2 protein was significantly repressed. A) immunoblot analysis of CHL-1 cells treated with 300 µg/ml protein of CBSC-exosomes alone and co-treated with 1.2mW/cm^2 UVA+B radiation. B) Bar graphs illustrating fold changes in protein expression levels. The data denotes the mean ± SEM of three experiments. (*$p < 0.05$, **$P < 0.01$).

4.4 Discussion

Genomic DNA is a fundamental molecule in maintaining the integrity and stability of the cells in all living organisms. The DNA is continuously exposed to various exogenous and endogenous insults on daily basis, affecting the genome stability (Williams and Schumacher, 2016). Under normal cues, cellular repair takes place in healthy cells that control the DNA damage. Any aberrations in the repair mechanisms may contribute to a wide range of maladies such as genetic disorders.

Any irregularity in genomic instability that may lead to carcinogenesis could result from certain acquired defects in the DNA repair mechanisms. These include BER, NER, MMR, HRR, and NHEJ (Broustas and Lieberman, 2014). Additionally, the genomic instability might be affected by genetic or epigenetic changes in oncogenes and tumour suppressor genes such as *BRCA1*, *BRCA2* and *TP53*. Malignant melanoma is an invasive skin cancer that occurs due to dysregulation in proliferation, migration, apoptosis and repair mechanisms in melanocytes (Tangella et al., 2021).

TP53 is a major tumour suppressor protein that plays a significant role in DNA damage control regulating the cell cycle to restore genome integrity (Aubrey et al., 2018). This tumour suppressor protein also plays an important role in maintaining cellular homeostasis by initiating the repair of damaged DNA and simultaneously monitoring the cell cycle (Moulder et al., 2018). P53 is impacted by the action of different DNA repair pathways. For instance, NER eliminates the helix distorting lesions generally caused by UV radiation. Similarly, BER targets the lesions caused by base modifications in oxidative stress. MMR eliminates the nucleotides mistakenly inserted during replication.

The NHEJ and HRR resolve the DSBs caused by ionising radiation (Whitaker et al., 2017).

Abnormalities in the normal functioning of p53 lead to distorted DNA integrity causing a rise in mutational rates (Rivlin et al., 2011). Moreover, it is well established that p53 activates p21 to promote the excision repair mechanism before replication (Abbas and Dutta, 2009). So far, investigations have highlighted the multi-functionality of p53 in regulating cellular proliferation, DNA repair, cell cycle arrest and cell death (Kamagata et al., 2020). Currently, there is no published study regarding the effect of CBSC exosomes on the mRNA expression of *p53*, *p21*, *BCL-2* and *caspase-3* in melanoma CHL-1 cells. Therefore, there is a need to identify the genes under *p53* control such as Bax or other p53-mediated apoptotic pathways.

In this study, our data illustrate that CBSC exosomes in CHL-1 cells up-regulate the level of p53 at both mRNA and protein levels. Our data is indicative of p53 mediated pathway that upregulates *p21* to stop the cell cycle to allow repairing. Our study is by a previous study that observed an increase in the level of p21 with an increased expression of p53 (Fischer et al., 2016). In contrast, BCL-2 was significantly repressed in CHL-1 cells treated with CBSC exosomes both at mRNA and protein levels (figures 16 & 17), while caspase 3 was also significantly up-regulated, both at mRNA and protein levels, thus favouring apoptosis. So far, we did not conclusively identify what causes the DNA damage. However, it is obvious from our data that exosomes trigger it in cancer cells and lymphocytes in melanoma patients.

Moreover, the current study also revealed an elevated expression of *p53*, *p21* and *caspase 3* in CHL-1 cells and a down-regulation of *BCL-2* expression in response to UVA+B radiation (figures 16 & 17). Interestingly, the expression of mRNA and proteins of interest in UV induced melanoma cells co-treated with exosomes could be due to the disruption of redox homeostasis by an increase in oxidative stress that causes DNA damage associated with UV radiation. Our results are strikingly consistent with a previous study that suggested UV radiation plays a significant role in the occurrence of programmed cell death, via p53 mediated apoptosis by inducing the inhibition of the BCL-2 family of anti-apoptotic proteins. It was also indicated that the apoptotic effects of UV depended on the duration of the irradiated cells which resulted in the upregulation of p53 dependent apoptotic pathway (Lee et al., 2013).

Chapter five: General discussion and future work

5 General discussion and Future work

5.1 General Discussion

The current study was piloted to determine the effects of CBSC exosomes in melanoma CHL-1 cells and in lymphocytes obtained from melanoma patients compared to those from healthy individuals, to have a better understanding of the exosomes' action and potential genotoxicity. In order to prevent cancer progression using anti-cancer drugs, it is crucial to evaluate the underlying mechanisms of carcinogenesis and the mode of action of chemotherapy drugs on tumour cells, but also their potential of causing no harm to the normal cells. Following this, the Comet assay is proved to be a well-established method to evaluate the genotoxicity of a particular compound compared to the other similar assays (Anderson et al., 2019). Therefore, the Comet assay was used to determine the induced DNA damage after treatment with CBSC exosomes in melanoma cells but more importantly in lymphocytes of melanoma patients and those of healthy volunteers. The DNA damage on CHL-1 cells was also evaluated by the Comet assay after treatment with CBSC-exosomes alone and in combination with UVA+B radiation. Furthermore, this report also looked at the apoptotic pathways by assessing the expression of p53, p21, BCL-2 and caspase 3 at mRNA and protein levels in melanoma cells by using the qPCR and the western blot techniques.

Cancer remains a global health concern, associated with serious health consequences.

Prevention and timely treatment of cancer is a promising approach to overcoming the growing number of carcinomas globally (Bray and Soerjomataram, 2015). Malignant melanoma is an invasive type of skin cancer caused by melanocytic malignancy. Besides other risk factors, the link between malignant melanoma and UV radiation is well established (Moran et al., 2018). Diagnosis of this malignancy depends upon the biopsy of the suspected lesion by evaluating its thickness, cellular growth, symmetry, size, shape and colour (Ahmed et al., 2020). Currently, the main therapeutic strategy in the treatment of melanoma involves surgical intervention, chemotherapy, radiotherapy, immunotherapy and targeted therapy (Ahmed et al., 2020). However, due to major adverse effects and resistance to chemotherapeutics, there is a growing urge to discover new drugs to combat these complications (Wang et al., 2019).

EVs belong to a heterogeneous group of nanoparticles released from different sources of cells into the extracellular space. They were initially considered cellular debris (Margolis and Sadovsky, 2019). Currently, they are highlighted to play a major role in cell-to-cell communication and cell signalling. Amongst them, exosomes are highlighted, as they allow the exchange of biomolecule present in their cargo to the surrounding cells. The release and biogenesis of exosomes have been demonstrated to be enhanced in conditions like oxidative stress and cancer (Chiaradia et al., 2021). Exosomes have been highlighted to exert cytotoxicity in cancer cells as a biological therapeutic. However, the effect of CBSC exosomes on melanoma cells and lymphocytes from melanoma patients and healthy individuals has hardly been reported.

Mesenchymal stem cells and mesenchymal stem cell-derived exosomes have shown promising cell-based therapies in tissue repair and regeneration (Zhang et al., 2019). Similarly, in cancer therapies, these EVs have been highlighted for their multifunctional properties that include inhibition of cancer cell proliferation and cancer cell apoptosis (Zhao et al., 2020). Currently, there has been a growing interest in the therapeutic effects and clinical applications of exosomes in the diagnosis and treatment of various chronic disorders including malignant melanoma (Dhondt et al., 2018).

The antitumour properties of exosomes in several types of carcinomas have been well documented. In T24 cells (urinary bladder carcinoma), exosomes derived from Wharton jelly suppressed the viability of these cells by exerting apoptotic effects through the downregulation of the Akt pathway and upregulation of cleaved caspase 3 (Karaoz et al., 2019). Similarly, a reduction in the proliferation of U87 glioblastoma multiform (GBM) cells was observed by Wharton jelly-derived exosomes (Sharif et al., 2018). Moreover, exosomes are also known to suppress tumour growth and angiogenesis in other tumours including Kaposi’s sarcoma, ovarian tumour and hepatoma cell lines (Bruno et al., 2013). This is in agreement with our data of the CCK8 assay that revealed a significant decrease in the viability of melanoma cells and lymphocytes obtained from melanoma patients, in a timely and dose-dependent manner.

Exposure to UV radiation results in the accumulation of ROS that results in DNA damage and protein modifications (Wu et al., 2021). Since H_2O_2 and UV are considered chemical and physical stressors that induce ROS in cells, therefore, melanoma CHL-1 cells were additionally co-treated with UVA+B to obtain a better consistency of results.

Under stressful conditions, exosomes are known to be capable of mediating protective or harmful signals, depending on their cargo and the requirements of the recipient cells (Chiaradia et al., 2021). For instance, exosomes isolated from mouse mast cells treated with H_2O_2 developed more tolerance to oxidative stress in target cells, compared to cells pre-treated with exosomes under normal conditions (Eldh et al., 2010). Therefore, these nanoparticles are indicated to play an important role in the pathophysiology of diseases associated with high levels of oxidative stress. Moreover, it is also reported that under cellular oxidative stress especially in combination with chemotherapeutics, exosomes can spread damage to the neighbouring cells by inducing inflammation. Furthermore, a recent report suggested that exosomes released under oxidative stress are able to protect the cells by making them tolerant to the same stimuli, by transferring ROS scavengers to the target cells (Borras et al., 2020). Controversial studies exist that suggest exosomes exert both protective and damaging roles in oxidative cellular stress conditions by either acting as scavengers of ROS or ROS inducing nanoparticles (Bodega et al., 2019). This is observed in cisplatin-induced acute kidney injury and gemcitabine-induced pancreatic cancer cells, where exosomes protected the cells from the cytotoxic effects of the chemotherapy drugs, but on the other hand induced oxidative stress to the surrounding healthy cells (Zhou et al., 2013). Although no definite biochemical feature of exosomes has been evaluated under oxidative stress, however, it is well established that the function of exosomes on target cells under oxidative stress depends upon the cells they are released from and their molecular cargo.

Similarly, exosomes derived from macrophages are depicted to induce high levels of ROS in damaged axons. NADPH oxidase (NOX) is an enzyme that is assumed to be transferred by exosomes to the damaged target cells to induce apoptosis (Hervera et al., 2018). Our Comet assay data with exosome treatment alone and co-treatment with UV showed similar results with the prevalence of high DNA damage in melanoma cells. No significant DNA damage was observed in cancer cells when exosomes were co-treated with UVA+B compared to the positive control group. This effect of exosomes could potentially favour genotoxicity in cancer cells with the possibility of exhibiting anti-cancer properties. Our results are in agreement with the study that is suggestive of increased ROS levels by exosomes that might lead to apoptotic effects in cancer cells (Hervera et al., 2018). Although the effect of exosomes in ROS-induced cancer cells has been investigated, nonetheless, more research is required to elucidate the molecular mechanisms and biochemical and biological properties of these nanoparticles in oxidative stress.

DNA damage is an important step in initiating apoptosis (Szabo et al., 2013). To metastasize and spread, cancer cells need to avoid apoptosis (Elgazar et al., 2018). Thus, to facilitate cancer cells from growing and proliferating, agents should be used to induce apoptosis (Attia et al., 2020). Regulated cell death is a complex mechanism triggered by external or internal insults that has a great impact on cellular homeostasis. Any deregulation in this pathway might lead to the development of certain maladies including cancer (Cavalcante et al., 2019). Hence, many chemotherapeutic interventions aim to focus on apoptosis as a treatment modality, either alone or as a combination therapy (Wong, 2011).

Evidence suggests that exosomes play a significant role in maintaining the healthy physiology of the cells (Isola and Chen, 2017). In normal tissues, exosomes play a considerable role in regulating normal physiological functions. For instance, in the brain, these nanovesicles potentiate the integrity of axons, microglia and myelination. Maintaining the integrity of axons is done through certain enzymes such as catalase and superoxide dismutase-1 that resist oxidative stress, suggesting that exosomes released from oligodendrocytes increase the oxidative stress tolerance of neurons. Although the exact mechanism involved in the enhanced survival of oligodendrocytes is unclear, however cellular phosphorylation of Akt and Erk1/2 kinases are identified to be activated by oligodendrocyte exosomes under normal cues and oxidative stress (Fröhlich et al., 2014). Our results were also consistent with the findings in studies where the exosomes derived from different sources showed apoptotic effects in various carcinomas including hepatocellular carcinoma, prostate cancer, lung cancer and breast cancer, while no effect on healthy lymphocytes was observed (El-Kattawy et al., 2021, Lorenc et al., 2020, Huang et al., 2019, Saeki et al., 2013).

P53 is a tumour suppressor gene displaying diverse regulatory properties in the cell cycle (Darcy et al., 2008). Depending on the extent of the DNA damage, the faith of the cell is decided as to whether enter G1/arrest or be subjected to programmed cell death. In the advent of minor damage, p53 activates the G1/arrest signal that instigates the repair mechanism of cells prior to entering the S-phase. Similarly, in the presence of major DNA damage *p53* induces a pro-apoptotic signal initiating the intrinsic apoptotic pathway.

The regulatory function of *p53* is mediated through the expression of *p21* (a cyclin-dependent kinase inhibitor (CDKI)), which can halt the cell cycle to allow time for repair (Haupt and Haupt, 2017). It is well documented that *p53* is a tumour suppressor gene that regulates the mechanisms involved in DNA repair processes and apoptotic pathways. In cell cycle arrest, this tumour suppressor gene induces the transcriptional activation of *p21* mRNA. Similarly, in programmed cell death, p53 induces several genes and proteins such as the BH3 domain-only proapoptotic proteins (Puma, Noxa, Bad, Bax and Bak) in addition to upregulation of caspase 3 and 7 respectively (Chen, 2016). *P53* has been reported to inhibit the expression of anti-apoptotic proteins such as BCL-2 while stimulating pro-apoptotic proteins like Bax (Kim et al., 2014). Similarly, once activated Bax proteins trigger the apoptotic executioner caspases such as caspase 3 leading to apoptosis (Vince et al., 2018).

In cancer, the tumour cells possess the ability to evade cell cycle checkpoints and apoptosis resulting in uncontrolled cellular proliferation. Since *p53* is a major cell cycle regulator, thus its absence or mutation can result in abnormal cellular proliferation and ultimately cancer. A large body of evidence suggests that *p53* is the most mutated gene in several types of carcinomas causing harmful effects (Kong et al., 2012). In malignant melanoma, wild-type p53 is indicated to be abnormally phosphorylated suggesting defective signalling pathways regulated by this protein. Mutation in this protein may fail to promote cell cycle arrest and programmed cell death (Avery-Kiejda et al., 2011).

Evidence suggests that an aberrant mRNA expression of p53 in melanoma cells might lead to deregulation in cell cycle control and apoptotic pathways. It is also predicted that this aberration could also result in melanoma progression and proliferation (Avery-Kiejda et al., 2011).

Studies indicate that p53 interacts with p21, BCL-2 and caspase 3 to instigate cell death (Cavalcante et al., 2019). Therefore, in this report, a few mediators involved in *p53* activation were investigated. These involved tumour suppressor gene *p53*, *p21*, anti-apoptotic *BCL-2* and pro-apoptotic *caspase 3*. The gene and protein expression in CBSC exosome-treated CHL-1 cells were assessed by RT-PCR and WB, respectively. An upregulation in the expression of p53 protein was observed. This result was consistent with the real-time PCR results where a significant up-regulation in the expression of *p53* at mRNA level was observed in both treatment groups. Similarly, the expression of p21 at protein and mRNA levels was also significantly upregulated in both treatment groups (Figures 16 & 17). These results were in accordance with a previous study that revealed an increase in the expression of *p53* is followed by an upregulation in the expression of the *p21* gene (Shih et al., 2007).

Significant repression in the expression of the *BCL-2* gene was observed in the CHL-1 cells treated with exosomes. This is suggestive of apoptosis where the anti-apoptotic BCL-2 is inhibited to induce apoptosis. This was confirmed by the expression of caspase 3 both at mRNA and protein levels. Caspase 3 is a pro-apoptotic protein that is induced in the advent of programmed cell death.

Our data from qPCR and western blot are compatible with a previous investigation that suggests melittin is capable of inducing apoptosis in leukemic cells via downregulation of BCL-2 and upregulation of caspase-3 (Moon et al., 2008). This indicates that CBSC exosomes induce apoptosis in cancer cells by triggering the p53 mediated pathway that inhibits cellular proliferation, subsequently leading to apoptosis.

In conclusion, the CBSC exosomes showed genotoxic and cytotoxic effects in melanoma cell lines, whereas no such effects were observed in the lymphocytes from healthy individuals. The decrease in the viability of lymphocytes in melanoma patients needs further investigation on more blood samples, as the number of samples were limited in this project. Genotoxicity was also observed in CHL-1 cells treated CBSC derived exosomes co-treated with UVA+B radiation. The anti-tumourigenic effect of CBSC-exosomes was also observed in the upregulated expression of P53, p21 and caspase 3 and inhibition of BCL-2 at protein and mRNA levels, respectively.

Moreover, it was also noted that the CBSC exosomes were highly effective in inhibiting the progression and growth of cancer cells, with no significant side effect on the survival rate and proliferation of healthy lymphocytes. Following the results of a previous study, the data of this investigation allude that exosomes derived from human cord blood stem cells effectively treat aggressive tumours by inducing cellular apoptosis while possessing no damage to the somatic cells (Zhou et al., 2017).

5.2 Research limitations

The initial aim of this project was to work on primary melanoma cells instead of cell lines. However, due to the impact of covid-19, access to samples was minimal. Therefore, this project was solely based on limited samples that were accessible at the time. Going forward, it would be advantageous to work on more sample numbers as well as primary cell lines.

Sample collection was a major limitation during the pandemic. The samples we had available were limited and were collected pre-covid. That is the main reason that qPCR and western blot assays were performed on melanoma (CHL-1) cells and not on lymphocytes from healthy individuals and melanoma patients.

Another limitation was receiving exosome samples. The exosomes were donated to us by our collaborators based in Tehran-Iran. Shipment restrictions halted the project for a year and a half; therefore, the whole project was conducted in only nine months.

5.3 Future work

The results presented in this study determine that CBSC exosomes increase the DNA damage in cancer cells. It also revealed that the exosomes exerted similar effects on cancer cell lines even after their co-treatment with UV in melanoma CHL-1 cells. Due to the limited number of samples, the effect on viability of lymphocytes from melanoma patients could not be fully illustrated, hence further investigation is required. Micronucleus assay should also be performed as an additional technique to evaluate chromosomal damage due to the exposure of exosomes as a genotoxic agent.

Depending on the cargo and origin of exosomes they are considered to possess antioxidant properties that inhibit ROS-induced DNA damage in healthy cells. This process can also be investigated in melanoma cells by treating them with vitamin E incorporated exosomes to have a better understanding of their efficacy in the presence of antioxidants. In addition, if the efficacy of antioxidants is up-regulated by exosomal treatment in healthy cells, therefore, this might be beneficial in the prevention of adverse effects caused by chemotherapeutic drugs on these cells. Moreover, melanoma cells and normal melanocytes can also be co-treated with exosomes and H_2O_2 to have a better understanding of their molecular mechanisms involved in oxidative stress. Furthermore, as it is suggested that exosomes regulate the catalase and superoxide dismutase enzymes in normal cells, hence it will be beneficial to inhibit these enzymes in healthy cells to observe their effects on the normal physiology of the cells.

The CBSC derived exosomes have been shown to affect the regulation of p53, p21, BCL-2 and caspase 3 proteins. Hence, more research is required to determine the role of other proteins such as ATM and ATR to distinguish the specific type of DNA damage that occurs after treatment with exosomes. In addition, as the exosomes did not exert any harmful effects on healthy cells, therefore their cargo needs to be fully examined.

Lastly, the effect of exosomes could also be observed in both cells and lymphocytes of other malignant cancers to further expand their multipotentiality for chemotherapeutic use including drug discovery.

Chapter six: References

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Appendices

Appendix A.1

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

Centre Number:

CONSENT FORM FOR PATIENTS

Title of Project: The anti-carcinogenic/therapeutic effect of cord blood-derived exosomes in malignant melanoma.

Reviewed by Leeds Central Research Ethics Committee (REC) (REC reference number: 12/YH/0464)

1. I confirm that I have read and understand the information sheet (version 3, 19- 06-09) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights beingaffected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust or the University of Bradford, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree that the sample I have given and the information gathered about me can be stored at the University of Bradford, as described in the attached information sheet. ☐

5. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
____________________	____________________	____________________
Name of Person	Date	Sign

Appendix A.2

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

School of Life Sciences

DATA COLLECTION FORM
(To be completed by the Doctor)

STUDY TITLE: Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints
REVIEWED BY LEEDS East RESEARCH ETHICS COMMITTEE (REC)
(REC REFERENCE NUMBER: 12/YH/0464)

PATIENT NUMBER		DATE OF SAMPLE	
AGE			
SEX (PLEASE TICK)	M / F	CONSENT	Y / N
ETHNIC GROUP		INFORMATION SHEET	Y / N
OCCUPATION			

CURRENT SMOKER Y/N PAST SMOKER Y/N HOW MANY/MUCH PER DAY?
CIGARETTES CIGARS PIPE
ALCOHOL Y/N UNITS PER WEEK

DIET	WESTERN	ASIAN	OMNIVORE	VEGETARIAN	VEGAN

VITAMINS / ANTI-OXIDANTS (PLEASE LIST)

PRESCRIBED DRUG USE (PLEASE LIST)

RECREATIONAL DRUG USE Y/N

IF YES PLEASE LIST
MEDICAL **Asthma** **COPD** **others**

CANCER
Inflammatory disease
EXTENT SITE HISTOLOGY SURGERY

CANCER
Inflammatory disease
Pre cancerous state
OTHER MEDICAL CONDITIONS (PLEASE LIST)
Family history of cancer and Inflammatory disease
Chemotherapy or radiotherapy

MOST RECENT MEASURE

	RESULT	DATE		OTHERS	RESULT	DATE
WEIGHT						
HEIGHT						
BMI						

Appendix A.3

Participant Information Sheet for patients (Version 4, 28/07/2020).

Study title: The anti-cancer effect of cord blood derives exosomes in lymphocytes from healthy individuals and melanoma patients

Reviewed by Leeds East Research Ethics Committee (REC)

(REC reference number: 12/YH/0464)

Invitation to the research study

We should like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish and you will be allowed around 24 hours to consider this.

(Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study).

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you want to take part.

Part 1

What is the purpose of the study?

In this study blood cells will be treated in a test tube with very small chemical particles or UVA (Ultraviolet A light) to determine if patients with different diseases are more at risk after exposure compared to healthy individuals. For example, chemicals and UV (Ultraviolet) can break and damage the DNA of white blood cells. Further examination of this resulting damage may improve our knowledge of cancers and other inflammatory diseases.

The tests are not predictive of any kind of disease and the test results will not impact you or the healthy volunteers with whom you are compared. Blood samples for the study will be taken during the clinic visit in which you are recruited, if possible, at the same time as the samples taken for your routine care.

A blood sample of around 2-4 teaspoons (20 ml) will be taken. Samples will be stored only until the end of the study (after 8 years) and used for studies of similar nature or to check original responses. The research is also used for some PhD programmes.

Why have I been invited?

Because you are a patient at the Clinic, and we should like to determine if these small chemical particles or UVA could be more harmful to you than to people without the disease.

Do I have to take part?

No, it is up to you to decide. We shall outline the study and go through this information sheet, which we shall then give to you. We shall ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

Part 2

What will happen to me if I take part?

Only a single blood sample will be taken for this research study. A brief questionnaire will need to be completed by the researchers.

Each individual will be given a coded study number so that clinical data will be linked anonymously with the research results.

The study tests are not predictive for you.

The data obtained will only be available to the research team and will not be returned

to you. Responses will be compared only on a group basis i.e. collective responses from patients with that individual disease compared to responses collected from people without that disease. Results could be published in the form of scientific papers. The work may benefit the medical and scientific community at large but will not be of direct benefit to you as an individual. If, however, you would like more information, the appropriate consultant will be prepared to talk to you individually about the study results.

The data will be stored until the study is completed at the end of 8 years.

People who cannot take part in the study.

People who are not well enough to take part will be excluded e.g. those with anaemia.

If you have any further questions, you could contact the research team:

Prof Andrew Wright NHS Trust, St Luke's Hospital

Bradford Teaching Hospitals NHS Foundation Trust, BD5 0NA.

Telephone: 01274 542200

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