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THE EFFECT OF MORPHINE ON ENDOTHELIAL DIFFERENTIATION OF VASCULAR MESENCHIMAL STROMAL CELLS (VMSC)

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Introduction

In the field of illicit drug use, heroin-related mortality is a complex phenomenon. There is a great variability in prevalence mortality rates attributable to heroin overdose, but most studies on longitudinal trends of overdose deaths, or overdose-related hospitalizations, show increases across time [1].

As well as other opiates, heroin and its metabolite morphine cause mental and grastrointestinal effects, as well as urinary retention; however the most serious effect of opiates is the respiratory depression and pulmonary edema, which is non-cardiogenic.

Indeed, the most common pulmonary findings in opiate-related fatalities are pulmonary congestion and edema, as well as acute/chronic alveolar hemorrhage. The cause is thought to be damage to the capillary endothelium: ischemic, related to ipoperfusion of the pulmonary vascular bed, and/or related to the direct toxic effect of the substance on entothelial cells.

Tissue regeneration and repair are essential after endothelial cell injury and are related to the activity of human vascular mesenchimal stromal/stem cells (vMSCs). vMSCs are a versatile class of multipotent adult stem cells capable of self-renewal and because of their trophic property in secreting a wide range of growth factors including vascular endothelial growth factor, along with their immunomodulatory, anti-apoptotic and anti-inflammatory properties exerted in response to a mechanical damage or flogosis, they play a fundamental role in tissue repair and regeneration. Holan et al. recently demonstrated the expression of opioid receptors on vMSCs and how these showed functional characteristics impairment following acute morphine exposition.

As a matter of fact, also an opiate-related impairment of vMSCs function might be a part of the pathophysiology of endothelial cell injury.

The aim of this study is to assess the effect of in-vitro opiate exposure on the phisiologial activity and maintenance of human vMSCs.

In the first chapter a general introduction to opioid abuse will be illustrated. As it is well known, opioids consist of a diverse group of drugs, among them derivatives of the naturally occurring opium (*i.e.* morphine, heroin, codeine), synthetic (*i.e.* methadone, fentanyl) and endogenous compounds (*i.e.* enkephalins, endorphins and dynorphins). In general, opioids cause analgesia and sedation, respiratory depression and slowed gastrointestinal transit; however, severe intoxication results in coma, respiratory depression and pulmonary edema, which may ultimately progress to apnea and death.

Among opioids, morphine and its synthetic derivative heroin (diacetyl-morphine) are still heavily abused in many countries around the world.

Opiate receptors are present in different concentrations in several regions of the body, namely in nervous cells where they are involved in analgesia or in control of autonomic activity. However, they can also be found on mesenchymal stem cells.

Human mesenchymal stem cells and their role in regenerative process are the topics of the second chapter of the thesis. In particular, an overview of stem cells will be reported. Stem cells can be classified according to their potential into pluripotent (embryonic stem cells, induced pluripotent stem cells), multipotent (mesenchymal stem cell, hematopoietic stem cells, neuronal stem cells, and endodermal stem cells), and oligopotent (monoblast, proerythroblast). While pluripotent stem cells have the potential to differentiate into all cell lineages of the three germ layers, multipotent stem cells have the capacity to commit towards several cell lineages that are originated from the same germ layer and oligopotent or unipotent stem cells can differentiate into a few or even one cell lineages. Mesenchymal stem cells (MSCs) belong to the adult stem cells that are able to repair body cells and maintain the normal turnover of regenerative processes. MSCs are able to

successfully differentiate towards a number of different cell lineages, including endothelial cells. Since vascular endothelial cells play a crucial role in various vascular functions, a damage or dysfunction of these cells leads to serious consequences; therefore mesenchymal stem cells have been considered as a favourable source to obtain a large amount of endothelial cells to replace the damaged ones.

The third chapter of the thesis will focus on the experimental study, evaluating morphine toxicity on endothelial differentiation of vascular Mesenchimal Stromal Cells.

Chapter 1

Opioid Abuse: General Introduction

The use of opioids has been documented as far back as 3500 BC in ancient Egypt [2], with crude opiate extracts from poppies used as potent analgesics. Over time this led to the development of morphine as the prototypical opioid for acute pain relief [3].

Every year, thousands of opioid users around the world suffer a fatal opioid overdose, with respiratory depression being one of the primary causes of death [4].

Addiction to opioids nowadays derives not only from the use of heroin, but also from a misuse of prescription opioids, often originating from a legitimate need for pain relief from chronic conditions [5]. This has led to more opioid addicted individuals than in the past, abusing a greater variety of opioids.

Even if it has been over 140 years since the development of heroin, the need to understand the underlying mechanisms of opioids and the physiological ramifications of opioid use is more important than ever before.

Opioids are abused for their mood-altering effects, and tolerance and physical and psychological dependence account for continued abuse.

They represent diverse group of drugs, either derived naturally from the poppy plant or synthesized, among them derivatives of the naturally occurring opium (morphine, heroine, codeine), synthetic (methadone, fentanyl) and endogenous compounds (enkephalins, endorphins, and dynorphins). Morphine-like analgesic drugs are also known as narcotics.

Opioids are most commonly used in therapeutic settings for their analgesic actions, which are mediated by both peripheral and central mechanisms.

Concerning their receptors, there are several subtypes of receptors, the most common of which are known as μ (mu), κ (kappa), and δ (delta). Those receptors differ in their affinity to different agonists and antagonists, and in their effects.

Opiate receptors are present in different concentrations in different regions of the body, especially but not limited to the nervous system.

In general, opioids produce a constellation of physiological effects that includes constipation, urinary retention, pupillary constriction, respiratory depression, pulmonary congestion and edema. It is the latter effects that typically accounts for the toxicity and lethal overdose from opioids.

Severe intoxication results in coma and respiratory depression, which may progress to apnea and death.

1.1. Abused Opioids

Heroin, morphine, methadone, oxycodone, and fentanyl derivatives account for about 98% of all hospital admissions and opiate deaths.

Heroin remains an ever-important drug of interest due to the continued use and abuse around the world.

It is a synthetic drug produced from the morphine contained in the sap of the opium poppy and it is synthesized from morphine in a relatively simple process.

Heroin was initially used in place of codeine and morphine for patients suffering from lung diseases such as tuberculosis. Additonally, the German Bayer Company marketed the drug as an analgesic in 1898 and also advertised it as a cure for morphine addiction. However, the addictive properties were quickly devastating.

Heroin is typically self-administered by intramuscular or intravenous injection and also by nasal insufflation or smoking.

After administration heroin is rapidly deacetylated to an active metabolite, 6-acetylmorphine (6-MAM), which is then hydrolyzed to

morphine. The conversion to morphine occurs within minutes. Spontaneous hydrolysis to 6-acetylmorphine may occur under various conditions.

Heroin itself does not bind to any opioid receptor, instead it functions as a very efficient, and highly lipid soluble pro-drug that penetrates the brain far quicker than its major metabolites would otherwise be able to do [6,7].

Heroin has been associated with cerebral arteritis and visceral polyarteritis; it may also have a direct toxic effect on the terminal hepatic veins, causing an acute lesion being described as an inflammatory infiltrate of neutrophils and mononuclear cells in sinusoidal lumina and terminal veins which progresses to fibrosis. Heroin is also associated with glomerular injury, which may result in malignant hypertension.

In addition to the effects common to all opiates, there have been reports of acute rhabdomyolysis with myoglobinuria, sometimes accompanied by muscle pains, weakness, and swelling, during heroin intoxication.

The supply of heroin is often affected by global events that implies that there are fluctuations in the availability and purity of street heroin [8].

Morphine is a naturally occurring opioid obtained from opium; it is believed that opium contains 10 to 17% morphine.

Morphine is the most recommended and most widely used opioid analgesic for chronic pain and therefore a relatively large volume of pharmacokinetic data exists.

Morphine can be administered by intravenous, subcutaneous, oral, and rectal routes. It undergoes significant first-pass metabolism when given orally, and thus requires high doses to achieve the desired effects.

While the oral route is the accepted route of administration for pain control in patients with chronic pain, it is usually not utilized by drug addicts.

Morphine receptors on the terminals of primary afferent nerves mediate inhibition of the release of neurotransmitters. Morphine also causes respiratory depression, due to a direct effect on the respiratory centers of the brainstem by reducing the responsiveness to carbon dioxide.

At the same time, nausea and vomiting caused by morphine and other opioids are due to direct stimulation of the chemoreceptor trigger zone in the central nervous system.

A single intravenous dose of 0.125 mg/kg of morphine to adult results in a peak serum concentration at 0.5 minute of 440 ng/mL, declining to 20 ng/mL by 2 hours. Intramuscular injection of the same dose results in an average peak serum concentration of 70 ng/mL, 10 to 20 minutes after administration, declining to 20 ng/mL after 4 hours. Epidural administration of a single dose of 0.1 mg/kg of morphine to surgical patients produces an average peak serum concentration of 80 ng/mL after 10 minutes, declining to approximately 10 ng/mL after 4 hours. The oral bioavailability of morphine is approximately 38% with a reported range of 15 to 64%. Oral doses of 20 to 30 mg of morphine administered to adult terminally ill cancer patients maintain morphine serum concentrations above 20 ng/mL for 4 to 6 hours [9,10].

Morphine is relatively hydrophilic and therefore distributes slowly into tissues. In the adult, small amounts cross the blood-brain barrier, with more lipophilic opioids such as heroin and methadone crossing rapidly. Morphine administered epidurally or directly into the spinal canal is effective in producing prolonged analgesia.

Metabolism occurs primarily in the liver with 90% of a dose excreted in the urine and 10% in the feces. Approximately 87% of a dose of morphine is excreted in the 72-h urine, 75% as morphine-3-glucuronide, 10% as free morphine, and the remainder as morphine-6-glucuronide, morphine-3-sulfate, normorphine, and conjugates.

Methadone is one of the major substitution therapies administered as a means of aiding heroin addicts in decreasing their heroin intake and

becoming abstinent to heroin use. It provide a means of preventing extreme withdrawal from heroin abstinence as well as providing tolerance to the effects of heroin in the case of relapse [11].

Methadone is a synthetic long-acting opiate agonist. Even if it is mainly used as a maintenance therapy for heroin addicts, it is also occasionally used to treat chronic pain. There have been reports of deaths associated with methadone treatment, mostly as a result of a too rapid dose increases in subjects who may have lost their tolerance.

However, also methadone overdose represents a problem among the methadone substitution population [11].

Oxycodone has become, over the past two decades, an important drug in fatal cases of accidental overdose deaths during illicit use in many countries of the world [12,13].

This compound is a codeine derivative, whose potency and half-life are comparable to those of morphine. Deaths due to respiratory depression following oxycodone ingestion have also been reported.

Fentanyl represents an emerging and challenging evolution of the opioid epidemic [14,15].

Fentanyl and related drugs are synthetic opioid agonists structurally related to meperidine. Fentanyl is 50 to 100 times more potent than morphine, and has a half-life of about 4 hours. It can be administered intravenously and transdermally, and it is used for anesthetic procedure.

Due to a very high potency of fentanyl and related drugs, respiratory depression may occur very rapidly.

The higher potency of fentanyl has allowed relatively easy trafficking of small fentanyl packages through legitimate national and international postage systems, which are then subsequently cut to a lower purity, or used to enhance the “quality” of heroin [16].

1.2. Common Opiate Effects

Opiate drugs exert their properties through dopaminergic neuron activation in specific area of the central nervous system.

Usually, the effect on the mood is relaxation and euphoria; where tolerance to euphoria-inducing effects develops rapidly.

Sedation, which is dose-dependent, is a first sign of opiate intoxication.

Nausea and vomiting are also common side effects of opiates, resulting from their actions on the chemoreceptor trigger zone in the central nervous system (i.e. the medulla).

However, not all opiates have same likelihood for causing nausea.

Opioid drugs decrease gastrointestinal motility and peristalsis, acting in the gastrointestinal tract, thus causing constipation.

Since tolerance does not develop to this effect, constipation persists even in chronic users.

Respiratory depression is the most serious adverse effect of opiates and respiratory arrest is almost always the cause of death from opiate overdose.

Respiratory arrest occurs within minutes of the intravenous overdose.

After overdose from oral, intramuscular or subcutaneous route, sedation almost always precedes respiratory arrest.

Opioid-induced respiratory depression can be caused by the effects of opioids at multiple sites.

For example, effects on the respiratory neurons located in the medulla can lead to suppression of the respiratory rate and inhibition of the respiratory drive; and effects on the peripheral chemoreceptors located in the carotid and aortic bodies and lungs can cause the suppression of ventilatory responses to hypoxemia and hypercapnia, which in turn can result in sedation [4,17].

Pulmonary edema occurs with several opioid drugs, and is non-cardiogenic.

The precise mechanisms are unknown but probably involve hypoperfusion with tissue injury and cytokineinduced pulmonary capillary endothelial injury.

Pulmonary edema is particularly common with heroin and morhpine intoxication.

Other opioids effects include miosis, which is invariably present in opiate intoxication, unless anoxic brain damage is present; pruritis, which is believed to be caused by histamine release mediated by the mu receptors; and urinary retention that is mediated through spinal cord opiate receptors.

The primary cause of death in instances of fatal opioid overdose remains pulmonary congestion and respiratory depression [4].

1.3. Development of Tolerance to Opioids

Tolerance is defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) as the requirement, on repeated drug taking, for markedly increasing the dose of a drug to achieve the same level of desired effect or a marked decrease in the effect of the drug when the original dose is administered.

Tolerance is an important pharmacological mechanism regarding opioid use. It is observed in opioid users, with large increases in the dose of opioid required to induce euphoria after repeated use [18].

Tolerance to opioids is important in their clinical use as analgesics, with reports of dose escalation required due to the development of tolerance [19].

There is mixed evidence in the literature as to whether tolerance develops to all opioidergic effects.

In the context of illicit opioid use, the two primary opioidergic effects of concern are euphoria and respiratory depression. Dose escalation of opioids in order to maintain the same level of euphoria is well noted in the addicted population [20].

There is some evidence that agree that tolerance to respiratory depression occurs, though requiring a longer period of opioid administration.

Repeated administration of morphine (2.5 mg/kg subcutaneous) each day for 10 days was shown to result in significantly attenuated respiratory depression in response to acute morphine [21].

Hill et al [22] demonstrated that tolerance to acute morphine respiratory depression occurred following prolonged morphine treatment with a 75mg morphine pellet implanted subcutaneously in mice for a period of 6 days.

Opioid tolerance is often investigated with regard to opioid-induced antinociception, given that in the clinical setting opioids are used primarily as analgesics.

The development of tolerance to opioid antinociception in animal models is an accepted phenomenon; however, it seems controversial regarding other opioidergic effects. With regards to opioid induced respiratory depression there are conflicting reports that tolerance does [21] or does not [23] occur.

1.4. Forensic Aspects Related to Opioids Abuse

Fatal opioid consumption may produce a variety of autopsy findings depending primarily on the duration of intravenous drug consumption.

In the case of drug-induced lethal intoxication, massive pulmonary edema frequently weighing more than 1 Kg per lung can be seen macroscopically [24].

Histopathological investigations of lung tissue in drug-related deaths can reveal a large number of findings, which are primarily considered the result of drug-induced apnea or hypoxia.

Initially, autopsy frequently reveals massive, relatively protein-rich pulmonary edema.

In the case of protracted drug-induced pre-death morbidity, purulent bronchopneumonia may also develop from a preexisting purulent bronchitis. Also, following many years of intravenous drug consumption, deposits of immunoglobulin and complement can be detected in the pulmonary interstitium, as well as in the glomeruli in the case of heroin-associated nephropathy [25].

The so-called toxic pulmonary edema associated with opioids-related deaths is distinguished from others, in particular cardiac pulmonary edema and is discussed as the cause of death, although its pathogenesis is not entirely understood [25]. It involves edema with a histologically light eosin-red fluid in the alveolar spaces, partially penetrated with erythrocytes, thus a hemorrhagic pulmonary edema. In the case of opioids-related death, this pulmonary edema is particularly rich in proteins and can induce an excretion of foaming liquids from the respiratory orifices (i.e. mouth and nose).

In particular, a considerable increase in pulmonary alveolocapillary permeability was observed under the influence of heroin and/or morphine, together with a loss of albumins in the edema fluid. In such cases, primary toxic damage to the alveolocapillary basal membrane is considered.

The excretion of erythrocytes into the alveolar space is also displayed and leads to a hemorrhagic pulmonary edema as observed in overodose victims. A potential explanation was given for this histopathological picture: apart from toxic damage, which cannot be proven microscopically, acute vascular congestion alone was thought to lead to ruptures of the basal membrane mechanically since identical ruptures have also been observed in a control group with cardiac pulmonary edema [25]. Taken together, however, numerous ruptures of the basal membrane lead to considerable alveolocapillary damage [26].

The so-called pulmonary granulomatosis is typical after many years of intravenous drug injection [27,28]. It is described as a fullblown

pulmonary granulomatosis, which are accumulations of birefringent foreign material, which entered the lung via the veins by means of embolism. This foreign material is surrounded by perivascularly localized granulomas [25]. The granulomas contain collagen fibers, a primarily lymphomonocytic inflammatory infiltrate and polynuclear foreign-body giant cells. In some cases, pulmonary granulomatosis can include perivascular granulomas with subsequent and considerable right heart overload, resulting in right ventricular hypertrophy of the myocardium, and the risk of microthrombosis formation is discussed [29]

However, it is always a condition that occurs after many years of intravenous drug consumption [30].

Apart from lungs disorders, other organs are also targets in opioid abuser. After many years of intravenous drug abuse, inflammatory as well as a toxic involvement of the myocardium must also be considered. Animal experiments have shown to differentiate the toxic effects of a substance from other influencing factors [31]. This also applies to the independent toxic effect of any accompanying substances injected [32]. Inflammatory processes are significant due to the permanently activated immune status. Toxic processes are significant since injected drugs and toxins can lead to allergic eosinophilic myocarditis or to drug-induced cardiomyopathy. In addition, varying degrees of myofibrillar degeneration have been described. Moreover, activation or degranulation of myocardial mastocytes and increased expression of TNF-alpha have also been cited [33].

Regular intravenous consumption of drugs may lead to a wide range of histopathological findings in the kidneys [34], including reversible drug-induced uremia [35]. The term used in the literature for such kidney disease is “heroin-associated nephropathy” (HAN).

Intravenous drug consumption is an important factor in kidney failure (endstage renal disease, ESRD) with subsequent dialysis treatment.

The spectrum of heroin-associated kidney diseases includes primarily acute kidney failure, glomerulopathies, such as focal segmental glomerulosclerosis (FSGS), and membraneproliferative glomerulonephritis (MPGN), frequently associated with immune complex glomerulonephritis as a result of bacterial endocarditis or sepsis [36]. In addition, interstitial nephritis can be seen in various forms.

Rhabdomyolysis is also tipically occurring after heroin consumption, first described in 1971 [37]. The prevalence of rhabdomyolysis in drug related deaths has been investigated. In an immunohistochemical investigation, Welte et al. [38] could show myoglobin deposits in renal tubules in comparison with a control group. A heroin overdose may result in myoglobin kidney failure. Even in H&E staining, protein cylinders can be proven in the renal tubules, the character of which can be classified by means of immunohistochemical staining for myoglobin [38].

With regards to neuropathological findings, there are several effects of intravenous drug consumption on the central nervous system. According to immunohistochemistry, axonal damage occurs [39] in addition to hypoxic or hypoxemic lesions [25], as well as neurovascular complications. Neurons of the central nervous system can express different kinds of opioid receptors, which, in the case of chronic opiate exposure, react adaptively with altered receptor density.

Opioid receptors can be displayed immunohistochemically and by means of in situ hybridization. By this means, both “downregulation” and “upregulation” of receptor density have also been observed.

Chapter 2

Stem Cells and Vascular Maintenance

2.1. Pericytes and Their Role in Regenerative Medicine

Pericytes were described more than 100 years ago as perivascular cells that wrap around blood capillaries.

Pericytes are also known as mural cells because of their location within the blood vessel [40].

They are usually named for the organ in which they reside; therefore in the liver they are known as Ito cells or hepatic stellate cells; in the kidney n as mesangial cells; and in the bone marrow they are called adventitial reticular cells.

Although it is well known that pericytes belong to the same lineage as vascular smooth muscle cells (vSMCs), no specific molecular markers are currently able to distinguish pericytes from vSMCs or other mesenchymal cells.

The basement membrane separates the majority of the pericyte-endothelial interface, although both cell types come in contact at certain points via micro-holes in the basement membrane.

Pericytes are fibroblast-like cells with distinguishable nuclei, low cytoplasmic content, and several long processes surrounding the endothelial wall.

Pericytes located on the outer surface of blood capillaries interact with underlying endothelial cells and are covered in the same basement membrane.

Pericytes processes are usually connected with more than one endothelial cell via adhesion plaques in addition to peg-and-socket contacts, which permit direct contact between the two cell types [41].

Concerning the origin of pericytes, during embryogenesis, blood vessel formation and development occurs via the following two mechanisms:

vasculogenesis, which is in vivo vessel formation; and angiogenesis, which is the formation of new blood vessels from pre-existing vessels. During vasculogenesis, pericytes originate either from mesenchymal stem cells (MSCs) residing in the abluminal side of the endothelial tube (mesodermal origin) or from the neural crest. Some studies have also proposed that pericytes may arise in addition directly from endothelial cells and the bone marrow.

Analysis of the anatomic relationship between pericytes and endothelial cells shows that they interact closely via juxtacrine or paracrine signalling, and possibile pathways for endothelial-pericyte signalling and the role of different intercellular signalling pathways in vascular stability have been described.

One of the most important functions of pericytes is their role in blood flow regulation and participation in the stability, remoulding, maintenance and permeability of blood vessels.

Similarly to vascular smooth muscle cells, pericytes regulate vasodilation and vasoconstriction within the blood capillaries to control vessel diameter and blood flow [40].

Mid-capillary pericytes play a limited role in blood flow regulation; however, since these pericytes are located at junctions with endothelial cells, they are believed to permit the fusion of cells and transfer of fluids and proteins through the walls of blood capillaries [40].

In sites between pericytes there are decreased amounts of proteins in the vascular basement membrane. Furthermore, these areas are invaded by neutrophils to allow their escape into the perivascular space.

The presence of such important areas allows local wound healing and the extravasation of blood vessels without causing any damage to membrane integrity.

In 2008, Caplan reported that all MSCs are pericytes, not only because all MSCs express pericytes marker, but also because $CD146^{+}$, $CD34^{-}$, $CD45^{-}$ and $CD56^{-}$ sorted pericytes demonstrate multipotent potential

and differentiate into adipocytes, chondrocytes, myocytes and osteocytes [42].

This differentiation potential of pericytes is highly associated with their lineage and the microenvironment.

However, the perivascular locations of neural and haematopoietic stem cells in foetal tissue indicate that other stem cells accommodate this perivascular environment [40], supporting the hypothesis that not all pericytes are MSCs.

With regard to pericytes contribution on wound healing, it is well known that wound healing is a complex multistep process that includes inflammation, re-epithelialization, angiogenesis, matrix formation and tissue regeneration and pericytes play an important role during each of these processes.

In addition to their different physiological roles, pericytes were shown to contribute to tissue regeneration and repair in various experimental settings.

In particular, it has been demonstrated that they hold reliable adeptness for vascular regenerative therapies, due to their physiological contribution to angiogenesis and maintenance of the blood vessel integrity [43]. Pericytes potential capacity to differentiate into cells of different lineages, in addition to cells of vascular tissue, suggests a potentially valuable contribution in vascular regenerative medicine.

2.2. Stem Cells

Stem cells represent a population of somatic cells that are present in nearly all tissue of the body and contribute to tissue regeneration and healing. The damage of stem cells or the impairment of their function is reflected in worsened regeneration.

Stem cells are undifferentiated biological precursors that have two unique properties: self-renewal and the ability to differentiate into specialized cells under the appropriate conditions [45]. According to

the differentiation potential, stem cells can be generally categorized, from high to low, as totipotent, pluripotent, multipotent, oligopotent, and unipotent.

Totipotent cells can differentiate into every cell type including the embryonic and the extra-embryonic tissues, and therefore have the ability to develop a complete organism.

Pluripotent cells (embryonic stem cells, induced pluripotent stem cells) can differentiate into the cells of all three germ layers, ectoderm, mesoderm and endoderm [45], but not to the extra-embryonic membranes and therefore cannot form a complete organism.

Multipotent cells (mesenchymal stem cell, hematopoietic stem cells, neuronal stem cells, and endodermal stem cells) are able to differentiate into several cell lineages, usually these lineages belong to the same germ layer where the multipotent cells are derived from.

Oligopotent cells (monoblast, proerythroblast) can differentiate into only a few cell lineages that are closely related to their original layer.

Finally unipotent cells can give rise to only one cell type and therefore are described as precursor cells or progenitors.

Since the pluripotent cells can differentiate towards cells from all germ layers, they also provide great hope for cell-based therapies in regenerative medicine. The first discovered and well-studied pluripotent stem cells were embryonic stem cells, which are isolated from the inner cell mass of embryos in the blastocyst stage [46].

Also induced pluripotent stem cells have been created and used as an alternative source of embryonic stem cells.

To date, any data on the effects of opioids on the properties and function of stem cells are absent or strongly limited.

Indirect observation from experimental and clinical studies indicate that tissue repair and regeneration are impaired after a systemic administration of opioids or in opioid drug addicts [47,48].

For example, it has been shown that morphine and other opioids inhibit neural stem cell proliferation and hippocampal neurogenesis [49,50]. Willner et al. [51] showed that a short-term morphine exposure of neural progenitors already induced the inhibition of their proliferation and promoted apoptosis. The prolonged use of morphine was also reported to impair angiogenesis and activation of endothelial progenitor cells and negatively influence wound repair [52].

2.2.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) belong to the adult stem cells, which are able to repair body cells and maintain the normal turnover of regenerative organs.

MSCs can be obtained from bone marrow [53,54], peripheral blood, umbilical cord as well as umbilical cord blood, skeleton muscle, placenta, and adipose tissue [55,56]

MSCs are multipotent and able to differentiate towards a number of different cell lineages. For example MSCs can be successfully differentiated towards chondrocytes, osteoblasts or adipocytes [45,57] but MSCs are also able to differentiate towards several additional mesodermal cell lineages.

For example MSCs can differentiate into skeleton muscle cells [58] cardiac muscle cells [59], and smooth muscle cells [60,61].

Interestingly, MSCs also show the capacity to differentiate into cells derived from other germ layers, such as neuron-like cells in vitro [62].

In addition MSCs also pose the ability to differentiate into endothelial cells [63,64].

This phenomenon can be explained due to the fact that MSCs, even if mainly originate from mesoderm, are also in parte originated from the other two germ layers.

To characterize MSCs, minimal criteria were suggested by the International Society for Cellular Therapy [65]. MSCs should therefore

display plastic adherence; show a multipotent differentiation potential in vitro towards the adipogenic, osteogenic and chondrogenic cell lineage as demonstrated by specific staining; express the mesenchymal markers CD73, CD90 and CD105, and be negative for markers CD34, CD45, CD14 or CD11b, CD79α or CD19 and HLA-DR.

Because of their trophic property in secreting a wide range of growth factors, including vascular endothelial growth factor, epithelial growth factor, insulin-like growth factor, transforming growth factor-alpha and -beta1 (TGF-β1), along with their immunomodulatory, anti-apoptotic and anti-inflammatory properties exerted in response to a mechanical damage or flogosis, they play a fundamental role in tissue repair and regeneration.

2.2.2. Stem Cells in Regenerative Medicine

Stem cells are widely considered and used to re-establish the function of damaged cells or tissues or to generate organs for tissue replacement [66].

Because of a dearth of transplantable organs, there is a growing hope that stem cells may represent an answer to replace tissues worn out by old age and ravaged by disease.

Patients with injuries from accidents or degenerative diseases always require a large amount of mature cells that can be generated from stem cells with sufficient numbers and quality.

The most known and widely used stem cell therapy is the transplantation of bone marrow hematopoietic cells to treat multiple myeloma or leukemia patients [67,68].

In addition, using skin stem cells growing skin grafts for patients with severe burns of the body is another clinical available stem cell therapy since long ago [69].

Several other stem cell applications are being studied in clinical trials such as spinal cord injury [70], reconstruction of large bone defects [71], or corneal disease [72].

In addition stem cells have also been considered as a supplemental source to treat diabetes [73], liver damages [74], Parkinson's disease as well as Alzheimer's disease [75].

The stem cell therapy has drawn an increasing attention in recent years due to their repair function capacities and the rising requirement of tissue replacement.

Taken together, stem cell therapy has been already applied into the clinic and provides a promising way to achieve patient-specific therapies for various diseases in the future.

However, basic research must be performed carefully to better elucidate stem cells function and proprietis as well as prevent undesired differentiations and optimize the long-term repair.

2.2.3. Endothelial Cell Differentiation of MSCs

Vascular endothelial cells play a crucial role in various vascular functions.

Damage or dysfunction of endothelial cells may lead to serious consequences.

Mesenchymal stem cells have been considered as a favourable source to obtain a large amount of endothelial cells to replace the damaged ones.

Differentiated endothelial cells are characterized with the major endothelial cell markers.

For example, vascular endothelial growth factor receptor 2 (VEGFR2) is one of the two major VEGF receptors and it is regarded as an early stage marker during endothelial cell differentiation [76].

Later stage markers include von Willebrand factor (vWF), platelet-endothelial cell adhesion molecule 1 (PECAM-1), endothelial nitric

oxide synthases (eNOS) and vascular endothelial-cadherin (VE-cadherin), which are used to identify mature differentiated endothelial cells [76].

Because the change of mRNA expression and surface marker profile is not sufficient to prove the success of differentiation, endothelial cell functional tests such as three-dimensional capillary-like tube formation assay on matrigel is used to further characterize the cells [44,77]. On this matrice cultured cells usually grow developing a tube-like structure because the matrix itself acts as a model of the basement membrane creating a physical support and ideal environment

The most used medium to induce endothelial cell differentiation contains normally VEGF and fibroblast growth factor (FGF).

VEGF is the most common supplementary agent and enhances the endothelial cell differentiation significantly [78].

FGF induces MSC differentiation towards ECs even without adding VEGF and promotes angiogenesis during the formation of large and mature blood vessels [79].

Other soluble factors such as endothelial growth factor (EGF) are also known to enhance the differentiation process.

Chapter 3

Morphine toxicity on Endothelial Differentiation of Vascular Mesenchimal Stromal Cells

3.1. Aim of the Study

In the field of illicit drug use, heroin-related mortality is a complex phenomenon. There is a great variability in prevalence mortality rates attributable to heroin overdose, but most studies on longitudinal trends of overdose deaths, or overdose-related hospitalizations, show increases across time [1].

As well as other opiates, heroin and its metabolite morphine cause mental and grastrointestinal effects, as well as urinary retention; however the most serious effect of opiates is the respiratory depression and pulmonary edema, which is non-cardiogenic.

Indeed, the most common pulmonary findings in opiate-related fatalities are pulmonary congestion and edema, as well as acute/chronic alveolar haemorrhage [25]. The cause is thought to be damage to the capillary endothelium: ischemic, related to ipoperfusion of the pulmonary vascular bed, and/or related to the direct toxic effect of the substance on entothelial cells [25].

Tissue regeneration and repair are essential after endothelial cell injury and are related to the activity of human vascular mesenchimal stromal cells (vMSCs). As a matter of fact, also an opiate-related impairment of vMSCs function might be a part of the pathophysiology of endothelial cell injury.

The aim of this study is to assess the effect of in-vitro opiate exposure on the phisiologial activity and maintenance of human vMSCs.

3.2. Material and Methods

Sample collection and vascular mesenchymal stromal cell isolation and characterization

Abdominal aorta fragments were collected by the Unit of Vascular Surgery, "Santa Maria delle Croci" Ravenna Hospital (Italy) during repair surgery following the Code of Ethics of the World Medical Association from patients with abdominal aorta aneurism.

Explants were washed several times in phosphate buffer saline (PBS), dissected in small pieces and cultured in Minimal essential medium (MEM) (Gibco, Thermo Scientific, Monza, Italia) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Scientific, Monza, Italia), 10.000 U/ml Penicillin-Streptomycin at 37°C and 5% CO2. After two weeks, the cells obtained were transferred to T25 cell culture flasks and subcultured for the following experiments in the same growing conditions as previously described. Cells from passage 2 to passage 10 were utilized for this study.

The vascular mesenchymal stem/stromal cells (vMSCs) obtained have been previously characterized for the expression of mesenchymal markers such as CD90 and CD105, in agreement with the scientific literature.

Morphine sulphate treatment

Treated vMSCs consisted in cells exposed to 0,1 mM, 0,4 mM, 0,8 mM and 1 mM morphine sulphate (Molteni Farmaceutici S.p.A, Florence, Italy) diluted in MEM supplemented with 2% FBS for 7 days at 37°C and 5% CO2. Control samples consisted in vMSCs grown in MEM supplemented with 2% FBS and MEM supplemented in 10% FBS for 7 days.

Cell viability assessment

vMSCs were seeded in triplicate into a 96-well culture plate at the density of 8x103 cells/well for 24 h. Then, the medium was changed with a fresh one containing the morphine sulphate solutions to be tested.

After 7 days, 10 µl of tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) was added in each well and incubated for 3 h at 37°C in 5% CO2. Formazan crystals were dissolved by adding dimethylsulfoxyde (DMSO) in isopropanol (1:1). Optical density was measured, using a spectrophotometer microplate reader (LT-4000 Microplate reader, Labtech L.t.d, Heatfield, UK) at 578 nm (reference wavelength 690 nm).

Results were expressed in percentage as relative viability compared to control samples.

Cell proliferation assay

BrdU cell proliferation assay was performed by using Cell Proliferation ELISA BrdU (Roche Diagnostics GmbH, Mannheim, DE) according to manufacturer's protocol. In brief, vMSCs were seeded in triplicate into a 96-well culture plate at the density of 8x103 cells/well for 24 h. Then, the medium was changed with a fresh one containing the morphine sulphate solutions to be tested. At the end of the treatment, 10 µM BrdU labeling solution was added in each well for 24 h. Then, the samples were fixed and incubated with anti-BrdU antibody peroxidase conjugated, for 90 minutes at room temperature. Following three washes in PBS, tetramethyl–benzidine (TMB) substrate solution was added for 20 min at room temperature, and the reaction was stopped with 1M H2SO4. The optical density was measured using a spectrophotometer Microplate Reader (LT-4000, LabTech, Euroclone, Milan, Italy) at a wavelength of 450 nm and a reference wavelength of

690 nm. Results were expressed in percentage as relative values compared to control vMSCs.

Reactive Oxygen Species (ROS) detection assay

Carboxy-H2DCFDA fluorescent dye (Invitrogen, Carlsbad, CA, USA) was employed to quantitate the oxidative stress induced by seven days morphine exposition. vMSCs were seeded in triplicate into a 96-well culture plate at the density of 1x104 cells/well for 24 h. Then, the medium was changed with a fresh one containing the morphine sulphate solutions to be tested. At the end of the treatment, samples were washed in PBS and 5μM carboxy-H2DCFDA ROS detection probe (Thermo Fisher Scientific, Monza, Italy) diluted in MEM supplemented with 2% FCS was added for 3 h at 37°C and 5% CO2.

As positive ROS control samples, vMSCs treated with 200 mM H2O2 in MEM supplemented with 2% FCS were added. A fluorimeter microplate reader (Glomax, Promega Corporation, Madison, WI, USA) with excitation and emission wavelengths set respectively at 492 nm and 517 nm was used to detect the fluorescent signal. Results were expressed as relative percentage compared to control samples.

Senescence associated β-Galactosidase assay (SA-β-Gal)

vMSCs were seeded in triplicate into a 96-well culture plate at the density of 1x104 cells/well for 24 h. Then, the medium was changed with a fresh one containing the morphine sulphate solutions. At the end of the treatment, samples were washed in PBS, fixed with 2% formaldehyde for 30 minutes and stained with the cell event senescent green probe (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA), a sensitive fluorescent substrate for the β-galactosidase enzyme, diluted 1:1000 in the staining buffer, for 1h and 30 min at 37 °C, in a CO2 free environment. At the end of the incubation, samples were washed in PBS and and the fluorescent signal was measured using a fluorescent

microplate Reader (GloMax Discover System, GM3000, Promega Corporation, Madison, WI, USA) at an excitation wavelength of 490 nm and an emission wavelength of 514 nm.

Ultrastructural analysis by transmission electron microscopy (TEM)

vMSCs were seeded on cover glasses at the density of 1.5 x104 cells/glass for 24 h. Then, the medium was changed with a fresh one containing the morphine sulphate solutions to be tested. At the end of the treatment, samples were washed with PBS and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 minutes at 4°C, and they were post fixed with a solution of 1% OsO4 in 0.1M phosphate buffer for 30 minutes at room temperature. After some washes in 0.15M phosphate buffer, the samples were dehydrated in a graded series of aceton and embedded in Epon resin (Sigma Aldrich, St. Louis, Missouri, USA). Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed under a Philips CM100 (FEI Italia Srl, Milan, Italy). The images were digitally captured by SIS Megaview III CCD camera (FEI Italia Srl, Milan, Italy).

In vitro vascular differentiation

vMSCs were seeded on cover glasses at the density of 1.5 x104 cells/glass for 24 h. Then, the medium was changed with a fresh one containing 50 ng/ml of vascular endothelial growth factor (VEGF) for 7 days at 37° and 5% CO2, followed by morphine sulphate treatment for other 7 days at 37°C and 5% CO2. At the end of the treatment, the samples were fixed in 4% paraformaldehyde in PBS for 30 minutes at 4°C, followed by a permealization step in 0.1% Triton - X 100 for 5 minutes at 4°C. After some washes in PBS, the samples were covered with 2.5% bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, Missouri, USA) diluted in PBS (blocking reagent), for 30 minutes at room temperature (RT), followed by incubation in mouse anti-human

CD31 antibody (Origene, Thermo Fisher Scientific, Monza, Italy) diluted 1:100 in blocking reagent, over night at 4°C. Then, the samples were washed in PBS and incubated for 1 h and 30 minutes at 37°C with secondary antibody anti-mouse IgG – Cy3 conjugated (Sigma Aldrich, St. Louis, Missouri, USA), diluted 1:2000 in PBS. Samples were rinsed with PBS, counterstained with DAPI and mounted in vectashield medium (Vector Laboratories, Inc, Burlingame, CA, USA). The fluorescence microscopy Eclipse E800 Nikon (Nikon, Tokyo, Japan) was utilized to observe the samples.

Statistical analysis

Statistical analysis was carried out using GRAPH PAD PRISM 5.0 software (San Diego, CA, USA) applying a one-way ANOVA followed by Tukey's multiple comparison test. The differences were considered significant at $p < 0.05$.

3.3. Results

vMSC viability after morphine sulphate treatment

In vitro cellular toxicity induced by different concentrations of morphine sulphate was investigated by MTT assay. Results showed a decrease of cell viability dose dependent (figure 1A) after 7 days of treatment, reaching a reduction of 54% at 0.8 mM ($p < 0.05$) and 63% at 1mM ($p< 0.05$) compared to control samples. However, there was no statistical significant difference between samples exposed to 0,8 mM and 1mM of morphine sulphate.

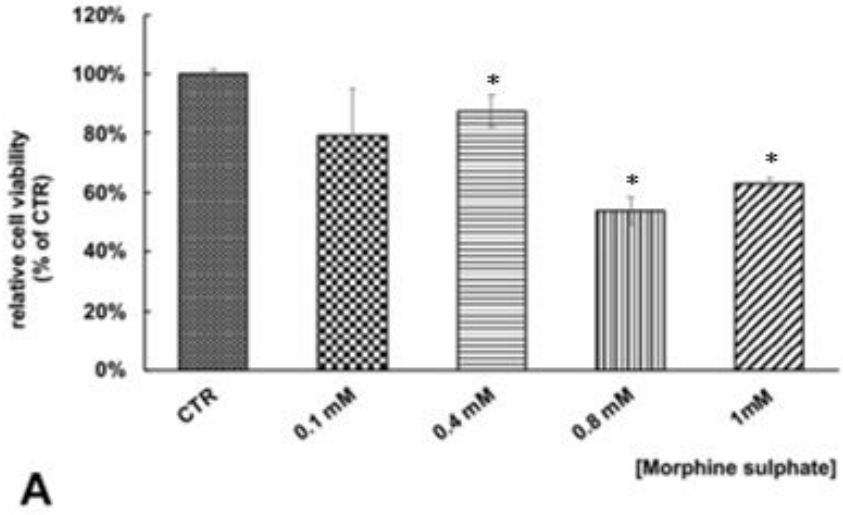


Figure 1A. Effect of morphine sulphate on the viability of vMSC exposed to different concentration (0 – 1mM) for 7 days. At the end of the treatment a MTT assay was carried out. Data are expressed in percentage as cell viability relative to control samples (100%) and they are expressed as mean ± SEM of three independent experiments run in triplicates. Statistical significance was set as *$p < 0.05$, vs. control samples (CRT).

vMSC proliferation after morphine sulphate treatment

Based on cell viability results, in order to verify a potential reduction of cell proliferation induced by morphine treatment, a BrdU assay was carried out on vMSCs exposed to different concentration of morphine sulphate. Results showed a significant growth inhibition compared to control samples at the concentration of 0.8 mM ($p < 0.05$) and 1mM ($p < 0.05$) of morphine sulphate (figure 1B), suggesting an influence of the drug on cell cycle distribution, a pro – oxidant and pro- senescence effect.

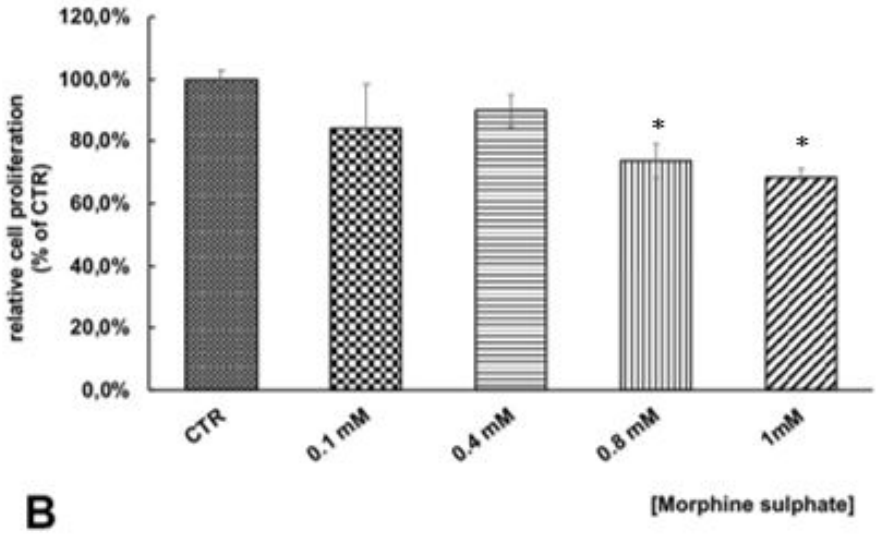


Figure 1B. Effect of morphine sulphate on the proliferation of vMSC exposed to different concentration (0 – 1mM) for 7 days. At the end of the treatment a BrdU assay was carried out. Data are expressed in percentage as cell proliferation relative to control samples (100%) and they are expressed as mean ± SEM of three independent experiments run in triplicates. Statistical significance was set as *$p < 0.05$, vs. control samples (CRT).

ROS generation in vMSCs after morphine sulphate exposition

In order to demonstrate an effect of morphine sulphate on ROS generation, a carboxy-H2DCFDA assay was carried out on vMSCs exposed to different concentration of the drug. Figure 1C showed a significant increase in intracellular ROS in vMSCs exposed to 0.8 mM ($p < 0.05$) and 1mM ($p < 0.05$) (figure 1C) compared to control samples, confirming a pro-oxidant effect of morphine sulphate.

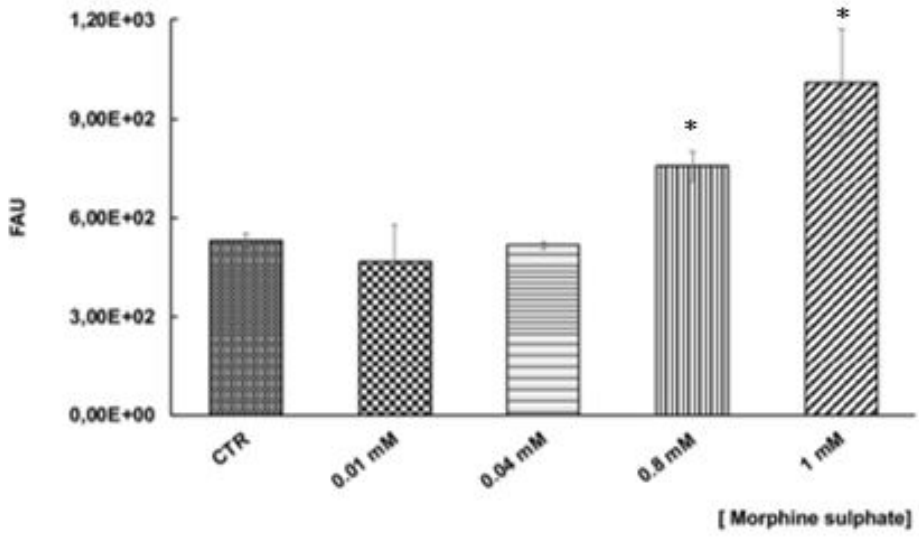


Figure 1C. Effect of morphine sulphate on the total amout of cellular ROS in vMSCs exposed to different concentration (0 – 1mM) for 7 days. At the end of the treatment a carboxy-H_2DCFDA assay was carried out. Data are expressed in fluorescent arbitrary units (FAU) and they are expressed as mean ± SEM of three independent experiments run in triplicates. Statistical significance was set as *$p < 0.05$, vs. control samples (CRT).

Evaluation of SA-β-gal biomarker on vMSCs exposed to morphine sulphate

In order to demonstrate a pro-senescent effect of morphine on vMSCs, a SA-β-gal assay was carried out. As shown in figure 1D, a light but constant increase of the fluorescence signal was observed in all the samples compared to control ones, suggesting a dose dependent relation between cellular senescence and morphine concentration.

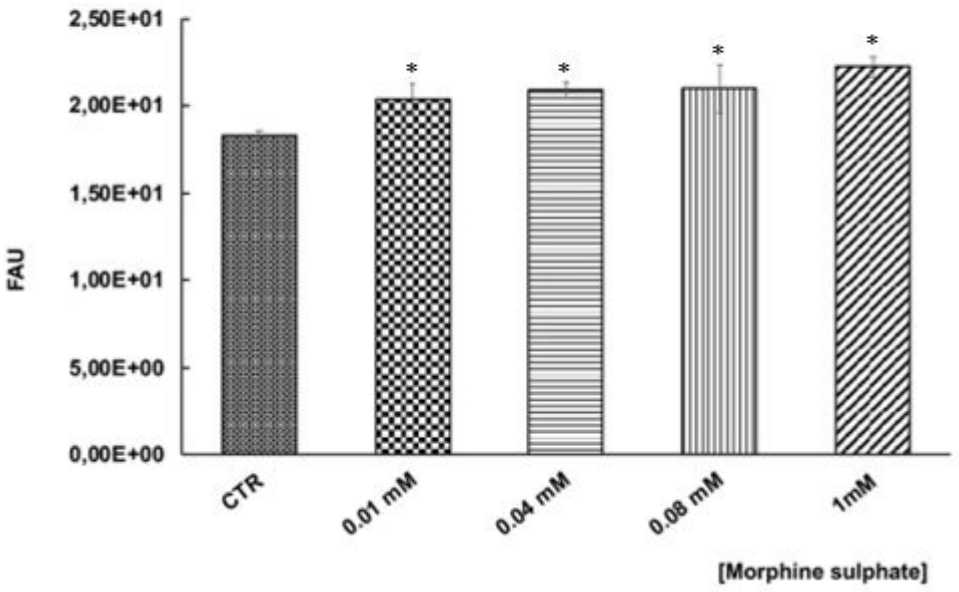


Figure 1D. Effect of morphine sulphate on induction of cellular senescence in vMSCs exposed to different concentration (0 – 1mM) for 7 days. At the end of the treatment a SA-β-gal assay was carried out. Data are expressed in fluorescent arbitrary units (FAU) and they are expressed as mean ± SEM of three independent experiments run in triplicates. Statistical significance was set as *$p < 0.05$, vs. control samples (CRT).

Ultrastructural analysis of vMSCs exposed to morphine sulphate

To better investigate the cellular behavior toward the toxicity induced by morphine sulphate, an ultrastructural analysis by TEM was carried out. The final aim was to detect any modification in nucleus and cellular organelles which could allow to understand the potential mechnisms underpinning celluler compensation to morphine sulphate treatment.

Control cells demonstrated a fibroblast shape morphology, with well preserved nucleus and nucleoli and plasma membrane (figure 2A). In the cytoplasm, several cellular organelles such as mitochondria, rough endoplasmic reticulum (RER) and Golgi complex were easily detected. They showed normal size and cytoplasmic distribution, compatible with an active and healthy condition of the cell (figure 2B). Some lysosomes, were also detected in the cytoplasm (figure 2B).

After 0.8 mM morphine exposition, vMSCs showed a round shape morphology (figure 2C). In the cytoplasm numerous long shaped mitochondria and several primary lysososmes were detected (figure 2D).

After 1 mM of morphine exposition, a strong proliferation of primary lysosomes and autophagic vesicles was detected in the cytoplasm of the vMSCs (figure 2E).

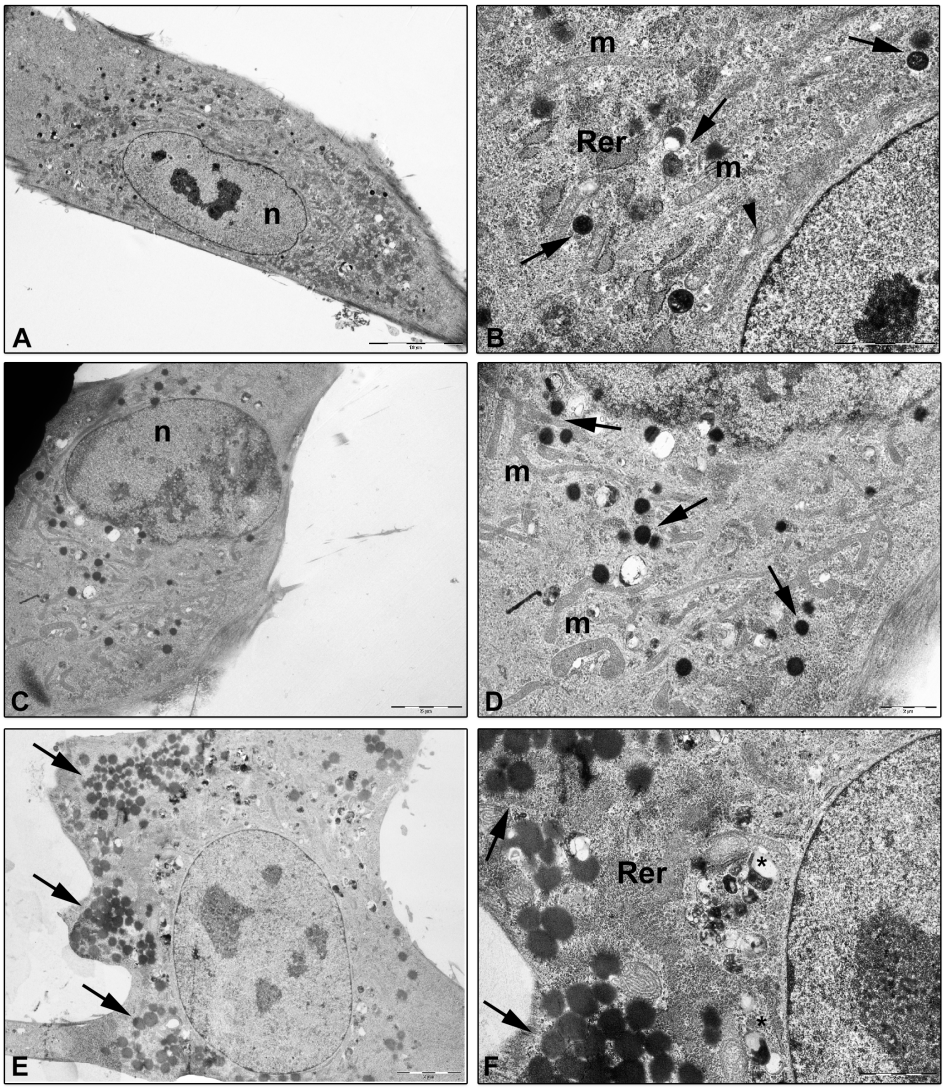


Figure 2. TEM ultrastructural analysis of vMSCs exposed to 0.8 and 1 mM of morphine sulphate for 7 days. (A) Control vMSCs. Cells showed a fibroblast shape morphology with nucleus (n) and nucleoli well detected (bar: 10 μm); (B) Detail of vMSC cytoplasm showing mitochondria (m), primary lysosomes (arrow), rough endoplasmic reticulum (Rer) and Golgi complex (arrowhead) (bar: 2 μm); (C) vMSCs after 0.8 mM of morphine sulphate treatment. Cells showed a round shape morphology. Nucleus (n) and cytoplasm organelles are still detected (bar: 5 μm); (D) Detail of vMSC cytoplasm showing numerous long shape mitochondria (m) and several primary lysosomes (arrow) (bar: 2 μm); (E) vMSCs after 1 mM of morphine sulphate treatment. A proliferation of primary lysosome (arrow) scattered in the cytoplasm is observed (bar: 5 μm); Primary lysosomes (arrow), secondary lysosomes, autophagic vesicles (*) and irregular Rer (Rer) are detected(F) (bar: 2 μm).

CD31 immunofluorescence

In order to demonstrate the effect of morphine sulphate in impairing the ability of vMSCs to differentiate towards a vascular phenotype, the expression of the endothelial marker CD31 was investigated in cells exposed to VEGF and/or morphine sulphate for 7 days by immunofluorescence.

vMSCs exposed to VEGF for 7 days, showed a strong fluorescence signal on their cellular surface corresponding to the endothelial marker CD31 (figure 3A). vMSCs exposed to 0.8 mM (figure 3C) and 1mM (figure 3D) of morphine sulphate showed a strong reduction of the expression of CD31 protein, suggesting an impairing of morphine sulphate on vascular differentiation. Control samples consisting in vMSCs, exposed or not to morphine sulphate, without any VEGF stimulation, showed a weak CD31 expression (figure 3B, D, F).

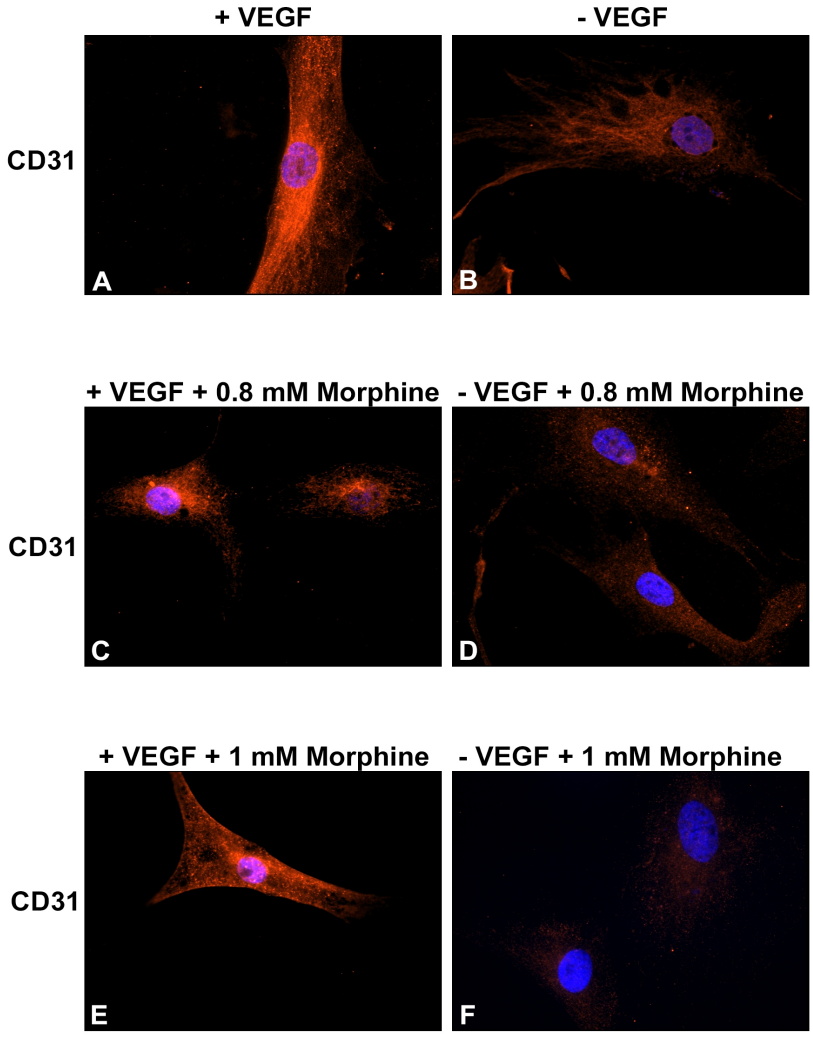


Figure 3. Immunofluorescence images of vMSCs exposed to different concentration of morphine sulphate and immunolabeled for the endothelial CD31 protein. (A) vMSCs exposed to VEGF for 7 days reveal a positive staining for CD31 protein marker (red); (B) vMSC grown in absence of VEGF for 7 days. A weak signal corresponding to CD31 marker is observed on cellular surface; (C) vMSCs exposed to VEGF and 0.8 mM morphine sulphate for 7 days. A reduced red fluorescent signal was observed on cellular surface; (D) vMSC grown in absence of VEGF and in presence with 0.8 mM of morphine sulphate for 7 days. Almost no fluorescent signal corresponding to CD31 marker is detected; (E) vMSCs exposed to VEGF and 1 mM morphine sulphate for 7 days. A weak signal is observed on cellular surface; (D) vMSC grown in absence of VEGF and in presence with 1 mM of morphine sulphate for 7 days. Almost no fluorescent signal is detected. Images are representative of three independent experiments. All the cells are co-stained with 4,6-diamidino-2-phenylindole (DAPI) (blue) for nuclei visualization. Magnification 600x for all the images.

In vitro vascular differentiation

To confirm the influence of morphine sulphate to impair vascular differentiation on vMSCs, a tubular in vitro assay was carried out on geltrex matrix, exposing the cells to VEGF for 7 days, with and without morphine sulphate.

vMSCs exposed to VEGF and in absence of morphine sulphate showed a thin and elongated cellular morphology, resembling an endothelial phenotype (figure 4A). Some of them connected each other, producing a network. After the exposition of VEGF and 0.8 mM (figure 4C) and 1 mM (figure 4E) of morphine sulphate, a few numbers of cells were characterized by an elongated shape, (figure 4C and 4E) while the majority of them were round shaped, suggesting cell death (figure 4C).

Control cells, not exposed to VEGF did not show any morphological modification, both in absence and presence of morphine sulphate (figure 4B, 4D and 4F).

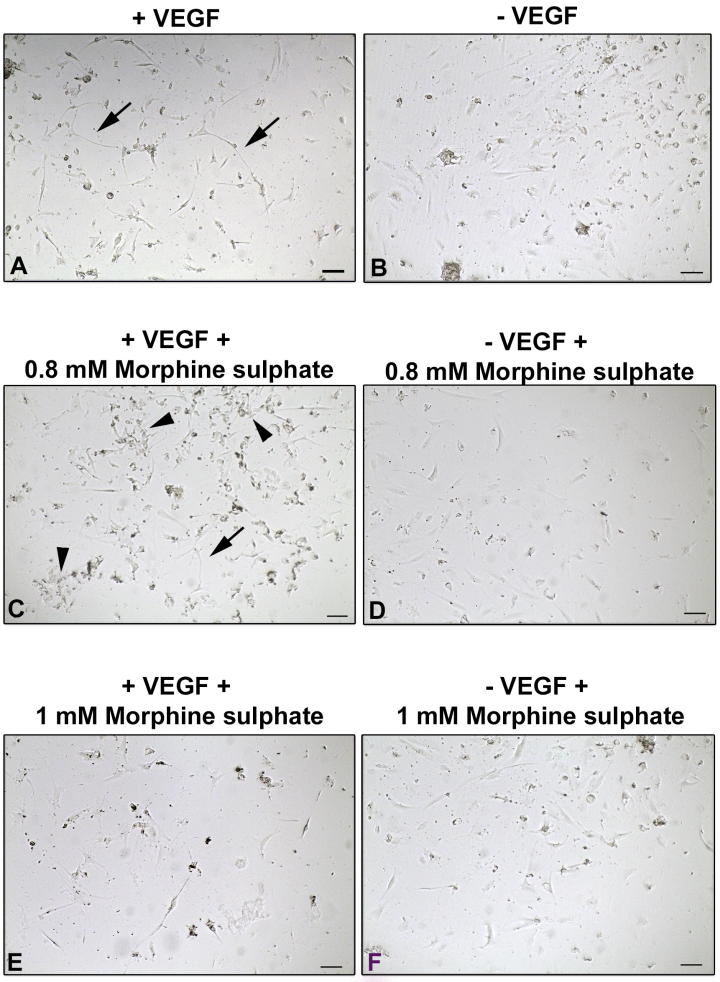


Figure 4. In vitro tubular assay of vMSCs, grown on geltrex matrix and exposed to different concentrations of morphine sulphate and/or VEGF for 7 days. (A) vMSCs exposed to VEGF show a thin and elongated cellular morphology, resembling an endothelial phenotype. Some cells connect each other producing tubular structures (arrows); (B) vMSCs grown in absence of VEGF. Cells showed a fibroblastic and polygonal shape morphology. No tubular structures are detected; (C) vMSCs exposed to VEGF and 0.8 mM morphine. Several cells are dead (arrowheads) while just a few of them showed an elongated morphology (arrows). No tubular structures are observed; (D) vMSCs grown in absence of VEGF and in presence with 0.8 mM of morphine sulphate. A few cells are detected showing a fibroblastic and polygonal like morphology; (E) vMSCs exposed to VEGF and 1 mM morphine. Dead and a few elongated cells are detected. Tubular structures are missing; (F) vMSCs grown in absence of VEGF and in presence with 1 mM of morphine sulphate. A few cells are observed showing a fibroblastic and polygonal like morphology. Light microscopic images representative of three independent experiments. Bar: 100 μm.

3.4. Discussion

In opiate-related fatalities common findings are pulmonary congestion and edema, as well as acute/chronic alveolar hemorrhage. The cause is thought to be damage to the capillary endothelium and also tissue regeneration and repair mechanisms, including vMSCs function, are supposed to be impaired. In this study the effect of in-vitro morphine sulfate exposure on the function and morphology of human vMSCs was investigated. Human mesenchymal stem/stromal cells are a versatile class of multipotent adult stem cells capable of self-renewal and osteogenic, chondrogenic, adipogenic as well as myogenic and endothelial differentiation [80,81]. Because of their trophic property in secreting a wide range of growth factors, including vascular endothelial growth factor, epithelial growth factor, insulin-like growth factor, transforming growth factor-α and -β1, along with their immunomodulatory, anti-apoptotic and anti-inflammatory properties exerted in response to a mechanical damage or flogosis, they play a fundamental role in tissue repair and regeneration [82,83].

Holan et al. demonstrated the expression of opioid receptors (OR) on hMSCs and how these cells show functional characteristics impairment following acute morphine exposition [84]; nonetheless, only limited knowledge currently exists about the impact of morphine and other opioids on stem cells [51].

Therefore, we designed the following in vitro study with the aim of assessing the effect on functional characteristics of vMSCs resulting from morphine sulphate treatment. In particular cell viability, proliferative potential, oxidative stress and pro-senescence effect, were evaluated in association with the ultrastructural analysis by transmission electron microscopy (TEM). In addition, we also focused our attention on the in vitro vascular differentiation: both by investigating the expression of the endothelial marker CD31 by immunofluorescence in cells exposed to VEGF and morphine sulphate; and by a tubular in vitro assay on geltrex

matrix, in order to confirm the influence of morphine sulphate to impair vascular differentation of vMSCs.

At first, cultured cells were exposed to concentrations suggested by the literature and established on the basis of different ranges (sub-toxic concentration responsable for impairment, toxic concentration, lethal concentration) [85]. However, since we did not observe an evident correlation between these concentration and phenotype modification or inhibition of proliferartion, we decided to test higher morphine concentration.

Cellular alterations were detected at concentrations of 0.1, 0.4, 0.8 and 1 mM of morphine sulphate. Such concentrations are higher than toxic and even lethal blood concentration of morphine (0.05-4 mg/L). Certainly an in-vitro study does not consider many confounding factors occurring in a living subject including pharmacokinetics. However, this high concentration requested to observe functional and morphologic alterations may be also related to the intrinsic ability of hMSCs to maintain their functional integrity [86]. As a matter of fact, stem cells are relatively resistant cells, and they can largely retain their functional abilities, stem cell traits and surface markers, and characteristic multi-lineage differentiation potential, after exposure to external insults [86].

Although a minimal impairment was observed above 0.1 mM concentrations, a significant correlation was evident only at the concentration of 0.8 mM and 1mM, revealing a decrease of cell viability, a significant growth inhibition, a pro-oxidant and pro-senescence effect.

Firstly, we evaluated cell viability after morphine sulphate treatment by MTT assay, which is an accurate and reproducible means of measuring living cells via mitochondrial dehydrogenase activity, where the key component is a tetrazolium bromide (MTT) which is cleaved by the mitochondrial dehydrogenase of viable cells, yielding purple crystals which are insoluble in water and result in purple solution that is spectrophotometrically measured [87]. Although we did not find an evident

alteration with concentration of 0.1 mM and 0.4 mM morphine exposition, we observed a cell viability decrease and a cytostatic effect for the highest concentrations tested.

This cytostatic effect was also confirmed with the cell proliferation assay, a colorimetric immunoassay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis. As expected, results showed a significant growth inhibition at the concentration of 0.8 mM and 1mM of morphine sulphate, suggesting a negative influence of the drug on cell cycle.

In order to elucidate the mechanism behind these impairing effects, we also assumed that a morphine exposure could generate biosynthesis of ROS, since it is typical of a cell with a blocked cell cycle to increase ROS production. Moreover, in vivo studies carried out in murine models have already reported a significant oxidative stress increase following morphine administration [88]. Therefore, in order to demonstrate an effect of morphine sulphate on ROS generation in our cultured cells, a carboxy-H2DCFDA assay was carried out, confirming a pro-oxidant effect in vMSCs exposed to 0.8 mM and 1mM of morphine sulphate.

This result was also in agreement with Senescence-Associated β-galactosidase (SA β-Gal) assay, which detects the activity of lysosomal enzyme β-galactosidase, the most widely used biomarker for senescent and aging cells [89]. In case of a cellular insult, in fact, excessive production of ROS causes oxidative damage to cellular constituents and autophagy is then induced to maintain cellular homeostasis, which is an intracellular lysosomal degradation process by which dysfunctional components are removed by the cell [90]. In our system, morphine exposition induced ROS production, which was balanced by an upregulation of the autophagic process. Indeed, results showed a constant increase in β-galactosidase activity, suggesting a dose dependent relation between cellular senescensce and morphine concentration.

These latter results were confirmed with ultrastructural analysis at Transmission Electron Microscopy, where numerous long shaped mitochondria and several lysososmes, together with autophagic vescicles, were clearly appreciable in the cytoplasm of vMSCs treated with 0.8 and 1 mM of morphine sulphate. According to our expectations, these results suggest a reaction of the cell to counteract drug toxicity.

Finally, in order to demonstrate the impairment of the ability of vMSCs to differentiate towards a vascular phenotype related to morphine exposure, we exposed cells both to morphine sulphate and to VEGF, essential to activate the differentiation process. Therefore, we investigated by immunofluorescence the expression of CD31, a specific biomarker of vascular endothelial cells, showing a strong reduction of its expression. Secondly, to confirm the influence of morphine sulphate in the impairment of vascular differentiation, a tubular in vitro assay was carried out on geltrex matrix. On this matrice cultured cells usually grow developing a tube-like structure because it acts as a model of the basement membrane creating a physical support and ideal environment. Indeed, control cells showed a thin, elongated cellular morphology and were partly connected one to each other, producing a network resembling capillary tube formation. However, after the exposure to 0.8 and 1 mM of morphine sulphate, only a few numbers of cells showed an elongated shape, whereas the majority remained round shaped, suggesting cell degeneration till death. These further findings are consistent with previuous in vivo and in vitro studies [52], in which matrigel assay showed impaired angiogenesis in animals and reduced tubular formation in cultured endothelial cells treated with morphine.

Taken together, although an evident relation between morphine exposition and cell death or apoptosis was not observed, the results of the study showed a clear cell impairment, including viability decrease, cytostatic and senescence effect as well as a state of cell cycle arrest and loss of replicative capacity for the highest concentrations tested.

Limits of the study and future perspectives

Our vMSCs were healthy and viable cells, obtained from uninjured aorta fragments and, as typical of human mesenchymal stem cells, resistant and capable of retaining their functional abilities and differentiation potential. Under morphine sulphate exposure for 7 days they displayed functional decline and impaired vascular differentiation, thus resulting in regeneration impairment [91].

In this context, we demonstrated a functional decline and a reduced proliferation capacity to the structures responsible for regeneration when the endothelium is damaged.

The results obtained in this experimental study must be considered only introductive to the related forensic issue.

Regarding the duration of morphine exposure, a protocol assessing vMSCs alteration for longer intervals must be developed, in order to test potential tolerance mechanism related to chronic opioid abuse.

Moreover, since our study was performed on a limited number of samples, testing larger samples on other cellular types would be beneficial to better outline the mechanism behind these detrimental effects. Moreover it is desirable to test cellular cultures deriving from different subjects. Indeed, a wide inter-individual variability has been widely reported in other in-vitro studies on cellular function and damage.

In addition, further studies considering different substances, including other drugs of abuse, are required. As a matter of fact, testing contaminated street heroin or opiated combined with cocaine (in the street formulation known as “speed”) would be of higher interest, as these formulation are commonly sold and made up of a mixture of diacetylmorphine and a variety of other drugs of abuse and/or chemical additives that can cause deadly side effects [92].

Furthermore, an endothelial cell dysfunction is also demonstrated in cocaine abusers, and it is thought to related to the increased tendency towards thrombosis and vasospasm observed in those subjects [93].

Previous studies suggested that cocaine may suppress endothelial cell growth and cause focal loss of endothelial cell integrity [94-96], but the underlying mechanism is only partially known.

Conclusion

In Forensic Pathology, when dealing with opiate-related fatalities, common findings at autopsy include pulmonary congestion and edema, as well as acute/chronic alveolar hemorrhage. These latters effects typically accounts for the toxicity and lethal overdose from opioids. The cause is thought to be damage to the capillary endothelium, which on the one hand is believed to be ischemic, related to ipoperfusion of the pulmonary vascular bed; and on the other hand is thought to be related to the direct toxic effect of the substance on entothelial cells.

Morphine and other opioids produce their pharmacological effects by binding to opioid receptors located throughout the body, whose presence has been recently demonstrated also on MSCs.

MSCs belong to the adult stem cells that are able to repair body cells and maintain the normal turnover of regenerative processes, as they successfully differentiate towards a number of different cell lineages, including endothelial cells.

Therefore, they have been considered as a favourable source to obtain a large amount of endothelial cells to replace the damaged ones.

A damage or dysfunction of capillary endothelium is believed of being responsible for the pulmonary congestion, edema and acute/chronic alveolar haemorrhage frequently observed at autopsy in opiate-related fatalities.

In this context, although confirmation studies are required, the results of this study seem to be encouraging and demonstrate that this approach, based on morphological and immunofluorescence methodologies, may have a high potential for being a useful tool or as an addition to other methods in forensic pathology.

Indeed, if pulmonary congestion commonly seen in fatal overdose from opioids is believed to be related to a direct toxic effect of the substance on entothelial cells, we demonstrated cell impairment also of MSCs, which are responsible for repairing damaged cells.

Even if an evident relation between morphine exposition and cell death or apoptosis was not clearly observed, the results showed viability decrease, cytostatic and senescence effect as well as loss of replicative capacity of MSCs after morphine exposition.

Moreover, the application of these tehniques may lead to the identification of new markers and morphological parameters that could become important complementary investigations to be used in traditional Forenisc Pathology.

The results obtained in this experimental study must be considered only introductive to the related forensic issue. In the future, further studies considering different substances, including other drugs of abuse such as street heroin or opiated combined with cocaine, are required.

Furthermore, since endothelial cells dysfunction is also demonstrated in cocaine abusers, and it is thought to be related to the increased tendency towards thrombosis and vasospasm, testing cocaine as well would be of higher interest. These substances are believed to suppress endothelial cell growth and cause focal loss of endothelial cell integrity, however the underlying mechanism is only partially known.

In conclusion, the perspective for adding new tools to the classical forensic autopsy reveal the role of the research on biochemical, genetics analysis also devoted to infere the cause of death and the pathological mechanism in drug acute narcotism casework.

In the future it will be useful to include the collection of biological samples to the procedure and guidelines for forensic autopsy.

The forensic perpsectives have to consider the value of experimental data to assurge toward scientific evidence for application in caseworks and to provide a robust interpretation to the judicial questions in the forensic arena.

References

1. Martins SS et Al.Worldwide prevalence and trends in unintentional drug overdose: a systematic review of the literature. *Am J Public Health*, 2015

2. Norn S et Al. History of opium poppy and morphine. *Dan Medicinhist Arbog*, 2005

3. Schung SA et Al. Adverse effects of systemic opioid analgesics. *Drug Safety*, 1992

4. White JM. Mechanisms of fatal opioid overdose. *Addiction*, 1999

5. Okie S. A flood of opioids, a rising tide of deaths. *N Engl J Med*, 2010

6. Crobetti AD et Al. 75 years of opioid research: the ecxiting but vain quest for the Holy Grail. *Br J Pharmacol,* 2006

7. De Gregori S et Al. Morphine metabolism, transport and brain disposition. *Open Access*, 2011

8. Ciccarone D. Impact of South American heroin on the US heroin market 1993-2004. *Int J of Drug Policy*, 2009

9. Davis T at Al. Comparative morphine pharmacokinetics following sublingual, intramuscular, and oral administration in patients with cancer. *The Hopsice J*, 1993

10. Hoskin PJ et Al. Morphine: pharmacokinetics and clinical practice. *Br J Cancer*, 1990

11. Hickman et Al. The impact of buprenorphine and methadone on mortality: a primary care cohort study in the UK. *Research Report*, 2018

12. Cicero TJ et Al. Multiple determinants of specific modes of prescription opioid diversion. *J Drug Issues*, 2011

13. Inciardi A et Al. Prescription drugs purchased through the internet: who are the end users? *Drug and Alcohol Dependence*, 2010

14. Mayer S et Al. Characterizing fentanyl-related overdose and implications for overdose response: findings from a rapid ethnographic study in Vancouver, Canada. *Drug and Alcohol Dependence*, 2018

15. Pichini S et Al. European Drug Users at risk from illicit Fentanyls mix. *Front. Pharmacol*, 2017

16. Dasgupta N et Al. Crowdsourcing black market prices for prescription opioids. *J of Medical Internet Research,* 2013

17. Scendoni et Al. Correlation study between anatomopathological data and levels of blood morphine concentrations in heroin-related death. *Legal Medicine*, 2021

18. Warner-Smith M et Al. Heroin overdose: causes and consequences. *Addiction*, 2002

19. Chapman RC et Al. Prolonged morphine self-administration and addiction liability. Evaluation of two theories in a bone marrow transplant unit. *Cancer*, 1989

20. Frank D et Al. High risk and little knowledge: overdose experiences and knowledge among young adult nonmedical prescription opioid users. *Int J Drug Policy*, 2015

21. Mohammed et Al. Comparison of tolerance to morphine-induced respiratory and analgesic effects in mice. *Toxicology Letters,* 2013

22. Hill S et Al. Effect of tamoxifen and brain-penetrant protein kinase C and c-Jun N-terminal kinase inhibitors on tolerance to opioid-induced respiratory depression in mice. *J of Pharmacology and Experimental Therapeutics*, 2017

23. Kishioka S et Al. Diltiazem enhances the analgesic but not the respiratory depressant effects of morphine in rhesus monkeys. *Eu J of Pharmacology*, 2000

24. Sporer KA et Al. Heroin-related noncardiogenic Pulmonary Edema: a case series. *Chest,* 2001

25. Dettmeyer RB. Histopathology and Drug Abuse. *Forensic Histopathology,* 2018

26. Matsubara O et Al. Alveolar basement membrane breaks down in diffuse alveolar damage: an immunohistochemical study. *Pathology International,* 1995

27. Karch SB. Karch's pathology of drug abuse, 4th edn, CRC Press, Boca Rotan, London, New York, 2009

28. Muller KM. Fixerpneumopathie. *Pneumologie*, 2004

29. Hopkins GB. Pulmonary angiothrombotic granulomas in drug offenders. *JAMA*, 1972

30. Pare JP et Al. Long-term follow-up of drug abusers with intravenous talcosis. *Am Rev Respir Dis,* 1984

31. Cerretani D et Al. Cardiac oxidative stress determination and myocardial morphology after a single ecstasy (MDMA) administration in a rat model. *Int J Leg Med,* 2008

32. Toth Ar et Al. Myocardium and striated muscle damage caused by licit or illicit drugs. *Leg Med*, 2009

33. Perskvist N et Al. Pathogenetic role of cardiac mast cell activation/degranulation, TNF-alpha, and cell death in acute drug related fatalities. *Vasc Health Risk Manag,* 2007

34. Perazella MA. Drug-induced nephropathy: an update. *Expert Opin Drug*, 2005

35. Friedman EA et Al. Disappearance of uremia due to heroin-associated nephropathy. *Am J Kidney Dis*, 1995

36. Hill GS. Drug-associated glomerulopathies. *Toxicol Pathol,* 1986

37. Richter RW et Al. Acute myoglobinuria associated with heroin addiction. *JAMA*, 1971

38. Welte TB et Al. Prevalence of rhabdomyolysis in drug deaths. *Forensic Sci Int*, 2004

39. Buttner A et Al. Widespread axonal damage in the brain of drug abusers as evidenced by accumulation of beta-amyloid precursor protein (beta-APP): an immunohistochemical investigation. *Addiction*, 2006

40. Hirschi KK et Al. Pericytes in the microvasculature. *Cardiovasc Res*, 1996

41. Bergers G et Al. The role of pericytes in blood vessels formation and maintenance. *Neuro-Oncology*, 2005

42. Caplan AI. All MSCs are pericytes? *Cell Stem Cell*, 2008

43. Birbair A et Al. Type-2 pericytes participate in normal and tumoral angiogenesis. *Am J Physiol Cell,* 2014

44. Chen CW et Al. Perivascular multi-lineage progenitor cells in human organs: regenerative units, cytokine sources or both? *Cytokine Growth Factor Rev*, 2009

45. McKay R. Stem cells - hype and hope. Nature, 2000

46. Thomson JA. Et Al. Embryonic stem cell lines derived from human blastocysts. *Science*, 1998

47. Barlass U et Al. Morhpine worsens the severity and prevents pacreatic regeneration in mouse models of acute pancreatitis. *Gut*, 2018

48. Beyer R et Al. Alteration of neuronal precursor cells in stages of human adult neurogenesis in heroin addicts. *Drug Alcohol Dependence*, 2015

49. Abdyazdani N et Al. The role of morphine on rat neural stem cells viability, neuroangiogenesis and neuro-steroidgenesis properties. *Neuroscience Letters*, 2017

50. Feizy N et Al. Morphine inhibited the rat neural stem cell proliferation rate by increasing neuro steroid genesis. *Neurochemical Research*, 2016

51. Willner D et Al. Short term morphine exposure in vitro alters proliferation and differentiation of neural progenitor cells and promotes apoptosis via mu receptors. *PLoS One*, 2014

52. Lam C et Al. Prolonged use of high-dose morphine impairs angiogenesis and mobilization of endothelial progenitor cells in mice. *Anesthesia and Analgesia*, 2008

53. Becker AJ et Al. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature*, 1963

54. Cudkowicz G et Al. An approach to the characterization of stem cells in mouse bone marrow. Ann. N. Y. Acad Sci, 1964

55. Zuk P et Al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell, 2002

56. Kern S et Al. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells*, 2006

57. Zippel N et Al. Purinergic receptors influence the differentiation of human mesenchymal stem cells. *Stem Cells Dev*, 2012

58. Gronthos S et Al. Surface protein characterization of human adipose tissue derived stromal cells. *J Cell Physiol*, 2001

59. Planat-Benard V et Al. Plasticity of human adipose lineage cells towards endothelial cells: physiological and therapeutic perspectives. *Circulation*, 2004

60. Rodríguez LV et Al. Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells. *Proc Natl Acad Sci USA*, 2006

61. Wang C et Al. A small diameter elastic blood vessel wall prepared under pulsatile conditions from polyglycolic acid mesh and smooth muscle cells differentiated from adipose derived stem cells. *Biomaterials*, 2010

62. Scuteri A et Al. Mesenchymal stem cells neuronal differentiation ability: a real perspective for nervous system repair? *Curr Stem Cell Res Ther*, 2011

63. Wosnitza M et Al. Plasticity of human adipose stem cells to perform adipogenic and endothelial differentiation. *Differentiation*, 2007

64. Zhang Y et Al. The role of purinergic receptors in stem cells in their derived consecutive tissues. In: di Nardo P (ed.), *Adult Stem Cell Standardization*. Roma: River Publishers, 73-98, 2011

65. Dominici M et Al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, 2006

66. Daley GQ. The promise and perils of stem cell therapeutics. *Cell Stem Cell*, 2012

67. Becker AJ et Al. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature*, 1963

68. Cudkowicz G et Al. An approach to the characterization of stem cells in mouse bone marrow. *Ann N Y Acad Sci*, 1964

69. Burke JF et Al. Immunosuppression and temporary skin transplantation in the treatment of massive third degree burns. *Ann Surg*, 1975

70. Cummings BJ et Al. Human neural stem cells differentiate and promote locomotor recovery in spinal cord injured mice. *Proc Natl Acad Sci*, 2005

71. Beyth S et Al. Stem cells in bone diseases: current clinical practice. *Br Med Bull*, 2011

72. Daniels JT et Al. Corneal epithelial stem cells in health and disease. *Stem Cell Rev*, 2006

73. El Badri N et Al. Mesenchymal stem cell therapy in diabetes mellitus: progress and challenges. *J Nucleic Acids*, 2013

74. Zhang Z et Al. Stem cell therapies for liver failure and cirrhosis. *J Hepatol*, 2013

75. Androutsellis Theotokis A et Al. Targeting neural precursors in the adult brain rescues injured dopamine neurons. *Proc Natl Acad Sci*, 2009

76. Bai K et Al. Endothelium oriented differentiation of bone marrow mesenchymal stem cells under chemical and mechanical stimulations. *J Biomech*, 2010

77. Huang NF et Al. Mesenchymal stem cells for vascular regeneration. *Regen Med*, 2008

78. Oswald J et Al. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. *Stem Cells*, 2004

79. Perets A et Al. Enhancing the vascularization of three dimensional porous alginate scaffolds by incorporating controlled release basic fibroblast growth factor microspheres. *J Biomed Mater Res*, 2003

80. Mattioli-Belmonte M et Al. Stem cell origin differently affects bone tissue engineering strategies. *Front Physiol*, 2015

81. Teti G et Al. The Hypoxia-Mimetic Agent Cobalt Chloride Differently Affects Human Mesenchymal Stem Cells in Their Chondrogenic Potential. *Stem Cells Int*, 2018

82. Murphy MB et Al. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp Mol Med*, 2013

83. Trohatou O et Al. Mesenchymal Stem/Stromal Cells in Regenerative Medicine: Past, Present, and Future. *Cell Reprogram*, 2017

84. Holan V et Al. The Impact of Morphine on the Characteristics and Function Properties of Human Mesenchymal Stem Cells. *Stem Cell Rev,* 2018

85. Schulz M et Al. Therapeutic and toxic blood concentrations of nearly 1000 drugs and other xenobiotics. *Crit Care,* 2012

86. Lopez-Perez R et Al. Mesenchymal stem cells preserve their stem cell traits after exposure to antimetabolite chemotherapy. *Stem Cell Res*, 2019

87. Denizot F et Al. *J. Immunol. Methods*, 1986

88. Lam, CF et Al. High-dose morphine impairs vascular endothelial function by increased production of superoxide anions. *Anesthesiology*, 2007

89. Lee BY et Al. Senescence-associated beta-galactosidase is lysosomal beta-galactosidase, *Aging Cell*, 2006

90. Yun HR et Al. Roles of Autophagy in Oxidative Stress. *Int J Mol Sci*, 2020

91. Sepúlveda JC et Al. Cell senescence abrogates the therapeutic potential of human mesenchymal stem cells in the lethal endotoxemia model. *Stem Cells*, 2014

92. Mars SG et Al. The textures of heroin: User perspectives on "black tar" and powder heroin in two U.S. cities. *J Psychoactive Drugs*, 2016

93. Eichhorn EJ et Al. Cocaine-induced alterations in prostaglandin production in rabbit aorta. *J Am Coll Cardiol,* 1992

94. Welder AA et Al. A primary culture system of rat heart-derived endothelial cells for evaluating cocaine-induced vascular injury. *Toxicol Methods*, 1993

95. Gilloteaux J et Al. Transplacental cardiotoxicity of cocaine: atrial damage following treatment in early pregnancy. *Scanning Microsc,* 1991

96. Jones LF et Al. Chronic cocaine treatment enhances the responsiveness of the left anterior descending coronary artery and the femoral artery to vasoactive substances. *J Pharmacol Exp Ther*, 1990

Abstract

In the field of illicit drug use, heroin-related mortality is a complex phenomenon that still shows increases across time. The most common pulmonary findings in opiate-related fatalities are pulmonary congestion and edema, as well as acute/chronic alveolar haemorrhage. The cause is thought to be damage to the capillary endothelium: ischemic, related to ipoperfusion of the pulmonary vascular bed, and/or related to the direct toxic effect of the substance on entothelial cells.

In this context, also tissue regeneration and repair mechanisms, including vascular mesenchimal stromal cells (vMSCs) function, are supposed to be impaired. As a matter of fact, tissue regeneration and repair are essential after endothelial cell injury and are related to the activity of human vascular mesenchimal stromal/stem cells (vMSCs). Therefore, an opiate-related impairment of vMSCs function might be a part of the pathophysiology of endothelial cell injury.

The aim of this study was to assess the effect of in-vitro morphine sulfate exposure on the function and morphology of human vMSCs.

Even if an evident relation between morphine exposition and cell death or apoptosis was not clearly observed, the results showed a definite cell impairment, including viability decrease, cytostatic and senescence effect as well as a state of cell cycle arrest and loss of replicative capacity of MSCs after morphine exposition.

The results obtained in this experimental study must be considered only introductive to the related forensic issue; however, although a confirmation on a wider number of samples is required to draw any definitive conclusion, they seem to suggest a negative impact on vMSCs regenerative potential and correct outcome in case of endothelial cells' injury in opiate-related fatalities.

The forensic perspectives have to consider the value of experimental data to assurge toward scientific evidence for application in caseworks and to provide a robust interpretation to the judicial questions in the forensic arena.

RELAZIONE ANNUALE

Dottorato in Scienze Mediche Generali e dei Servizi - XXXIV ciclo - A.A. 2020/2021 –

NOME DOTTORANDA	Maria Carla Mazzotti
TUTOR	Prof.ssa Susi Pelotti
CICLO	XXXIV ciclo - A.A. 2020/2021
SSD	MED/43 Medicina Legale
DIPARTIMENTO	Dipartimento di Scienze Mediche e Chirurgiche (DIMEC)

Titolo del progetto di Dottorato

"The effect of morphine on endothelial differentiation of vascular Mesenchimal Stromal Cells (vMSCs)" → Analisi dell'influenza della tossicità della morfina e sue ripercussioni sull'alterazione della differenziazione endoteliale su cellule vascolari mesenchimali (vMSCs)

Attività svolte nel terzo anno

Quadro di sintesi

Nel periodo intercorso tra novembre 2020 e ottobre 2021, è stata costantemente svolta l'attività di revisione della letteratura e di stesura di articoli scientifici.

Più dettagliatamente, l'attività di ricerca si è concentrata in particolar modo sulla revisione della letteratura inerente il progetto di ricerca e nello specifico sulle cellule vascolari mesenchimali, che hanno un ruolo cruciale nel processo di riparazione dei vasi sanguigni. Negli assuntori cronici di sostanze stupefacenti come la morfina, il danneggiamento dei capillari alveolari con la conseguente compromissione della permeabilità capillare polmonare contribuisce all'insorgenza/sviluppo di edema polmonare e da queste premesse lo scopo del progetto di ricerca è stato proprio quello di analizzare le alterazioni morfologiche e ultrastrutturali indotte da morfina a differente concentrazione su colture di cellule vascolari mesenchimali al fine di valutarne la citotossicità.

Un altro tema affrontato come proseguo della ricerca pregressa ha riguardato lo studio degli artefatti prodotti dagli insetti quali mosche nella scena del crimine, i cosiddetti *fly artifacts.* Il lavoro scientifico fa seguito ad una pubblicazione del primo anno di dottorato, centrata sulla microscopia elettronica a scansione come metodo di analisi dei *fly artifacts* prodotti sperimentalmente dalla mosca *Callipohra Vomitoria* su diverse

tipologie di superfici (comunemente riscontrabili sulle scene del crimine), che sono state poi confrontate con le macchie di sangue di controllo.
Inoltre, tra le attività dell'ultimo anno si segnala anche la continua attività di revisione della letteratura sul tema dell'abuso sull'anziano, sul quale è stata riservata una parte significativa della ricerca di tutti i tre anni di dottorato. In particolare, in questo ultimo anno è stato completato e pubblicato sulla rivista internazionale *American Journal of Forensic Medicine and Pathology* un articolo relativo a cinque casi autoptici eseguiti per conto dell'Autorità Giudiziaria, selezionati in quanto caratterizzati da uno stesso denominatore comune legato ad una diagnosi di *neglect* emersa soltanto post-mortem. In tutti i casi riportati la diagnosi è stata possibile grazie ad un approccio combinato dell'analisi della scena con il sopralluogo giudiziario, degli elementi circostanziali e delle risultanze autoptiche e la raccolta di tutte le informazioni ha consentito di individuare la presenza di fattori di rischio non preventivamente valutati o segnalati da nessuno. Tale lavoro scientifico ha permesso di evidenziare, sia per l'isolamento delle vittime anziane sia per l'assenza di un adeguato supporto socio-sanitario, la necessità di ricerche ulteriori anche nel campo della patologia forense con metodi standardizzati per contribuire a strategie di prevenzione ed evitare che le vittime di *neglect* degli anziani rimangano nascoste anche dopo la morte.

Di seguito sono elencati i convegni e *webinar* scientifici a cui si è partecipato:

- Prochild Winter School dell'Università di Bologna. Formazione specifica multi professionale in tema di prevenzione, diagnosi e cura delle diverse forme di maltrattamento infantile, violenza domestica e di genere, della durata di 10 giorni per 60 ore totali svoltesi tra gennaio e febbraio 2021.
- Convegno GIPF/SIMLA (Gruppo Italiano Patologi Forensi/Società Italiana Medicina Legale e delle Assicurazioni): "Il sopralluogo e l'esame necroscopico al tempo della infezione da SARS-CoV-2", 21 gennaio 2021.
- Convegno GIPF/SIMLA: "Sicurezza, procedure operative e protocolli diagnostici al tempo della infezione da SARS-CoV-2", 11 febbraio 2021.
- Convegno GIPF/SIMLA: "La virtopsy del cadavere positivo al SARS-CoV-2 ed esperienze casistiche", 25 febbraio 2021.
- Seminario Università di Bologna: "RNA, Biological Functions and Therapeutic Potential", 26 gennaio 2021.

- Seminario Università di Bologna: "The legacy of Caster Semenya: Unfair Advantages, Equality of Opportunity and The Construction of Categories in Sport Lecture" by Silvia Camporesi, King's College, 23 febbraio 2021.
- Convegno Congressare-Promise Group: "A confronto sulla nuova responsabilità sanitaria e sulla tutela del professionista della salute", 9 aprile 2021.
- Convegno FAMLI (Federazione delle Associazioni dei Medici Legali Italiani): "Valutazione medico-legale del Covid-19", 9 aprile 2021.
- Convegno Congressare-Promise Group: "Banca dati DNA: il ruolo dei laboratori nella sua implementazione". Pro Presence Live, 22 aprile, 6 maggio e 20 maggio 2021.
- Seminario AFMEL (Associazione Felsinea di Medicina Legale): "La valutazione medico-legale del danno nella persona anziana dopo la Consensus Conference", 14 maggio 2021.
- 44° Congresso Nazionale SIMLA: "Nell'era della complessità negata: autocoscienza ed impatto sociale della medicina legale", 18-19 giugno 2021.
- Convegno SIMLA: "Follow the money", Corinaldo 30 settembre-2 ottobre 2021.

Abstract esposti come poster a convegni (2021)

- Nuova mutazione del gene RYR2 in un caso di morte cardiaca improvvisa a seguito di aggressione: implicazioni medico-legali. Pelletti G, Gavelli M, **Mazzotti MC**, Fais P, Pelotti S. 44° Congresso Nazionale SIMLA, 18-19 giugno 2021.

Pubblicazioni

Articoli su riviste *peer-reviewed*

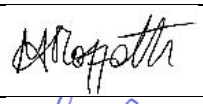
- Palazzo C, Pascali JP, Pelletti G, **Mazzotti MC**, Fersini F, Pelotti S, Fais P. Integrated multidisciplinary approach in a case of occupation related planned complex suicide-peticide. Leg Med (Tokyo), Feb. 2021

- **Mazzotti MC**, Fais P, Amadasi A, Pelletti G, Giovannini E, Giorgetti A, Pelotti S. When the hidden issue of elder abuse leads to death: do not neglect elder neglect. Am J Forensic Med Pathol, Jul. 2021

- Pelletti G, Martini D, Ingrà L, **Mazzotti MC**, Giorgetti A, Falconi M, Fais P. Morphological characterization using scanning electron microscopy of fly artifacts deposited by Calliphora Vomitoria (Diptera: Calliphoridae) on household materials. Int J Leg Med, Jul. 2021

- Carano F, Teti G, Ruggeri A, Chiarini F, Giorgetti A, **Mazzotti MC**, Fais P, Falconi M. Assessment of the structural and functional characteristics of human mesenchymal stem cells associated with a prolonged exposure of morphine. Sci Rep, Sep. 2021

Si segnala, in ultimo, che nel corso di questo anno è tuttora in prosecuzione il lavoro sperimentale oggetto dell'elaborato finale per la valutazione morfologica delle alterazioni indotte da sostanze stupefacenti e psicotrope sulle cellule endoteliali ottenute da colture primarie di parete vascolare, le cui prospettive future implicano l'utilizzo di sostanze alternative alla morfina, quali l'"eroina da strada" (i.e. il cosiddetto "*speed"*) e la cocaina, al fine di valutare la citotossicità di dette sostanze a concentrazioni analoghe a quelle ematiche in corso di intossicazione acuta e cronica.

Firma della Dottoranda Dott.ssa Maria Carla Mazzotti	
Firma del Tutor Prof.ssa Susi Pelotti