Potential therapeutic alternatives for smokers with osteoarthritis – an *in vitro* study for preclinical application

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To my parents
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Ascorbic acid</td>
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<tr>
<td>AC</td>
<td>Articular cartilage</td>
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<tr>
<td>Ace</td>
<td>Acetaminophen</td>
</tr>
<tr>
<td>ACI</td>
<td>Autologous Chondrocyte Implantation</td>
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<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CS</td>
<td>Cigarette smoke</td>
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<tr>
<td>CSE</td>
<td>Cigarette smoke extract</td>
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<tr>
<td>CSs</td>
<td>Corticosteroids</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>DCFH-DA</td>
<td>Dichlorfluorescein-diacetate</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DFO</td>
<td>Distal femoral osteotomy</td>
</tr>
<tr>
<td>Dic</td>
<td>Diclofenac</td>
</tr>
<tr>
<td>DMMB</td>
<td>Dimethyl-Methylene Blue</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's Phosphate Buffered</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetatic Acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GBD</td>
<td>Global Burden of Disease</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen Chloride</td>
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<td>HTO</td>
<td>High tibial osteotomy</td>
</tr>
<tr>
<td>IA</td>
<td>Intra-articular</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KL</td>
<td>Kellgren and Lawrence</td>
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<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MSS</td>
<td>Mainstream smoke</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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</table>
OARIS  Osteoarthritis Research International
OD    Optical density
P/S   Penicillin/Streptomycin
PBS   Phosphate-buffered saline
PY    Pack-year
RCTs  Randomized controlled trials
ROM   Range of motion
ROS   Reactive oxygen species
SRB   Sulforhodamine B
SSS   Sidestream smoke
TGF-β Transforming growth factor β
TKA   Total knee arthroplasty
TNF   Tumor necrosis factor
UKA   Unicompartmental knee arthroplasty
WHO   World Health Organization
YLD   Years lived with disability
Chapter 1

1. Introduction

1.1. Cigarette smoke and cigarette smoke-related diseases

1.1.1. Cigarette smoke composition

Cigarette smoke (CS), is the dominant cause of preventable deaths that endangers the longevity and life quality of humans (Chen et al., 2020). It is well known that more than 5000 detrimental chemical compounds and at least 55 carcinogens are involved in the CS (National Center for Chronic Disease et al., 2012, Chen et al., 2020). CS is an aerosol of liquid droplets generated by the combustion of tobacco during the smoking, containing about $10^{10}$ particles/mL (Valavanidis A et al., 2009). In general, CS consists of mainstream smoke (MSS) and sidestream smoke (SSS) (Fig. 1.1) (Thielen et al., 2008). The former is directly inhaled by the smoker, and the latter is absorbed from the surroundings of the burning cigarette. Two phases are involved in the MSS, namely a tar (particle) phase and a remaining gas phase. The gas phase (0.4 – 0.5 g/cigarette) is mainly comprised of air constituents, oxygen and nitrogen. The extent of particles in fresh MSS ranges between 0.1 and 1 μm in diameter, and the weight is around 4.5% of the whole smoke (Thielen et al., 2008). A great number of mutagenic and carcinogenic agents, such as PAHs, N-nitrosamines, and phytosterols, are mostly found in the particulate phase only (Valavanidis A et al., 2009). The compounds of SSS both in the gas and particle phase are similar to those of MSS. The gas phase can infiltrate and transit the lung alveolus and damage tissues remote from the lung, suggesting it is more vital for human health (Yamaguchi et al., 2007).
Figure 1.1 The sidestream smoke and mainstream smoke of a burning cigarette. (Reproduced with permission from Thielen et al., 2008).

1.1.2. Cigarette smoke-related diseases

It is well established that CS could affect negatively most human body systems (Fig 1.2), such as respiratory, cardiovascular and musculoskeletal diseases. The most common CS-related diseases are cardiovascular disease (CVD) (McEvoy et al., 2015), chronic obstructive pulmonary disease (COPD) (Laniado-Laborin, 2009), and diverse types of cancer, in particular lung cancer (Warren and Cummings, 2013). Cigarette smoking can damage the airway and pass through the lung alveolus, resulting in lung diseases and injury to other tissues. Smokers have a higher possibility (12 - 13 times) of dying from COPD than non-smokers, and CS is a major risk that triggers an attack in patients with asthma (National Center for Chronic Disease et al., 2014). Moreover, smokers have as much as a 30-fold increased risk of developing lung cancer or dying from lung cancer compared with non-smokers (National Center for Chronic Disease et al., 2014). Besides, exposure to cigarette smoke predisposes the individual to several diseases that affect heart and blood vessels (Barua et al., 2015). CS has been reported to cause approximately 140,000 premature deaths annually from CVD (National Center for Chronic Disease et al., 2014). Furthermore, exposure to the environment of smoked tobaccos among non-smokers also increases the health risk of respiratory (Oberg et al., 2011) and cardiovascular (Raghuveer et al., 2016) problems, as well as lung cancer (Manning et al., 2017).
However, owing to the loose regulation of tobacco products and difficulty in quitting smoking, the global tobacco control efforts are disappointing.

Figure 1.2 The effects of smoking on the human body. Smoking can damage every part of the body.

1.1.3. Cigarette smoke and musculoskeletal disorders

Although the detrimental effects of smoking for human health have been well accepted, less attention has been paid by researchers to the relevance of CS to musculoskeletal disease. The musculoskeletal system, which provides support and motion to the body, is made up of skeletal bones, connective tissues (ligaments, tendons, and cartilage), as well as skeletal muscles (Al-Bashaireh et al., 2018). Several preclinical and clinical researches have examined the association between CS and the musculoskeletal system, its impact on the prognosis of several orthopaedic disorders, surgical complications and prolonged hospital stays (Ehnert et al., 2019, Greenberg et al., 2017, Sloan et al., 2010, Chen et al., 2020). Our lab has previously assessed and compared the fracture healing capacities of smokers and non-smokers among 1585 patients. The outcome of the study showed that smoking significantly increased surgical complications and delayed healing, which resulted in prolonged hospital stays in smokers when compared to non-smokers (Fig 1.3). Recent evidence has demonstrated that smoking could cause an imbalance between bone
mineralization and absorption, resulting in lower bone mass and bone mineral density (BMD) (Ward and Klesges, 2001) and predisposing bone to osteoporosis and fracture (Law and Hackshaw, 1997).

Figure 1.3 Effect of smoking on the hospital stay and surgical complication during fracture healing. Data from 1585 patients of the BG Unfallklinik Tübingen shows smokers encounter significantly more complications and longer hospital stays than non-smokers (*p < 0.05) (Reproduced with permission from (Ehnert et al., 2019).

1.1.4. Cigarette smoke and osteoarthritis

The relationship between CS and osteoarthritis (OA) has been assessed by several epidemiological surveys, but the results are controversial. CS has been thought to have a defensive effect against OA, through reducing body weight (Chiolero et al., 2008) and stimulating anabolic action of chondrocyte by nicotine (Ying et al., 2012). In one follow-up study, Felson et al. (Felson et al., 1997) found that smokers had a lower incidence (28%) of OA than that of non-smokers (37.5%), after adjustment for multiple risk factors (age, sex, and weight). Similarly, in a retrospective study, Cerhan et al. found consistent results with those of the aforementioned study (Cerhan et al., 1996). However, the protective effect of smoking is minimal, and the methods of evaluation and selection bias may be inaccurate, leading to the results of these studies not being conclusive.

Recently, more and more researches have shown a positive relationship between CS and cartilage loss with the assistance of Magnetic Resonance Imaging (MRI) (Chen et al., 2020). Davies-Tuck and his colleagues indicated that smokers were related to increased medial knee cartilage loss, and a positive relationship between pack-year
(PY) smoked and the loss of cartilage bulk was observed (Davies-Tuck et al., 2009). Furthermore, in a cross-sectional analysis, Ding et al. suggested that smoking leads to an increase in knee cartilage loss and defects in subjects with a family history of knee OA (Ding et al., 2007). Nevertheless, these studies are only based on radiographic images (MRI, X-ray) or post-operative outcomes (Ding et al., 2007, Dube et al., 2016), and no direct research yet has evaluated the effects of CS on human cartilage or primary human chondrocytes. Thus, these results may be not conclusive, since the structural changes of cartilages within the smoker’s joints are still not known (Chen et al., 2020).

1.2. Osteoarthritis and cartilage

1.2.1. Osteoarthritis epidemiology

OA is a type of chronic disabling disease that affects millions of people all over the world (El-Tawil et al., 2016) (Fig. 1.4). It is a common form of arthritis and affects both large and small joints in the body, including hips, knees, hands, and feet (Allen and Golightly, 2015). Over the past decades, along with the increasing rate of joint injuries and obesity, the prevalence and burden of OA have consistently risen (March et al., 2014). In the recent Global Burden of Disease (GBD) 2015 Study, OA and diabetes accounted for the largest increases in burden disability of years lived with disability (YLDs) worldwide, when comparing the data of 1990–2005 with 2005–2015 (Collaborators, 2016). OA accounted for 3.9% of YLDs in 2015 in the global population, and it is predicted to climb to the fourth leading cause of YLDs by 2020, while it ranked sixth in 2003 (Silverwood et al., 2015). Overall, the incidence of OA is higher in women compared with men, as well as increasing with age. Approximately 15% of females and 9% of males aged over 60 have symptomatic knee OA (Hunter and Bierma-Zeinstra, 2019).

In the clinic, the most susceptible site of OA is the knee, followed by the hand and the hip (Prieto-Alhambra et al., 2014). The knee and hip, two pivot joints in humans, are more prone to cause disability and comorbidity compared with other joints. Throughout the world, the prevalence of OA has been estimated to be 0.85% and 3.8%
for hip OA and knee OA, respectively (March et al., 2014). Approximately 85% of the burden of OA is occupied by knee OA, causing billions in medical costs every year (Collaborators, 2016).

Figure 1.4 OA epidemiology and demographics. Age-standardized disability-adjusted life year rates for osteoarthritis by country (per 100,000 inhabitants). (Reproduced with permission from (El-Tawil et al., 2016).

1.2.2. Cartilage structure and morphology

Human articular cartilage (AC) is a smooth, viscoelastic tissue, which coats and protects the ends of long bones. This specially designed structure possesses the function of decreasing friction and distributing loading, owing to its high content of components, including the incompressible water as well as the cartilaginous matrix (proteoglycans, collagen fibrils, etc.) (Archer, 2003). In healthy cartilage, water is estimated to account for 65% to 80% of the mass in the deep and surface layer, respectively (Sophia Fox et al., 2009, Akkiraju and Nohe, 2015). Water content increases with OA, leading to increased permeability, decreased strength as well as a decreased Young’s modulus of elasticity (Otero et al., 2012). Approximately 10% - 20% of cartilage gross mass is made of collagen, and 90% - 95% of the collagen is occupied by collagen II (Akkiraju and Nohe, 2015). Some small amounts of collagens (type V, VI, IX, X, and XI) and additional macromolecules are also present. The function of collagen is to provide a cartilaginous framework and tensile strength (Akkiraju and Nohe, 2015). Proteoglycans comprise 10 to 15% of cartilage and possess the functions of water condense and compressive strength support (Sophia
Fox et al., 2009). Large proteoglycan, also refers to aggregan, attached through a link protein and is mostly responsible for hydrophilic behavior (Sophia Fox et al., 2009). In general, AC is structured in four laminar zones: superficial, transitional, deep and calcified zones (Davies and Kuiper, 2019) (Fig 1.5). Chondrocytes are derived from mesenchymal stem cells (MSCs) and exclusively found in the AC (Akkiraju and Nohe, 2015). They possess the properties of proliferating and synthesizing extracellular matrix (ECM) to support the functionality and stability of AC (Archer, 2003, Chen et al., 2020). Owing to the high volume of matrix, only 1%–5% of the total cartilage is occupied by chondrocytes (Akkiraju and Nohe, 2015). Chondrocytes are entrapped in lacuna and separated by ECM, leading to their not being able to migrate to the lesion sites (Archer, 2003). Since cartilage is a tissue without a blood supply, chondrocytes are nourished through diffusion from synovium and subchondral bone (Hugle and Geurts, 2017). Moreover, these cells live in an environment with low oxygen content, resulting in their low metabolic turnover. Chondrocytes are metabolically active cells and respond to outside stimuli such as cytokines and mechanical changes. These factors are of great necessity to the degradation and regeneration of ECM (Scanzello and Goldring, 2012).
1.2.3. Pathophysiology of OA

OA is a whole-joint disease, involving cartilage breakdown, osteophytes formation, synovial inflammation, degeneration of ligaments and menisci, as well as weakness of periarticular muscles (Hunter and Bierma-Zeinstra, 2019, Chen et al., 2020). The degeneration of cartilage is highly correlated with systemic risk factors (gender, aging, genetic heritability, nutrition, and smoking) and local risk factors (obesity, joint mechanics, occupational stress, physical activity, and injury) (Silverwood et al., 2015). In the traditional concept, OA was thought to be a condition of cartilage deterioration owing to increased mechanical loading. It was speculated that persistent mechanical loading disrupted ECM, which overruns the regeneration of cartilage by itself (Chen et al., 2017). Therefore, OA was defined simply as the damaged cartilage due to wear
and tear. Nevertheless, the onset and mechanism of OA are still unclear according to this explanation. Recently, this concept is being challenged, as the pathogenesis of OA is not so simple. The current view is that OA is a condition with a multifaceted etiology, including biomechanical factors (Guilak, 2011), cytokines (Fernandes et al., 2002), and proteases (Troeborg and Nagase, 2012), and this change is more nuanced. During the process of OA, the earliest change that occurs in cartilage is the shift of chondrocytes from a normally quiescent condition to activated cells as a compensatory mechanism, characterized by cell proliferation, cluster formation and increased synthesis of matrix molecules (collagen, aggrecan, and hyaluronan) (Goldring and Goldring, 2007). Nevertheless, in the end, the death of chondrocytes along with the alteration in the ECM prevail and change the development of OA. The initial degenerative switch in the AC brings about cartilage weakening and increased production of fragmented particles, which stimulate the release of pro-inflammatory cytokines, like TNF α, IL-1, and IL-6 (Scanzello and Goldring, 2012). Once secreted, these cytokines are able to affix to the receptors of chondrocytes or synoviocytes, contributing to the release of metalloproteinases such as matrix metalloproteinases (MMPs) and aggrecanases, which in turn accelerates cartilage degradation and fragmentation (Hwang and Kim, 2015). Increased cartilage fragments further induce the release of pro-inflammatory cytokines and proteases, forming a vicious cycle. Changes in the subchondral bone include the increase in bone turnover, development of bone marrow lesions, and vascular invasion from the subchondral bone through the tidemark into the cartilage (Hunter and Bierma-Zeinstra, 2019). In later stages, significant aseptic bone necrosis takes place and synovial fluid diffuses into the bone marrow, leading to bone cysts (Man and Mologhianu, 2014).
1.2.4. Symptoms of OA

Symptoms of OA often appear slowly and worsen over time. Pain is the hallmark of OA and a major driver of seeking clinical advice (Hunter et al., 2008). Swelling, morning stiffness, limited range of motion (ROM), muscle weakness, joint instability and crepitus are also observed frequently in OA patients (Hunter and Bierma-Zeinstra, 2019). Radiographic evidence of the disease includes synovial thickening, joint space narrowing, and the formation of osteophytes (Braun and Gold, 2012). Sadly, neither complete cure nor satisfactory diagnostics are currently available to reverse the condition.

1.2. Current treatments of OA

Nowadays, treatment designed for OA is variable (Fig 1.7). In general, conservative treatment strategies of OA include non-pharmacological as well as pharmacological
treatments. They are employed for patients with early or less advanced stages (Kellgren and Lawrence, K-L Grade 1–3) of OA for relieving pain, increasing joint mobility, and improving life quality (Ringdahl E, 2011). In the end stage (K-L Grade 4) of OA, surgery is the definitive option (Ronn et al., 2011).

![Figure 1.7 Ladder of treatment for knee OA.](image)

Treatments strategy varies from the mild (bottom), moderate to the advanced (top).

1.3.1. Non-pharmacological treatment
To date, all guidelines recommend that non-pharmacological treatments should play a core role in managing OA patients (Zhang et al., 2008, Silverwood et al., 2015, Nelson et al., 2014). Non-pharmacological methods such as patient education, regular exercise, weight control, physiotherapy, and assistive devices are recommended and treated as first-line treatment (Zhang et al., 2008). Most guidelines strongly recommend education and self-management as part of the administration of OA, including information about the pathophysiology of the disease, joint protection strategies, different treatment approaches and also about surgery when it is necessary (Zhang et al., 2008, Block, 2014). Performing moderate exercise is helpful in
improving joint flexibility, reducing pain and strengthening muscle in the leg and knee. Patients should be advised to participate in endurance or strengthening exercises and low-impact aerobic exercise (running, swimming, etc.), and lose weight if they are overweight or obese (Jordan et al., 2003). For obese persons, losing weight and augmenting physical health may help to alleviate the stress on the knees, and reduce symptoms. Several trials have clearly demonstrated a positive association between weight loss and symptom amelioration (Allen and Golightly, 2015). The use of braces and assistive devices are often recommended but not well defined, as they may be effective for reducing physical stress on the knees and relieving symptoms (Nelson et al., 2014). Other alternatives and complementary therapies, like acupuncture, taichi, transcutaneous electrical nerve stimulation and therapeutic ultrasound are still controversial in different guidelines, as there is insufficient evidence to support their efficacy (Block, 2014, Jordan et al., 2003).

1.3.2. Pharmacological treatment
Pharmacological treatments mostly often recommended in the guidelines are acetaminophen (Ace), non-steroidal anti-inflammatory drugs (NSAIDs), opioid analgesics, and intra-articular (IA) injections (Ringdahl E, 2011). As pain is the main symptom of and influence on the life quality of OA patients, analgesics are widely performed as a vital remedy for moderate-to-severe OA in clinic. Ace (Hunter and Bierma-Zeinstra, 2019) and NSAIDs (Dougados, 2006) are essential medicine and recommended as the first-line pain medication for OA by most guidelines. Although the efficacy of Ace has been well documented, the dosage should be limited since overdosing with Ace may be toxic to the liver (McGill and Jaeschke, 2014). Compared with Ace, NSAIDs have a stronger anti-inflammatory effect. Some studies have administered randomized controlled trials (RCTs) and meta-analysis to compare the safety and efficacy of Ace and NSAIDs, suggesting NSAIDs are more effective than Ace regarding pain relief (Towheed et al., 2006, Pavelka, 2004). However, side effects regarding gastrointestinal and cardiovascular complications should be considered in selecting these drugs. The dose and frequency of oral analgesic agents
are preferably restricted at the minimum effective level (Sostres et al., 2010). Additionally, as adjunctives or alternatives, topical NSAIDs can be as effective as oral analgesic agents in treating knee OA (R. and RL, 2009). There was no evidence that topical NSAIDs could cause serious gastrointestinal or renal adverse events in the general population. Some local reaction such as itching, skin irritation and burning are observed more frequently (Hunter and Bierma-Zeinstra, 2019). Recently, it was well established that IA injection of Ace (Arun et al., 2013) and NSAIDs (Thing et al., 2014) were effective in suppressing inflammation and alleviating pain in joints, indicating that it would be a promising alternative to delay joint demolition for patients with OA. When patients with refractory pain or Ace and NSAIDs are ineffective or contraindicated, more potent drugs are necessary, such as opioids. Opioids, both oral and transdermal administration, have potent effects on pain alleviation and function promotion of patients with hip or knee OA (Ringdahl E, 2011). Benefits obtained from the use of opiates, however, can be outweighed by the frequent adverse effects, such as nausea, dizziness, vomiting, constipation and sleepiness (Fuggle et al., 2019). In addition, dependence or addiction to opiates is another potential risk, therefore low effective and tolerated doses are recommended (Lipman, 2001).

Nowadays, IA injections of corticosteroids (CSs) and hyaluronic acid (HA) have been widely and successfully applied in treating knee OA (Wernecke et al., 2015, Concoff et al., 2017). IA injections of CSs, such as dexamethasone (Dex), hydrocortisone, and methylprednisolone, have been frequently used in alleviating joint symptoms and inhibiting inflammation, and their efficacy has been evaluated in many clinical trials (Grodzinsky et al., 2017, Stove et al., 2002). Osteoarthritis Research International (OARIS) recommended that IA injections of CSs should be performed after patients failing to respond, or having an unsatisfactory response, to oral analgesic/anti-inflammatory agents (Zhang et al., 2008). HA is the main component of cartilage ECM and synovial fluid in both healthy and OA joints (Akmal M, 2005, Temple-Wong et al., 2016, Chen et al., 2020). Most of the guidelines recommend that IA injection of HA (Wernecke et al., 2015, Baron et al., 2018) as a
viscosupplementation for OA joints, owing to HA concentration is decreased in the synovial liquid of pathologic joints compared with healthy joints (Akmal M, 2005, Temple-Wong et al., 2016, Chen et al., 2020). The characteristics of IA injection of HA are delayed onset and prolonged duration of symptomatic benefits in comparison with CSs injections (Trueba Davalillo et al., 2015).

Other drugs, such as chondroitin, glucosamine, antidepressants, sex hormones, herbal remedies, and vitamins are recommended by some guidelines, but little detail was given and there no consensus achieved (Nelson et al., 2014).

1.3.3. Surgical treatments

1.3.3.1. Arthroscopic lavage and debridement

Arthroscopic surgeries including lavage and debridement have been extensively applied in the management of OA. In theory, arthroscopic lavage could relieve joint pain secondary to OA by way of clearing up the debris as well as inflammatory cytokines that may lead to synovitis and pain (Ronn et al., 2011). Nevertheless, there is no evidence to support the benefit of arthroscopic lavage. In a systematic review, Reichenbach et al. evaluated data from 567 patients in seven randomized trials, and found that joint lavage was no more effective in pain relief or function improvement than control groups (a sham intervention, a placebo injection, and a nonintervention control) (Reichenbach et al., 2010).

Arthroscopic debridement should be used to treat patients with torn meniscal fragments, and isolated OA without meniscal tear should be avoided. Dervin et al. showed that patients with evident meniscus lesions or cartilage flaps might benefit from this surgery (Dervin et al., 2003). This result was similar to another study, in which middle-aged patients were selected, and arthroscopic debridement was found to be beneficial to transient relief of symptoms (Hubbard, 1996). In a systematic review, after two years, insignificant difference was observed in pain relief in the arthroscopic intervention groups compared with control treatments (sham surgery, exercise, or medical treatment) (Thorlund et al., 2015).
1.3.3.2. Cartilage repair techniques

1.3.3.2.1 Microfracture surgery

Microfracture surgery is a technique that works by drilling a hole to penetrate the underlying bone. Theoretically, this technique causes MSCs derived from the subchondral bone marrow to differentiate into chondrocytes, promoting chondrogenesis in the defective area (Ronn et al., 2011). This is a relatively quick and simple operation and can be done through arthroscopy. The peculiarity of mini-incision and ease of handling of this technique results in its broad use (Erggelet and Vavken, 2016). On the other hand, forming fibrocartilage rather than hyaline cartilage and possible functional deterioration are the drawbacks one should be aware of (Mithoefer et al., 2009).

1.3.3.2.2 Osteochondral graft transplantation

Osteochondral graft transplantation is one of the most established techniques for reconstruction of a cartilaginous surface. Osteochondral grafts are made up of cartilage and bone from other parts of the body (osteochondral autograft) or from a tissue donor (osteochondral allograft), and replace both the AC and the subchondral bone (Ronn et al., 2011). This allows the defective area to be refilled immediately with mature and intact hyaline cartilage by using an arthroscopic or mini-invasive procedure (Richter et al., 2016b). The superiority of this technique is the lesion of cartilage can be substituted with a similar tissue. Minor integration, possible disease transmission, restricted donor sites, and complexity of handling are the disadvantages of this procedure (Ronn et al., 2011). According to the recent literature, a lesion with dimensions < 2 cm² is the best indication that can be treated through microfracture (first-line option) or osteochondral graft transplantation (Richter et al., 2016a). Compared with microfracture, osteochondral graft transplantation showed results of more longevity and durability, especially among patients with high functional demand (Karmali et al., 2019).

1.3.3.2.3 Autologous Chondrocyte Implantation (ACI)
ACI technique includes two-stage surgical procedures with laboratory processing, which was first reported clinically by Brittberg et al in 1994 (Brittberg et al., 1994). In this technique, cartilage is sampled from non-weight-loading regions and then digested enzymatically to isolate chondrocytes, subsequently, these cells are cultured in vitro for several weeks and reimplemented on the damaged area of cartilage (Brittberg, 2008). Although chondrocyte repairs the defect with tissue resembling hyaline cartilage, some issues remain, including low proliferation in their nature, dedifferentiation during in vitro expansion and limited cells being available (Schnabel et al., 2002). Additionally, donor-site morbidity caused by cartilage harvest, multiple surgical procedures, high cost and the lack of definitive scientific evidence to justify its large-scale use all limit the usage of ACI (Brittberg, 2008).

Figure 1.8 Schematic illustration of ACI. The procedure consists of two steps: (1) cartilage harvested from non-weight-loading regions and cultured in in vitro environment; and (2) reimplantation of the cells by injecting them into the lesion. (Reproduced with permission from (Bauge and Boumediene, 2015).

1.3.3.3. Osteotomy

It has been widely noted that joint malalignment plays a fundamental role in OA onset and progression. Osteotomy is performed to relieve pain (particularly in patients with symptoms) and delay the onset or progression of OA through realigning the joints (Ronn et al., 2011). Different surgical techniques are used to adjust the load axis, including high tibial osteotomy (HTO) and distal femoral osteotomy (DFO) (Fig 1.9). HTO is commonly performed in patients with varus deformity, either by wedging
open bone from the proximal tibia to reconfigure the knee joint (Lee and Byun, 2012). Similarly, DFO is usually performed in patients with valgus deformity by closing the medial wedge of bone or wedging open lateral femur (Feucht et al., 2017). Osteotomies around the knee are effective, particularly in young patients or middle-aged active patients with predominantly unicompartmental OA. However, postoperative complications such as fracture, nonunion, nerve injury, as well as the risk of additional surgery following osteotomy should be taken into account (Sherman et al., 2018). Thus, appropriate patient selection, correct osteotomy types, and proper surgical techniques are essential to guarantee the success of osteotomy.

![Postoperative X-ray images of HTO and DFO](image)

**Figure 1.9 Postoperative X-ray images of HTO and DFO.** (a) Open-wedge HTO in unicompartmental OA of the medial knee compartment (Lee and Byun, 2012) and (b) open-wedge DFO in unicompartmental OA of the lateral knee compartment. (Reproduced with permission from Feucht et al., 2017).

### 1.3.3.4. Joint arthroplasty

It is well accepted that joint arthroplasty is a safe and highly effective procedure for patients with severe OA (Grayson and Decker, 2012). In the light of its irreversible nature, this surgery is only recommended for patients who have failed to respond, or having an unsatisfactory response, to other treatments (Ronn et al., 2011). Owing to the limited endurance of prosthetic components, which is normally 15 to 20 years, arthroplasties are normally performed in patients older than 60 years old (Hunter and Bierma-Zeinstra, 2019). In general, knee joint arthroplasties contain
unicompartmental knee arthroplasty (UKA) and total knee arthroplasty (TKA) (Fig 1.10). The former is indicated in cases of OA with merely one side of damaged cartilage, and the latter is executed in patients with more than one lesion compartment involved or after the failure of other surgeries (Ahmad et al., 2015). Moreover, UKA is usually performed in patients with well-preserved lateral compartments, including intact meniscus, cartilage and cruciate ligaments (Arirachakaran et al., 2015). TKA is recommended in patients with more than one lesion compartment involved, and considerable improvements in the function and life quality have been shown (Aujla and Esler, 2017). The long-term follow-up study of TKA indicated that the survival rate was 92.7% and 90.4% at 10 years and 15 years, respectively (Feng et al., 2013). The main reasons for failure were aseptic loosening of components and infections (Feng et al., 2013).

Figure 1.10 Plain radiographs of the TKA and UKA. (a) Anteroposterior (AP) view after the TKA surgery. (b) Lateral view after the TKA surgery. (c) AP view after the UKA surgery. (d) Lateral view after the UKA surgery (Reproduced with permission from Ronn et al., 2011).
1.4. Aim of the study

The objective of this thesis was to perform comparative analysis of 2-D monolayer and 3-D culture of primary human chondrocytes to establish an optimal cell culture model. The following points shall be addressed:

1. To evaluate the effects of cigarette smoke extract (CSE) with different concentrations on the viability, function, and gene expression of primary human chondrocytes.

2. “To research the possible mechanism by which CSE affects primary human chondrocytes.” (Chen et al., 2020)

3. To evaluate whether the pharmacologic treatment of dexamethasone is beneficial to chondrocytes impaired by CSE, and if not, whether it could be substituted by other treatments, such as acetaminophen or NSAIDs.

4. To evaluate the effects of HA and HA combinations (dexamethasone, acetaminophen or diclofenac) on chondrocytes impaired by CSE.
2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and reagents

Table 2.1: List of used chemicals and reagents.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Article No.</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid Glacial (100%)</td>
<td>20104.298</td>
<td>VWR</td>
</tr>
<tr>
<td>1,9-Dimethyl-Methylene Blue Zinc Chloride Double Salt</td>
<td>341088</td>
<td>Sigma</td>
</tr>
<tr>
<td>(DMMB)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2', 7'-Dichlorfluorescein-Diacetate (DCFH-DA)</td>
<td>21884</td>
<td>Sigma</td>
</tr>
<tr>
<td>4-Nitrophenyl Phosphate Disodium Salt Hexahydrate (pNPP)</td>
<td>4165.1</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>4-Nitrophenyl Sodium 10 mM</td>
<td>N7660</td>
<td>Sigma</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>A5000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Agarose</td>
<td>2267.4</td>
<td>Roth</td>
</tr>
<tr>
<td>Alcian Blue</td>
<td>3082.2</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Ammonium Thiocyanate</td>
<td>221988-100g</td>
<td>Sigma</td>
</tr>
<tr>
<td>Antibiotic/Antimycotic Stock Solution</td>
<td>P11-002</td>
<td>PAA</td>
</tr>
<tr>
<td>Boric Acid 99.8% p.a.</td>
<td>6943.1</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Bromophenol</td>
<td>A512.1</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Calcein Acetoxyethylmethyl Easter (Calcein AM)</td>
<td>ABD-22002</td>
<td>ATT Bioquest</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Y015.1</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Chondroitin Sulfate A Sodium Salt from bovine trachea</td>
<td>C9819</td>
<td>Sigma</td>
</tr>
<tr>
<td>Collagenase II</td>
<td>17454.01</td>
<td>Serva</td>
</tr>
<tr>
<td>Deoxyribonucleic Acid from Calf Thymus</td>
<td>D4522</td>
<td>Sigma</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle Medium (DMEM)</td>
<td>D6546</td>
<td>Sigma</td>
</tr>
<tr>
<td>Dexamethason Water Soluble</td>
<td>D2915</td>
<td>Sigma</td>
</tr>
<tr>
<td>Diclofenac Sodium Salt</td>
<td>D6899</td>
<td>Sigma</td>
</tr>
<tr>
<td>Diethylypyrocarbonate (DEPC)</td>
<td>K028.3</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide (DMSO)</td>
<td>4720.2</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Disodium Hydrogen Phosphate</td>
<td>7876.7</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Dulbecco's Phosphate Buffered Saline (DPBS)</td>
<td>D8537</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ethanol 99.9% p.a. (EtOH)</td>
<td>20821.33</td>
<td>VWR</td>
</tr>
<tr>
<td>Ethidium Bromide 1%</td>
<td>2218.1</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Fetal Calf Serum (FCS)</td>
<td>41G7141K</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>First Strand cDNA Synthesis Kit</td>
<td>K1621</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>Formaldehyde, 37% solution</td>
<td>A0823.1000</td>
<td>AppliChem</td>
</tr>
<tr>
<td>Glycerol, &gt;99% p.a.</td>
<td>G6376-100G</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
2.1.2. Buffers, medium and solutions

Table 2.2: List of buffers, medium and solutions.

<table>
<thead>
<tr>
<th>Compounds and handling</th>
<th>Buffers/Mediums/Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>502 µl DMSO</td>
<td>Calcein AM stock solution</td>
</tr>
<tr>
<td>1 mg Calcein AM</td>
<td></td>
</tr>
<tr>
<td>62.5 mg Trypan blue</td>
<td>Trypan blue solution</td>
</tr>
<tr>
<td>50 ml Dulbecco’s PBS</td>
<td></td>
</tr>
<tr>
<td>500 mg Alcian blue (8 GX)</td>
<td>Alcian blue solution (1%, PH 2.5)</td>
</tr>
<tr>
<td>50 ml Acetic Acid (3%)</td>
<td></td>
</tr>
<tr>
<td>28.65 mg Guanidine Hydrochloride</td>
<td>Guanidine Hydrochloride (6 M)</td>
</tr>
<tr>
<td>50 ml ddH₂O</td>
<td></td>
</tr>
<tr>
<td>Solution/Buffer</td>
<td>Composition</td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Safranin-O solution (0.1%)</td>
<td>50 mg Safranin-O &lt;br&gt;50 ml ddH₂O</td>
</tr>
<tr>
<td>Formaldehyde (4%)</td>
<td>Formaldehyde (37%) in ddH₂O</td>
</tr>
<tr>
<td>AP Activity Assay buffer (PH 10.5)</td>
<td>2 g 4-nitrophenyl-phosphate (0.2%) &lt;br&gt;3.75 g Glycine (50 mM) &lt;br&gt;12.11 g Tris-Base (100 mM) &lt;br&gt;95.21 mg MgCl₂ (1 mM) &lt;br&gt;1 L ddH₂O</td>
</tr>
<tr>
<td>PCR loading buffer</td>
<td>25 mg Bromophenol blue &lt;br&gt;5 ml 10X TBE &lt;br&gt;5 ml Glycerol (20%)</td>
</tr>
<tr>
<td>10 X TBE buffer</td>
<td>108 g TRIS &lt;br&gt;55 g Boric acid &lt;br&gt;40 ml EDTA (0.5 M, PH 8) &lt;br&gt;1 L ddH₂O</td>
</tr>
<tr>
<td>Ethanol solution (70%)</td>
<td>99% Ethanol in ddH₂O</td>
</tr>
<tr>
<td>Sodium Acetate solution (3M, PH 5)</td>
<td>12.3 g Sodium Acetate in 50 ml ddH₂O</td>
</tr>
<tr>
<td>Acetic Acid Solution (1%)</td>
<td>100% acetic acid in ddH₂O</td>
</tr>
<tr>
<td>Acetic Acid Solution (3%)</td>
<td>100% acetic acid in ddH₂O</td>
</tr>
<tr>
<td>TRIS Solution (10 mM)</td>
<td>1.2 g TRIS in 1L ddH₂O</td>
</tr>
<tr>
<td>SRB Solution</td>
<td>0.4% SRB in 1% acetic acid</td>
</tr>
<tr>
<td>Resazurin stock solution</td>
<td>0.025% in DPBS</td>
</tr>
<tr>
<td>Resazurin working solution</td>
<td>10% Resazurin stock solution in DPBS</td>
</tr>
<tr>
<td>PBE Buffer (PH 6.5)</td>
<td>6.5 mg N-Acetyl-L-Cyteine &lt;br&gt;138 mg Disodium hydrogen phosphate &lt;br&gt;14.9 mg EDTA &lt;br&gt;Adjust PH to 6.5 with NaOH &lt;br&gt;Adjust volume to 20 ml with ddH₂O</td>
</tr>
<tr>
<td>Papain stock solution (5 mg/ml)</td>
<td>5 mg papain from papaya latex &lt;br&gt;1 ml PBE buffer</td>
</tr>
<tr>
<td>Papain working solution (25 µg/ml)</td>
<td>5 mg/ml stock solution in PBE buffer</td>
</tr>
<tr>
<td>DMMB solution buffer (PH 3)</td>
<td>304 mg Glycine &lt;br&gt;160 mg sodium chloride &lt;br&gt;9.5 ml 0.1 M Acetic acid &lt;br&gt;90.5 ml ddH₂O</td>
</tr>
<tr>
<td>DMMB stock solution (8 mg/ml)</td>
<td>8 mg DMMB in 1 ml buffer</td>
</tr>
<tr>
<td>DMMB working solution (16 µg/ml)</td>
<td>8 mg/ml stock solution in DMMB buffer</td>
</tr>
<tr>
<td>Consumable</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Chondroitin Sulfate Standards stock solution (1 mg/ml)</td>
<td>10 mg Chondroitin Sulfate Standards in 10 ml PBE buffer</td>
</tr>
<tr>
<td>Chondroitin Sulfate Standards working solution (100 µg/ml)</td>
<td>1 mg/ml stock solution in PBE buffer</td>
</tr>
<tr>
<td>TE buffer (PH 7.5-8.0)</td>
<td>10 mM Tris base</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td>Calf Thymus DNA stock solution (1 mg/ml)</td>
<td>1 mg Calf Thymus DNA in 1 ml TE buffer</td>
</tr>
<tr>
<td>TNE buffer (PH 7.4)</td>
<td>121.1 mg Tris Base</td>
</tr>
<tr>
<td></td>
<td>37.2 mg EDTA</td>
</tr>
<tr>
<td></td>
<td>1.17 g sodium chloride</td>
</tr>
<tr>
<td></td>
<td>Adjust PH to 7.4 with HCl</td>
</tr>
<tr>
<td></td>
<td>Adjust volume to 100 ml with ddH₂O</td>
</tr>
<tr>
<td>Chondrocyte Cells Culture Medium</td>
<td>500 ml DMEM</td>
</tr>
<tr>
<td></td>
<td>+500 ml Ham's F12</td>
</tr>
<tr>
<td></td>
<td>+50 ml FCS</td>
</tr>
<tr>
<td></td>
<td>+10 ml Penicillin/Streptomycin</td>
</tr>
<tr>
<td></td>
<td>+50 µl L-Ascorbic-2-Phosphate</td>
</tr>
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</table>

2.1.3. Consumables

**Table 2.3: List of consumables**

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Manufacturer</th>
<th>Type</th>
<th>Serial number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture plate</td>
<td>Greiner bio-one</td>
<td>96-well, flat bottom</td>
<td>655180</td>
</tr>
<tr>
<td>Cell culture plate</td>
<td>Greiner bio-one</td>
<td>96-well, V bottom</td>
<td>651101</td>
</tr>
<tr>
<td>Cell culture plate</td>
<td>Corning Inc.</td>
<td>48-well, flat bottom</td>
<td>3548</td>
</tr>
<tr>
<td>Cell culture plate</td>
<td>Greiner bio-one</td>
<td>24-well, flat bottom</td>
<td>662160</td>
</tr>
<tr>
<td>Cell culture plate</td>
<td>Greiner bio-one</td>
<td>6-well, flat bottom</td>
<td>353046</td>
</tr>
<tr>
<td>Cell Star Tubes</td>
<td>Corning Inc.</td>
<td>50 ml</td>
<td>227261</td>
</tr>
<tr>
<td>Cell Star Tubes</td>
<td>Greiner bio-one</td>
<td>15 ml</td>
<td>188271</td>
</tr>
<tr>
<td>Eppendorf tube</td>
<td>SARSTEDT AG</td>
<td>0.5 ml, white</td>
<td>72.699</td>
</tr>
<tr>
<td>Equipment</td>
<td>Manufacturer</td>
<td>Type</td>
<td>Serial number</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----------------------------------</td>
<td>-----------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Eppendorf tube</td>
<td>Carl Roth GmbH + Co.KG</td>
<td>1.5 ml, white</td>
<td>4182.1</td>
</tr>
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<td>Eppendorf tube</td>
<td>Carl Roth GmbH + Co.KG</td>
<td>1.5 ml, blue</td>
<td>4190.1</td>
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<td>1.5 ml, green</td>
<td>4209.1</td>
</tr>
<tr>
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<td>1.5 ml, red</td>
<td>4189.1</td>
</tr>
<tr>
<td>Eppendorf tube</td>
<td>Carl Roth GmbH + Co.KG</td>
<td>1.5 ml, yellow</td>
<td>4204.1</td>
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<tr>
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<td>Eppendorf</td>
<td>2.0 ml, white</td>
<td>2549</td>
</tr>
<tr>
<td>Pipette Tips</td>
<td>Sorenson BioScience, Inc.</td>
<td>0.1 - 10 μl</td>
<td>Colorless</td>
</tr>
<tr>
<td>Pipette Tips</td>
<td>Sarstedt AG &amp; Co.</td>
<td>2 - 200 μl</td>
<td>Yellow</td>
</tr>
<tr>
<td>Pipette Tips</td>
<td>Ratiolab GmbH</td>
<td>100 - 1000 μl</td>
<td>Blue</td>
</tr>
<tr>
<td>Single-channel Pipette</td>
<td>Corning Inc.</td>
<td>10-100 μl</td>
<td>158240031</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Corning Inc.</td>
<td>100-1000 μl</td>
<td>058261237</td>
</tr>
<tr>
<td>Single-channel Pipette</td>
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<td>Fluostar Omega</td>
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<tr>
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<td>Lauder Dr. R. Wobser GmbH</td>
<td>Al 25</td>
<td>0727-11-0094</td>
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<tr>
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<td>Lauder Dr. R. Wobser GmbH</td>
<td>ECO ET 20</td>
<td>LY 06.1</td>
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### Table 2.4: List of used equipment.

2.1.4. Equipment

#### Table 2.4: List of used equipment.
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<th>Equipment</th>
<th>Manufacturer</th>
<th>Model/Part Number</th>
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<td>3523-21L</td>
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<td>HKMT 040-01 CC00412514</td>
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<td>100 ml 5500537</td>
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<tr>
<td>Gas washing bottle</td>
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<td>Scotsmen</td>
<td>AF 80 DD 8837 11 X</td>
</tr>
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<td>Thermo Fisher Scientific</td>
<td>Heratherm OMS 60 41296334</td>
</tr>
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<td>Incubator</td>
<td>Binder GmbH</td>
<td>9040-0078 11-22649</td>
</tr>
<tr>
<td>Incubator</td>
<td>Binder GmbH</td>
<td>9040-0081 11-22190</td>
</tr>
<tr>
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<td>Carl Roth GmbH + Co.KG</td>
<td>Cyclo 2 1109-065</td>
</tr>
<tr>
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<td>PeqlabBiotechnologie GmbH</td>
<td>EVOS-fl 91-AF-4301</td>
</tr>
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<td>Vortex Mixer 804995</td>
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<td>Maxisave S20201.8 41293949</td>
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<tr>
<td>Scale</td>
<td>Kern &amp;Sohn GmbH</td>
<td>ABJ 120-4M WB 1140084</td>
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</table>
2.2 Ethics Statement
All human studies were performed in accordance with the latest revision of the Helsinki declaration. All human tissues were obtained in accordance with the ethical vote (653/2018BO2) of Eberhard-Karls University and the patients’ written consent. The average age of donors for primary chondrocytes isolation was 69.64 ± 8.82 years (10 males and 4 females).

2.3 Methods
2.3.1. Generation of Cigarette Smoke Extract (CSE)
We used two commercial cigarettes (Marlboro, Philip Morris, New York City, USA) to prepare fresh CSE. In brief, the cigarettes were constantly combusted into a standard gas washing bottle with a 50 ml pre-warmed DMEM medium. Here two cigarettes were bubbled at a speed of 95 bubbles/min. The concentration of CSE was measured by a plate reader at \( \lambda = 320 \text{ nm (OD}_{320} \). An \( \text{OD}_{320} \) of 0.7 was regarded as 100% CSE. The fresh CSE was passed through a 0.22 \( \mu \text{m} \) pore filter to ensure sterility, and then it was diluted further (0.1, 0.5, 1, 5, 10, 20, 50%) with chondrocyte culture.
medium. The CSE concentrations correspond to exposure associated with smoking from 0.01 pack (0.1%) to 1 pack (10%) cigarettes/day (Sreekumar et al., 2017, Chen et al., 2020).

2.3.2. Isolation and culture of primary human chondrocytes
We obtained samples of primary human chondrocytes from osteoarthritic patients undergoing total knee arthroplasty. Isolation and culture of primary human chondrocytes were mentioned as previously described (Tendulkar et al., 2019). In short, the cartilages were chopped into pieces and washed with DPBS (w/o Ca\(^{2+}/\)Mg\(^{2+}\)) thoroughly. Subsequently, these pieces were subjected to collagenase (1500 U/mL) digestion in a 37 °C shaker incubator overnight, then the supernatant of the mixture was obtained by centrifugation to eliminate impurities. Finally, these cells were expanded in culture medium at 37 °C and 5% CO\(_2\). The cell culture medium with or w/o CSE was changed every 24 hours until 3, 7 and 14 days post-inoculation (Chen et al., 2020).

2.3.3. Chondrocyte pellet culture
Chondrocyte pellets were formed in polypropylene 96-well conical plates with 2.5 × 10\(^5\) cells per pellet (Solchaga et al., 2011). In brief, after centrifugation at 600 g for 10 min, pellets were resuspended at a density of 1.25 × 10\(^6\) cells/ml in the chondrocyte culture medium. Subsequently, 200 µl aliquots of the cell suspension (2.5 × 10\(^5\) cells/well) were dispensed into 96-well conical plates and spun in a benchtop centrifuge at 600 g for 10 min. Afterward, the pellets were cultured at 37 °C under a gas mixture of 95% air/5% CO\(_2\). Medium change was performed twice a week.

2.3.4. Resazurin Conversion Assay
To measure cell viability (mitochondrial activity) of primary human chondrocytes, a resazurin conversion assay was performed. In short, after half an hour incubation with 0.0025% (w/v) Resazurin working solution (in PBS) in a 37°C incubator, the fluorescence (ex/em = 540/590 nm) was evaluated by a plate reader, as the protocol
described before (Ehnert et al., 2018, Chen et al., 2020).

2.3.5. Sulforhodamine B (SRB) Staining to assess total protein content
To assess the total protein content of primary human chondrocytes, SRB staining was performed as reported before (Ehnert et al., 2018, Chen et al., 2020). In short, cells were fixed with ice-cold ethanol for 30 min at -4°C, and then stained with SRB working solution (0.4% w/v in 1% v/v acetic acid) at RT for 30 min followed by 3 washing steps with acetic acid (1% v/v) to eliminate unbound SRB. After dissolving the bound SRB in unbuffered TRIS solution (10 mM, pH ~10.5), the absorbance was recorded at $\lambda = 565$ nm with a plate reader.

2.3.6. Determination of sulfated glycosaminoglycan (sGAG) content
The content of sGAG was determined to assess the matrix formation of chondrocytes. Briefly, the pellets were transferred into 1.5-ml micro-centrifuge tubes (one pellet per tube) and washed with PBS. One ml papa in working solution was added into each tube, then the mixtures were incubated at 60°C overnight. DMMB dye (1,9 - Dimethyl - Methylene Blue zinc chloride double salt) was performed to quantify the sGAG content with chondroitin sulfate A (0–50 mg/mL) as a standard. The amount of sGAG was quantified by measuring the color shift (blue to purple) at $\lambda = 530$ nm and $\lambda = 590$ nm, respectively, in a plate reader (Babur et al., 2013).

2.3.7. DNA Quantification
DNA content was determined by Calf thymus DNA (ct-DNA) assay, as the manufacturer’s protocol described before. In brief, 100 µl of standard or sample was mixed with 100 µl of Hoechst 33342 dye working solution in a fluorescence plate and analyzed (ex/em = 355/460 nm) in a plate reader (Solchaga et al., 2011).

2.3.8. Live/dead staining
Calcein AM (living cell staining ) and Ethidium homodimer (dead cell staining ) were considered as indirect parameters of cell viability. Following the washing with PBS 3
times, chondrocytes exposed to the culture medium with or w/o CSE were stained with a mixture of 2 µM Calcein AM, 4 µM Ethidium homodimer and 1 mg/ml Hoechst 33342 at RT for 30 min. The stained cells were visualized under a fluorescence microscope (Mi et al., 2016, Chen et al., 2020).

2.3.9. Assessing ECM production by Alcian blue and Safranin-O staining
In order to evaluate the generation of glycosaminoglycans (GAGs) and collagen, Alcian blue and Safranin-O staining were performed (Tendulkar et al., 2019). In short, cells were fixed with 4% \( \text{v/v} \) formaldehyde for 30 min at RT followed by a washing step in PBS. Subsequently, cells were labeled with 1% \( \text{w/v} \) Alcian blue and 0.1% \( \text{w/v} \) Safranin-O staining at RT for 30 min, respectively. Afterwards, distilled water was added to eliminate the unbounded dye solution. All images were photographed with an EVOSfl microscope. After dissolving the bound Alcian blue staining with 6 M guanidine HCl in distilled water, the optical density was recorded at \( \lambda = 620 \) nm using a plate reader (De Bari et al., 2001, Chen et al., 2020).

2.3.10. Determination of Reactive oxygen species (ROS) production
ROS production was measured using \( 2',7' \)-dichlorofluorescein-diacetate (DCFH-DA). In short, 10 µM DCFH-DA dissolved in serum-free culture medium was supplemented to cells after washing with PBS and incubated for at 37 °C for 30 min. Then cells were exposed to CSE after washing with PBS. The measurement of fluorescence intensity was obtained by a plate reader (ex/em = 485/520 nm) following 15 min of incubation (Sreekumar et al., 2017, Chen et al., 2020).

2.3.11. Alkaline Phosphatase (AP) Activity Assay
AP activity is considered as a hypertrophic marker of chondrocytes (Nadzir et al., 2011). In short, AP reaction buffer was added to cells after washing with PBS and incubated in a 37°C incubator for one hour. Then, absorbance was determined using a plate reader (\( \lambda = 405 \) nm / OD405) as described (Sreekumar et al., 2017). The normalization was performed by Resazurin conversion (Chen et al., 2020).
2.3.12. Semi-Quantitative RT-PCR

The TriFAST reagent was used to collect total RNA based on the manufacturer’s instructions (Tendulkar et al., 2019). Complementary DNA (cDNA) was synthesized with the First Strand cDNA Synthesis Kit after the measurement of RNA concentration. Then, 10 ng of cDNA was used as a template to perform semi-quantitative RT-PCR by using Biozym Ready Mix. Details of primer used are provided in Table 2.5. Afterward, 1.5% agarose gel electrophoresis and ethidium bromide were utilized to separate and visualize the PCR products. Moreover, the pUC19/Msp1 marker was used as a size reference. Internal control was GAPDH. The data obtained was quantified using the ImageJ software (NIH, Bethesda, MD, USA).

Table 2.5: Summary of primers sequences and PCR conditions for the genes.

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<th>Gene</th>
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<th>Forward primer (5’-3’)</th>
<th>Reverse primer (3’-5’)</th>
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<th>Annealing temperature (°C)</th>
<th>Cycles (N°)</th>
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<td>30</td>
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Statistics

Graph Pad Prism software (El Camino Real, CA, USA) was used for statistical analyses. Results are expressed as bar or line diagrams (mean ± SEM). All the experiments were performed at least 3 times (N≥3) and measured as triplicates or more (n≥3). Data were analyzed by Mann–Whitney U-test or Kruskal–Wallis H test followed by a Dunn’s test. Minimum level of significance: p ≤ 0.05. The statistical analyses were advised by PD Dr. Sabrina Ehnert.
Chapter 3

3. Result

3.1. Comparison of 3-D culture and 2-D monolayer culture of primary human chondrocytes

Firstly, in order to establish the optimal model for chondrocyte cell culture, we compared different cell culture methods, namely 3-D pellet (Fig. 3.1.a), 2-D monolayer (Fig. 3.1.b) and knitted titanium scaffold (Fig. 3.1.c) culture. Viability, matrix formation and function of chondrocytes in these culture methods were evaluated to decide the optimal model. As shown in the table (Fig 3.1.d), 2-D monolayer culture has the advantage of simplicity and ease of operation compared with 3-D culture.

![Figure 3.1 Comparison of 3-D culture and 2-D monolayer culture of primary human chondrocytes. (a) Morphology and size of primary human chondrocytes in pellet culture and (b) morphology of primary human chondrocytes in monolayer culture after 24 h. (c) Illustration of chondrocytes seeded on the knitted titanium scaffolds after 24 h. Calcein-AM was used to show the living cells in the scaffold. The black arrowhead indicates the pellet. Different experimental methods were compared and listed in the table (d). Tick (✓) means the method is available and cross (x) means the method is not available.](image-url)

<table>
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<th></th>
<th>Methods</th>
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<td>✓</td>
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<td></td>
<td>SRB</td>
<td>x</td>
<td>✓</td>
<td>x</td>
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<td></td>
<td>Live/Dead</td>
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<td>✓</td>
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<td>x</td>
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<td>(COL II, X, SOX9, ACAN)</td>
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</table>

Figure 3.1 Comparison of 3-D culture and 2-D monolayer culture of primary human chondrocytes. (a) Morphology and size of primary human chondrocytes in pellet culture and (b) morphology of primary human chondrocytes in monolayer culture after 24 h. (c) Illustration of chondrocytes seeded on the knitted titanium scaffolds after 24 h. Calcein-AM was used to show the living cells in the scaffold. The black arrowhead indicates the pellet. Different experimental methods were compared and listed in the table (d). Tick (✓) means the method is available and cross (x) means the method is not available.
3.2. CSE exposure induces the reduction in viability of primary human chondrocytes after 24 h

Afterward, in order to assess the toxicity of CSE on the chondrocytes, cells were exposed to the CSE with increasing concentrations (0.1, 0.5, 1, 5, 10, 20, 50%) for 24 h. Mitochondrial activity (Fig. 3.2.a) and total protein content (Fig. 3.2.b) were used to determine the viability of cells after 24 h incubation (Chen et al., 2020), respectively. Mitochondrial activity (20%, **\( p \leq 0.01 \) and 50%, ***\( p \leq 0.001 \)) and total protein content (20% and 50%, ***\( p \leq 0.001 \)) of chondrocytes were significantly reduced in the concentrations of CSE over 20% compared to the control group. Similarly, the number of living cells was significantly reduced by 20% CSE, as determined by Calcein-AM staining (Fig. 3.2.c).

![Figure 3.2 CSE exposure induces the reduction in viability of primary human chondrocytes after 24 h.](image)

After 24 h, (a) Resazurin conversion and (b) SRB staining were performed for cell viability. (c). Representative microscopic images for Calcein-AM staining. Cells were visualized with Calcein-AM (green) and Hoechst 33342 (blue) for living cells and nuclear, respectively. Data are presented as bar diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. *\( p \leq 0.05 \), **\( p \leq 0.01 \), ***\( p \leq 0.001 \) as compared to the control group. Scale bar = 1000 µm.

3.3. Comparison of the effect of CSE exposure twice a week and once a day on the primary human chondrocytes
For the next step, in order to determine the optimal smoking condition, we compared two different exposure pathways to chondrocytes, namely, exposing twice a week (Fig. 3.3.a, b, c) and once a day (Fig. 3.3.d, e, f). Cells were exposed to increasing concentrations of CSE (0.1, 0.5, 1, 5, 10%) and stopped on days 1, 3, 7 and 14, respectively. On day 3 and day 7, mitochondrial activity (Fig. 3.3.a) and total protein content (Fig. 3.3.b) of chondrocytes were not affected significantly by exposure to CSE twice a week, which was consistent with Calcein-AM staining (Fig. 3.3.c) on day 7. On day 14, however, CSE exposure significantly decreased the mitochondrial activity (Fig. 3.3.a, 5% and 10%, **$p \leq 0.01$ and ***$p \leq 0.001$, respectively) and total protein content (Fig. 3.3.b, 0.5%, **$p \leq 0.05$ and 1%, 5%, 10%, **$p \leq 0.01$) of chondrocytes compared to the control group. Nevertheless, on day 3, mitochondrial activity (Fig. 3.3.d) and total protein content (Fig. 3.3.e, 10%, **$p \leq 0.01$) of chondrocytes were inhibited significantly by exposing to CSE once a day. On days 7 and 14, CSE-exposed chondrocytes showed a strong reduction in mitochondrial activity (Fig. 3.3.d, 5% and 10%, **$p \leq 0.01$) and total protein content (Fig. 3.3.e, 5% and 10%, ***$p \leq 0.001$), respectively. Similarly, the number of living cells was decreased by CSE in a dose-dependent manner on day 7, as determined by Calcein-AM staining (Fig. 3.3.f). Therefore, CSE exposure every day is more pronounced than exposure twice a week. Based on the above, chondrocytes exposed to CSE once a day were selected for further experiments.
Figure 3.3 Comparison of the effect of CSE exposure twice a week and once a day on the primary human chondrocytes. On days 1, 3, 7 and 14, (a) Resazurin conversion and (b) SRB staining were performed for cell viability. (c). Representative microscopic images for Calcein-AM staining. Cells were visualized with Calcein-AM (green) and Hoechst 33342 (blue) for living cells and the corresponding nuclei, respectively. Data are presented as bar diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 as compared to the control group. Scale bar = 1000 µm.
3.4. CSE exposure inhibits the accumulation of ECM in primary human chondrocytes

Aggrecan and collagen II are major components of ECM and can be stained specifically by Alcian blue and Safranin-O staining (Akkiraju and Nohe, 2015). We performed Alcian blue and Safranin-O staining to investigate the effect of CSE on the functionality of primary human chondrocytes. CSE-exposed chondrocytes showed a dose-dependent decline in matrix accumulation on day 7 (Fig. 3.4.a, 10%, ***<i>p</i> ≤ 0.001) and day 14 (Fig. 3.4.b, 5% and 10%, **<i>p</i> ≤ 0.01 and ***<i>p</i> ≤ 0.001, respectively), respectively. Similarly, the stains of Alcian blue (Fig 3.4.c) and Safranin-O (Fig 3.4.d) dye were decreased on day 7, respectively.

![Matrix formation day 7](image1) ![Matrix formation day 14](image2)

**Figure 3.4** CSE exposure inhibits the accumulation of ECM in primary human chondrocytes. Matrix formation was quantified on day 7 (a) and day 14 (b) by Alcian blue staining. Representative microscopic images for Alcian blue (c) and Safranin-O (d) staining were performed on day 7. Data are presented as bar diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. **<i>p</i> ≤ 0.01, ***<i>p</i> ≤ 0.001 as compared to the control group. Scale bar = 400 µm.

3.5. CSE exposure down-regulates the anabolic gene expression of primary human chondrocytes

Since after 7 days of treatment with CSE, the matrix accumulation of chondrocytes
was inhibited significantly by 10% CSE, we wanted to know whether the inhibition was due to the down-regulation of gene expression. On day 7, the mRNA expression analysis showed that Collagen II (Fig. 3.5.a, **\(p \leq 0.01\)), a major component of ECM protein (Archer, 2003), was significantly down-regulated to 0.2-fold in 10% CSE-exposed group. Similarly, gene expression of Aggrecan (Fig. 3.5.b, **\(p \leq 0.01\)), an ECM marker for cartilage (Archer, 2003), and SOX-9 (Fig. 3.5.c, **\(p \leq 0.01\)), which is the main transcription factor for particularization and sustentation of cartilage (Tew et al., 2008), was significantly suppressed to 0.5-fold and 0.75-fold, respectively. Intriguingly, gene expression of Collagen X (Fig. 3.5.d), a marker of hypertrophic chondrocyte (Nejadnik et al., 2015), was not affected by 10% CSE. Therefore, CSE exposure negatively affected the anabolic gene expression (Collagen II, Aggrecan and SOX-9) of chondrocytes, which might interrupt the formation of the chondrocyte matrix and result in cartilage demolishment.

Figure 3.5 CSE exposure down-regulates the anabolic gene expression of primary human chondrocytes. Semi-quantitative RT-PCR was performed using primers for gene expression. The gene expression of (a) Collagen II, (b) Aggrecan, (c) Sox9, and (d) Collagen X was normalized to the GAPDH (housekeeping gene). Data are presented as bar diagrams (mean ± SEM); analyzed by Mann Whitney test. **\(p \leq 0.01\) as compared to the control group.

3.6. CSE exposure increases oxidative stress and accelerates the death of primary human chondrocytes

It is believed that ROS in or induced by CS is one of the critical risk factors in producing adverse effects on the human body (Kamceva et al., 2016, Chen et al., 2020). The production of ROS was increased when chondrocytes were exposed to
CSE at the concentration of 1% and above, and 10% CSE induced significantly high amounts of ROS (2-fold) as compared to corresponding untreated cells, (Fig. 3.6.a, **p ≤ 0.01). Meanwhile, the death of chondrocytes occurs during the process of OA and is connected with the accumulation of ECM (Valavanidis A et al., 2009, Thomas et al., 2007). Increased oxidative stress is known to induce cell death (Collins et al., 2016, Chen et al., 2020). Therefore, it is interesting to assay whether the CSE could induce chondrocyte cell death through the generation of ROS production. Incubation of chondrocytes with 10% CSE for 3 days showed an increase in dead cells and a decrease in living cells, and treatment of chondrocytes with 0.01% hydrogen peroxide for 20 min, which represents a principal ROS in cigarette smoke (Valavanidis A et al., 2009, Chen et al., 2020), significantly induced cell death of chondrocytes (Fig. 3.6.b), thus associating the high ROS production by CSE with the increase of cell death in chondrocytes.

Figure 3.6 CSE exposure increases oxidative stress and accelerates the death of primary human chondrocytes. (a). ROS production was measured by DCFH-DA assay. (b). Living cells (green) and dead cells (red) were visualized by Calcein AM and ethidium homodimer, respectively (Representative figure for day 3. H$_2$O$_2$ (0.01% v/v) was performed as a positive control. Data are presented as line diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. **p ≤ 0.01, ****p ≤ 0.0001 as compared to the control group. Scale bar = 400 µm.

3.7. Clinical doses of Dex are toxic to the primary human chondrocytes
Steroid injections, such as hydrocortisone, Dex, and methylprednisolone, have been frequently and successfully used in alleviating joint symptoms and inhibiting inflammation (Wernecke et al., 2015, Chen et al., 2020). CSs act both immune-
suppressive and anti-inflammatory (Huebner et al., 2014). Besides, it is also reported that CSs could inhibit ROS generation (Dandona et al., 1999, Chen et al., 2020). On the other hand, many preliminary studies reported the adverse effects of Dex on cartilage integrity and chondrocyte viability (Tu et al., 2013, Zhao et al., 2014). In order to test the non-toxic concentrations of Dex to the chondrocytes, cells were treated with a series of doses of Dex (4-4000 μg/ml) for 24 h, in which clinical dosage (4000 μg /ml) was included (JR, 1996, Grodzinsky et al., 2017, Chen et al., 2020). Mitochondrial activity (Fig. 3.7.a, ≥ 1000 μg/ml, ****p ≤ 0.0001) and total protein content (Fig. 3.7.b, 2000 μg/ml and 4000 μg/ml, **p ≤ 0.01 and ***p ≤ 0.001, respectively) showed a dose-dependent inhibition in Dex-treated chondrocytes, suggesting detrimental effects of Dex on chondrocytes (Chen et al., 2020).

![Figure 3.7 Clinical doses of Dex are toxic to the primary human chondrocytes.](image)

After 24 h, (a) Resazurin conversion and (b) SRB staining were performed for cell viability. Data are presented as line diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 as compared to the untreated controls.

### 3.8. Clinical doses of HA increases the viability of primary human chondrocytes

HA is the main component of cartilage ECM and synovial fluid in both healthy and OA joints (Akmal M, 2005, Temple-Wong et al., 2016, Chen et al., 2020). It is used with IA injection and is extensively recommended in most guidelines (Wernecke et al., 2015, Baron et al., 2018) as a visco-supplementation, owing to HA concentration is decreased in the synovial liquid of pathologic joints compared with healthy joints (Akmal M, 2005, Temple-Wong et al., 2016, Chen et al., 2020). In addition to its role
in visco-supplementation, an important biological aspect of HA is its benefits to the chondrocytes, such as chondro-protection (Akmal M, 2005), scavenging of reactive oxygen-derived free radicals (Yu et al., 2014) and inhibition of inflammation (Fioravanti et al., 2005). In order to determine the non-toxic concentrations of HA to the chondrocytes, cells were treated with HA (0.1–5 mg/ml) for 24 h, in which clinical dosage (5 mg/ml) was included (Trueba Davalillo et al., 2015, Chen et al., 2020). Mitochondrial activity (Fig. 3.8.a) and total protein content (Fig. 3.8.b, 5 mg/ml, ***p ≤ 0.001) showed a dose-dependent improvement of cell growth in HA-treated chondrocytes, indicating the applied clinical doses of HA are beneficial to the primary human chondrocytes.

![Figure 3.8 Clinical doses of HA increases the viability of primary human chondrocytes.](image-url)

After 24 h, (a) Resazurin conversion and (b) SRB staining were performed for cell viability. Data are presented as line diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. ***p ≤ 0.001 as compared to the untreated group.

3.9. Cytotoxicity assessment of Vitamin C on the primary human chondrocytes

Vitamin C, also known as ascorbic acid (AA), has been extensively applied in orthopedic applications for its role in wound healing (Bikker et al., 2016), bone formation (Aghajanian et al., 2015) and chondro-protection (Chiu et al., 2016). Furthermore, AA is also a robust antioxidant and cofactor, which provides protection against oxidative stress and regulates cellular development (Graeser et al., 2009). Several studies have demonstrated that AA supplementation has the potential to inhibit the degeneration of chondrocyte morphology and biochemistry (Ibold et al., 2009, Stabler and Kraus, 2003). These findings suggest that AA may be a promising
drug or antioxidant in protecting oxidative stress damaged chondrocyte. In order to evaluate the concentrations that were non-toxic to the chondrocytes, cells were treated with a series of concentrations of AA (50 µM – 10 mM) for 24 h. Mitochondrial activity (Fig. 3.9.a, 10 mM, ****p ≤ 0.0001) and total protein content (Fig. 3.9.b) showed that the concentrations of AA over 1 mM were toxic to the chondrocytes.

![Graph](image)

Figure 3.9 Cytotoxicity assessment of Vitamin C on the primary human chondrocytes. After 24 h, (a) Resazurin conversion and (b) SRB staining were preformed for cell viability. Data are presented as line diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001 as compared to the untreated group.

3.10. Effects of AA on the CSE-exposed primary human chondrocytes

Subsequently, three concentrations of AA (50, 100, 200 µM) were performed in the following experiments. Mitochondrial activity (Fig. 3.10.a, 50, 100, 200 µM, ***p ≤ 0.001), total protein content (Fig. 3.10.b, 50, 100, 200 µM, ****p ≤ 0.0001), and matrix formation were increased (Fig 3.10.c, 50 µM, **p ≤ 0.01 and 100, 200 µM, *p ≤ 0.05) when the cells were solely incubated with AA on day 7. However, mitochondrial activity (Fig. 3.10.a, 200 µM, oooop ≤ 0.0001), total protein content (Fig. 3.10.b, 200 µM, ooop ≤ 0.01) and matrix formation showed a dose-dependent decrease (Fig. 3.10.c; 200 µM, ooop ≤ 0.01) when the chondrocytes were co-incubated with CSE and AA on day 7.
Figure 3.10 Effects of AA on the CSE-exposed primary human chondrocytes. On day 7 of treatment, (a) Resazurin conversion and (b) SRB staining were performed for cell viability. Matrix accumulation was quantified by (c) Alcian blue staining. Data are presented as bar diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 vs. the untreated group, °°p ≤ 0.01, °°°°p ≤ 0.0001 as indicated.

3.11. Evaluation of low doses of Dex and HA on the CSE-impaired primary human chondrocytes

HA injection is used to improve the functional mobility of pathologic OA joints, since HA is capable of improving viscoelastic properties to the synovial liquid (Temple-Wong et al., 2016, Chen et al., 2020). In addition to its role in viscosupplementation, an important biological aspect of HA is its benefits to the chondrocytes, such as chondro- protection (Akmal M, 2005), scavenging of reactive oxygen-derived free radicals (Yu et al., 2014) and inhibition of inflammation (Fioravanti et al., 2005). Subsequently, we attempted to evaluate whether the detrimental effects of CSE on cells could be retrieved by HA or low doses of Dex. Three different concentrations of HA (0.1, 1, 5 mg/ml) and Dex (4, 40, 400 μg/ml) based on the above data were chosen for the following experiments. On day 7, a
dose-dependent decrease in mitochondrial activity (Fig. 3.11.a, 400 μg/ml, **p ≤ 0.01), total protein content (Fig. 3.11.b, 400 μg/ml, **p ≤ 0.01) and matrix formation (Fig. 3.11.c, 400 μg/ml, **p ≤ 0.01) was observed in Dex-treated chondrocytes. Interestingly, there was no differential variation was observed when CSE-impaired cells were incubated with Dex for 7 days. On day 14, AP activity increased significantly (Fig. 3.11.d, 4μg/ml, *p ≤ 0.05, 40μg/ml, **p ≤ 0.01 and 400 μg/ml, **p ≤ 0.01) in the Dex-treated cells, indicating that Dex was prone to modifying the morphology of chondrocytes after long-term treatment. In contrast to Dex, a significant increase in mitochondrial activity (Fig. 3.11.e, 1 mg/ml and 5 mg/ml, °°°°p ≤ 0.0001, respectively) and total protein content (Fig. 3.11.f, 5 mg/ml, °°°°p ≤ 0.0001) was observed after HA exposure of CSE-impaired cells after 7 days. Besides, the accumulation of chondrocyte matrix was enhanced significantly (Fig. 3.11.g, 1 mg/ml, °p ≤ 0.05 and 5 mg/ml, °p ≤ 0.05) on day 7.
3.11 Evaluation of low doses of Dex and HA on the CSE-impaired primary human chondrocytes. On day 7 of treatment, (a, e) Resazurin conversion and (b, f) SRB staining were performed for cell viability. Matrix accumulation was quantified by (c, g) Alcian blue staining. (d) AP activity was measured on day 7 and day 14, respectively. Data are presented as bar and diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ vs. the untreated group, °$p \leq 0.05$, °°°$p \leq 0.001$ as indicated.

3.12. Effects of Ace and Dic on the CSE-impaired primary human chondrocytes

Severe adverse effects of oral analgesic/anti-inflammatory agents, like Ace and NSAIDs, led to the emergence of IA injections of these drugs, which proved to be effective in suppressing inflammation and alleviating pain in joints (Arun et al., 2013, Cannava et al., 2013, Mertz et al., 2016). Considering OA is a chronic disabling disease, pharmacological treatments would require a prolonged time-span (Chen et al., 2020). We evaluated the effects of Ace (10 μg/ml) and Dic (1 μg/ml) on the CSE-impaired chondrocytes, showing that these drugs did not promote the detrimental effects of CSE on the mitochondrial activity (Fig 3.12.a) and the total protein content (Fig 3.12.b) of chondrocytes on day 7. Similarly, matrix formation of CSE-impaired chondrocytes was unaffected by Ace and Dic (Fig 3.12.c).
Figure 3.12 Effects of Ace and Dic on the CSE-impaired primary human chondrocytes. On day 7 of treatment, (a) Resazurin conversion and (b) SRB staining were performed for cell viability. Matrix accumulation was quantified by (c) Alcian blue staining. Data are presented as bar diagrams (mean ± SEM); analyzed by Mann Whitney test. ****p ≤ 0.0001 vs. the untreated group, °p ≤ 0.05, °°p ≤ 0.01, °°°p ≤ 0.001 as indicated.

3.13. Evaluation of HA combined with anti-inflammatory drugs on the CSE-impaired chondrocytes

Lastly, we investigated the effects of HA combined with Dic (1 μg/ml), Ace (10 μg/ml) or low dose of Dex (4 μg/ml) on CSE-impaired chondrocytes. On day 7 of treatment, the groups of HA + Dic and HA + Ace significantly increased mitochondrial activity (Fig. 3.13.a, HA+Dic and HA+Ace, °°°p ≤ 0.001), and matrix accumulation (Fig. 3.13.c, HA+Dic and HA+Ace, °p ≤ 0.05, °°p ≤ 0.01, respectively) in CSE-impaired chondrocytes when compared with 10% CSE-exposed chondrocytes without treatment. Although HA combined with Dex did not significantly increase the mitochondrial activity and matrix accumulation in CSE-impaired chondrocytes, a slight increase trend was observed. All HA combination groups significantly promoted the total protein content (Fig. 3.13.b, HA+Dex and HA+Dic, °°p ≤ 0.01, °°°p ≤ 0.001, °°°°p ≤ 0.0001 as indicated.)
HA+Ace, p ≤ 0.001) of CSE-exposed chondrocytes, bringing it up to nearly the level of chondrocytes unexposed to CSE. The generation of ROS was suppressed by HA alone and HA combinatory treatments (Fig. 3.13.d, HA+Dex and HA+Die, p ≤ 0.01, HA+Ace, p ≤ 0.001), indicating that HA alone and HA combinatory treatments retrieved the chondrocyte impairment caused by CSE through suppressing and/or quenching the production of ROS.

Figure 3.13 Evaluation of HA combined with anti-inflammatory drugs on the CSE-impaired chondrocytes. On day 7 of treatment, (a) Resazurin conversion and (b) SRB staining were performed for cell viability. Matrix accumulation was quantified by (c) Alcian blue staining. (d) ROS production was measured by DCFH-DA assay. H2O2 (0.01% V/V) was performed as a positive control. Data are presented as bar diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 vs. the untreated group, °p ≤ 0.05, °°p ≤ 0.01, °°°p ≤ 0.001 as indicated.
Chapter 4

4. Discussion

CS is a poisonous and carcinogenic combination, which more than 5,000 chemicals and at least 55 carcinogens are involved (Talhout et al., 2011, Chen et al., 2020). Several preclinical and clinical studies have demonstrated the detrimental effects of CS in musculoskeletal disorders (Sreekumar et al., 2017, Ehnert et al., 2019, Chen et al., 2020). However, the association between CS and symptomatic OA is still unclear. Racunica et al. demonstrated that tibial cartilage volume was increased in smokers, but tibiofemoral cartilage defects were not present (Racunica et al., 2007). In a cross-sectional study, Kang et al. found that there was a weak association between indirect smoking and the prevalence of knee OA, while direct and former smoking was not associated with OA prevalence (Kang et al., 2016). On the contrary, the finding of Davies-Tuck and colleagues suggested that smoking was associated with increased medial knee cartilage loss (medial: difference=13.4 µl, P=0.03). In addition, a positive relationship between pack-year (PY) smoked and the loss of medial cartilage bulk was also observed (P=0.04) (Davies-Tuck et al., 2009). Similarly, Amin et al. found that male smokers with knee OA suffered the greater risk of cartilage loss as well as more severe pain than non-smokers (Amin et al., 2007). Nevertheless, these studies are only based on the radiographic images (MRI, X-ray) or post-operative outcome, and no direct research yet has evaluated the effects of CS on human cartilage or primary human chondrocytes. Thus, these results may be not conclusive, since the structural changes of cartilages within the smoker’s joints are still not known (Chen et al., 2020). Ying et al. (Ying et al., 2012) and Gullahorn et al. (Gullahorn et al., 2005) reported that nicotine increased proliferation, collagen synthesis, as well as gene and protein expression of chondrocytes from both normal human and OA patients. Nevertheless, cigarettes contain plenty of toxins, such as nicotine, cotinine, carbon monoxide and tar (Talhout et al., 2011). Toxins involved in tobaccos have been shown to increase oxidative stress (Kamceva et al., 2016, Chen et al., 2020), inflammatory responses (Barua et al., 2015), or hypoxia (Fricker et al., 2018), which
all could lead to cartilage damage. Recently, we have reported that CSE could damage the TGF-β signaling pathway and negatively affect migration, proliferation, and chondrogenesis of MSCs (Aspera-Werz et al., 2019). However, it is still unknown how cigarette smoke affects human cartilage and primary chondrocytes. Chondrocytes are derived from MSCs and exclusively found in AC, they possess the properties of proliferating and synthesizing ECM to maintain the functionality and integrity of AC (Sophia Fox et al., 2009, Chen et al., 2020). Chondrocytes lose their phenotype and dedifferentiate when they are expanded on conventional 2D culture surfaces (Babur et al., 2013), and this shift is activated both by morphological changes and by alteration in gene expressions (Schnabel et al., 2002). In this context, pellet culture and biomaterial-based scaffold, supplemented with or without various growth factors (TGF-β, BMP, FGF-2), were developed to avoid chondrocyte dedifferentiation (Solchaga et al., 2011, Herlofsen et al., 2011). In our previous studies, a high biocompatible knitted titanium scaffold was utilized as a pattern of the 3D cell culture method in intervertebral disc (IVD) treatment (Tendulkar et al., 2019). Thus, we compared these three different methods (monolayer culture, pellet culture, and scaffold) to establish an optimal cell culture method for chondrocytes. We found that the traditional 2D monolayer culture had the advantages of simplicity and ease of operation compared with 3D culture in our lab.

Nicotine is known as the primarily pharmacologically active and addictive component involved in smoking (Benowitz et al., 2009). The levels of nicotine in the blood plasma can reach to 100 ng/ml gradually after smoking a cigarette, but usually fluctuate between 20 and 60 ng/ml (Benowitz et al., 2009). The plasma half-life of nicotine averages about 2 h after cigarette smoking (Benowitz et al., 1988). Moreover, the concentrations of nicotine in skeletal muscle are in line with those of whole blood. In our study, the concentrations of CSE were correspond to the nicotine concentration in the cigarettes (Sreekumar et al., 2017). In addition, among “smokers,” the smoking history for patients can differ from a few cigarettes a day for a few years to packs of cigarettes daily for decades (Janjigian et al., 2010). In order to mimic this situation, we compared two different exposure pathways to chondrocytes, namely, exposing...
twice a week and once a day. We found that CSE exposure every day is more pronounced than that of twice a week.

In our study, we observed an inhibited effect of CSE on the metabolic activity of primary human chondrocytes. However, the mechanisms leading to these detrimental effects are still unknown. Thus, we subsequently attempt to investigate how CSE negatively affected chondrocytes. It is believed that oxidative stress induced by CS is one of the critical risk factors in producing adverse effects on the human body. Kamceva and co-workers reported that smoking is a key factor in promoting oxidative stress and inhibiting antioxidant defense in Coronary Artery Disease (CAD) patients, and the number of cigarettes smoked was positively associated with the level of oxidative damage (Kamceva et al., 2016). Recently, we have reported that nicotine and cotinine led to the accumulation of ROS by impairing antioxidant defense activity in bone cells, although they did not directly produce ROS (Aspera-Werz et al., 2018).

In our study, ROS production of chondrocytes was significantly elevated in culture supernatants following an exposure of 10% CSE, which corresponds to smoking approximately one pack cigarettes/day (Aspera-Werz et al., 2019, Chen et al., 2020). Considering that chondrocytes are quiescent cells and only proliferate under pathological conditions, the survival of chondrocytes is crucial for the functionality and integrity maintenance of AC (Charlier et al., 2016, Chen et al., 2020). Furthermore, the death of chondrocytes occurs during the process of OA and is connected with the accumulation of ECM (Thomas et al., 2007). Collins et al. reported that pro-death signaling pathways in chondrocytes could be initiated by increased oxidative stress, resulting in cell death and consequently compromising the integrity of AC (Collins et al., 2016). Therefore, we hypothesized that CS would induce chondrocyte cell death by increasing oxidative stress. We found that 10% CSE and 0.01% hydrogen peroxide (a principal ROS in cigarettes) equally caused a significant growth in the number of dead cells, thus linking the increased oxidative stress by CSE to the induction of cell death in chondrocytes. However, multiple modes are involved in the chondrocyte cell death, including apoptosis (Hwang and Kim, 2015), necrosis (Chen et al., 2001), autophagy (Chang et al., 2013), or a
combination of these processes (Almonte-Becerril et al., 2010). In the future, it is necessary to investigate which form of death occurs in the CSE-induced chondrocytes (Chen et al., 2020).

Vitamin C, also known as L-ascorbic acid (AA), has been extensively applied in orthopedic applications for its role in wound healing (Bikker et al., 2016), bone formation (Aghajanian et al., 2015) and chondro-protection (Huang et al., 2018). Furthermore, AA is also a robust antioxidant and cofactor, which provides protection against oxidative stress and regulates cellular development (Chiu et al., 2016). Several studies have demonstrated that AA supplementation is able to inhibit the degeneration of chondrocyte morphology and biochemistry (Chiu et al., 2016, Chang et al., 2015).

These findings suggest that AA may be a promising drug or antioxidant in protecting oxidative stress damaged chondrocyte. Furthermore, smokers showed lower AA concentrations in blood plasma than those of nonsmokers (Kelly, 2003, Smith and Hodges, 1987). Therefore, higher daily consumption of AA is required for smokers to reach the normal plasma concentration, owing to their elevated metabolic rate and defective AA recycling (Smith and Hodges, 1987, Schectman, 1993). In our study, AA inhibited the metabolism of chondrocytes co-incubated with CSE, resulting in a decreased viability and matrix formation on day 7. The concentrations of AA used in our study were lower than the peak concentration (200 μM) of AA that can be obtained by oral administration (AA 3 g every day 4 h administrated orally) (Padayatty et al., 2004), suggesting that AA supplementation was not an appropriate treatment for smokers with OA.

IA injections of CSs, such as hydrocortisone, dexamethasone (Dex), and methylprednisolone, have been frequently used in alleviating joint symptoms and inhibiting inflammation, and their efficacy has been evaluated in many preclinical and clinical trials (Zhang et al., 2008, Grodzinsky et al., 2017, Stove et al., 2002). Former studies have demonstrated the advantageous effects of Dex on suppressing pro-inflammatory cytokine accumulation, such as IL-1β, in the affected joint (Huebner et al., 2014, Chen et al., 2020). Besides, it is reported that Dex unleashes immune-suppression and anti-inflammation by means of suppressing the generation...
of ROS production (Dandona et al., 1999, Chen et al., 2020). However, the negative effects of these drugs on AC remain a concern, and many guidelines suggest that it should be used with caution (Grillet and Dequeker, 1990, Chen et al., 2020). Many preliminary studies also reported the adverse effects of Dex on cartilage integrity and chondrocyte viability (Song et al., 2012, Su et al., 1996). Our study showed that clinical doses of Dex were noxious to chondrocytes, and lower doses seemed to be nontoxic (Chen et al., 2020). Results of our present study are similar to findings by Busse et al., showing that Dex with diluted concentrations has minor effects on the viability of primary human chondrocytes (Busse et al., 2019, Chen et al., 2020). In addition, our study found that treatment with Dex for 14 days significantly increased the AP activity of primary human chondrocytes, suggesting that Dex might modify the function of chondrocyte after long-term exposure. Similarly, Stewart and colleagues reported that AP activity and AP mRNA were increased when the equine MSCs treated with Dex, resulting in cells differentiation toward bone (Stewart et al., 2008, Chen et al., 2020). In summary, the beneficial effects of IA injection of Dex occur at low doses and short-time treatment duration. Therefore, for clinical application in smokers with OA, several factors such as indication, dose, and treatment duration should be taken into consideration.

HA, also called hyaluronan, is a form of polyanionic, nonsulfated glycosaminoglycan with high molecular weight (Sirin et al., 2018). In the clinic, it is used with IA injection to improve the functional mobility of pathologic OA joints, since HA is capable of improving viscoelastic properties to the synovial liquid (Temple-Wong et al., 2016, Chen et al., 2020). In addition to its role in viscosupplementation, an important biological aspect of HA is its benefits to the chondrocytes, such as chondro-protection (Akmal M, 2005), scavenging of reactive oxygen-derived free radicals (Yu et al., 2014) and inhibition of inflammation (Fioravanti et al., 2005). The volume of synovial fluid in most of the knee joints is around 0.5-4.0 ml, and the pH is between 7 and 8 (Kraus et al., 2007). In general, IA injections of HA (2-3 ml) with a concentration of 10 mg/ml are used in treating the affected joints (Concoff et al., 2017, Chen et al., 2020). According to that, the dilution of HA with synovial fluid is
approximately 1:2 (Chen et al., 2020). In our study, high doses of HA (2.5 mg/ml and 5.0 mg/ml) promoted the viability and matrix accumulation of CSE-impaired chondrocytes. On the contrary, Akmal et al. found that HA had a beneficial effect on the metabolic activity of bovine articular chondrocytes in low doses (0.1 mg/ml and 1.0 mg/ml) (Akmal M, 2005, Chen et al., 2020). It is likely that CSE down-regulated the expression of CD44 (a HA receptor at the chondrocyte cell surface) and limited the interaction of chondrocytes with their surrounding ECM (Responte et al., 2012), resulting in a decreased cell response to HA (Chen et al., 2020).

In the past decades, oral NSAIDs or Ace have been widely used for relieving pain in OA patients (Ringdahl E, 2011, Sostres et al., 2010) and recommended as the first-line pain medication for OA by most guidelines (Hunter and Bierma-Zeinstra, 2019, Dougados, 2006). However, limited delivery to the inflamed joints (Wongrakpanich et al., 2018) and side effects regarding gastrointestinal and cardiovascular complications of oral administration (Sostres et al., 2010, McGill and Jaeschke, 2014) restrict their efficacy and application. These limitations have hence led to the emergence of IA injections of these drugs, which proved to be effective in suppressing inflammation and alleviating pain in joints (Arun et al., 2013, Cannava et al., 2013, Mertz et al., 2016). Considering OA is a chronic disabling disease, pharmacological treatments would require a prolonged time-span (Chen et al., 2020). Additionally, the nature of short biological half-life of Ace (approx 3 h) (Hodgman and Garrard, 2012) and Dic (approx 2 h) (Miyatake et al., 2009) requires frequent injections to obtain effective concentration (Chen et al., 2020). Therefore, we used these drugs with identical therapeutic levels observed in plasma and exposed primary human chondrocytes to them every day. We found that therapeutic doses of Dic (1 μg/ml) and Ace (10 μg/ml) did not augment the detrimental effects of CSE on the overall metabolism of chondrocytes (viability, proliferation, and matrix accumulation). Similarly, in the findings of Blot et al., 0.3–3 μg/ml of Dic unaffected the synthesis of proteoglycan and HA within the cartilages of moderate and severe OA (Blot et al., 2000). Qi et al. and Arun et al. found that IA injection of Dic had analgesic and anti-inflammatory effects on rats (Qi et al., 2016, Arun et al., 2013), indicating their
 possibility for IA injection. Accordingly, IA injections of NSAIDs and Ace would be a promising alternative for Dex and might delay joint deterioration in smokers with OA. It is critical to note that OA is a chronic disease that affects the whole joint, involving cartilage breakdown, inflammation, and osteophytes formation (Hunter and Bierma-Zeinstra, 2019, Chen et al., 2020). Therefore, in addition to pain alleviation and inflammation inhibition should be addressed, another important aspect is cartilage repair (Chen et al., 2020). In recent, several studies evaluated the effects of HA combined with anti-inflammatory drugs (CSs or NSAIDs) (Siengdee et al., 2015, Euppayo et al., 2017), for the sake of developing more effective OA treatments (Chen et al., 2020). Euppayo et al. compared the efficacy of IA injection of HA with or without anti-inflammatory (AI) drugs (CS or NSAIDs) in OA patients, and found that HA combined with AI had greater efficacy than HA alone in terms of pain alleviation (Euppayo et al., 2017). We investigated the effects of HA combined with different anti-inflammatory drugs (Ace, Dic, and Dex) on CSE-impaired chondrocytes. Our results demonstrated that HA combined with Dic, Ace, or low doses of Dex had a protective effect on the CSE-exposed chondrocytes, as they significantly inhibited the generation of free radicals and promoted the viability and ECM accumulation of cells. It is reported that synovial fluid levels of ROS (H$_2$O$_2$ and O$_2^-$) and H$_2$O$_2$-induced chondrocyte cell death could be suppressed by IA injection of HA (Yu et al., 2014). Our present study shows that an inhibition of ROS production with HA or its combinations with anti-inflammatory drugs, exhibiting chondro-protective effects by scavenging the generation of free radicals (Chen et al., 2020). In addition, the detrimental effects of cigarette smoke on chondrocytes in vitro are, at least partially, mitigated to the in vivo situation. Therefore, further studies are needed to elucidate whether the metabolic changes of cells in vitro would also appear in articular cartilage (Chen et al., 2020). Moreover, further in vivo work should be performed to determine the optimal drug dose and frequency before clinical application (Chen et al., 2020). In conclusion, the data presented herein are the first time to evaluate the effects of CSE on the metabolisms of primary human chondrocytes. CSE inhibited
chondrocytes viability, ECM accumulation, as well as it decreases their anabolic gene expression (Collagen II, Aggrecan, Sox9). Oxidative stress was associated with CSE and generated when chondrocytes were exposed to it. Increased oxidative stress induced chondrocyte cell death. Dex had a dose- and time- dependent negative effect on primary human chondrocytes, however, with favorable effects occurring at low doses (< 400 μg/ml) and short treatment intervals. Clinical dose (4 mg/ml) of Dex was toxic to the cells, and long-term duration would modify the function and morphology of cartilage. In contrast to Dex, therapeutic doses of Dic (1 μg/ml) and Ace (10 μg/ml) did not augment the detrimental effects of CSE on the overall metabolisms of chondrocytes. Additionally, a clinical dose of HA (5 mg/ml) or HA combined with Dic, Ace, or low doses of Dex had a protective effect on the CSE-exposed chondrocytes, as they significantly inhibited the generation of free radicals and promoted the viability and ECM accumulation in cell cultures.
Chapter 5

5. Summary

Although the adverse effects of smoking for human musculoskeletal system have been well accepted, less attention has been paid by researchers to the relevance of cigarette smoke to the onset of osteoarthritis (OA). Here, we investigated the effects of cigarette smoke extract (CSE) on human primary chondrocyte function. In addition, we investigated whether the pharmacologic treatment of dexamethasone was beneficial to chondrocytes impaired by CSE, and if not, whether it could be substituted by other treatments, such as acetaminophen and NSAIDs. Finally, we evaluated the effects of hyaluronic acid (HA) and HA combinatory treatments (dexamethasone, acetaminophen or diclofenac) on the chondrocytes impaired by CSE, in order to determine a potential therapeutic alternative for clinical application to smokers undergoing symptomatic OA.

All human tissues were obtained in accordance with the ethical approval of the University Hospital Tübingen and with patients’ written consent. Human primary chondrocytes were exposed to increasing concentrations (0%, 0.1%, 0.5%, 1%, 5%, 10%) of CSE (containing 3.6 ng/ml to 72 ng/ml nicotine and 40 ng/ml to 800 ng/ml tar). Cell viability was analyzed by resazurin conversion assay and SRB staining, matrix formation was stained using Alcian blue and Safranin-O staining. The generation of free radical was evaluated by DCFH-DA assay. Semi-quantitative RT-PCR was performed to analyze gene expressions.

Our present study demonstrated that the mitochondrial activity, total protein content and the accumulation of matrix were dose- and time-dependently inhibited by CSE in primary human chondrocytes. Moreover, increased oxidative stress led to cell death by 10% CSE, which is associated with approximately smoking one pack a day. As an anti-inflammatory treatment strategy, traditional pharmacologic therapy with dexamethasone (Dex) was evaluated. Clinical doses of Dex were toxic to the cells, and long-time incubation with lower doses (4–400 μg/ml) of Dex would lead to a hypertrophic chondrocyte phenotype. To substitute dexamethasone, a clinical dosage
of diclofenac (Dic) and acetaminophen (Ace) was tested on chondrocytes. Interestingly, therapeutic doses of Dic (1 \( \mu \text{g/ml} \)) and Ace (10 \( \mu \text{g/ml} \)) did not augment the detrimental effects of CSE on the overall metabolisms of chondrocytes. Additionally, a clinical dose of HA (5 mg/ml) and/or HA combined with Dic, Ace, or doses of Dex had protective effects on the CSE-exposed chondrocytes, as they significantly inhibited or trap the generation of free radical and promoted the viability and ECM accumulation of cells. Our study demonstrates that cigarette smoke induces cell death through elevating oxidative stress and demolishes cartilage formation. Intra-articular (IA) injection of HA combined with therapeutic doses of analgesic/anti-inflammatory agents (Ace or Dic) could reverse the detrimental effects of CSE on primary human chondrocytes, thus opening up potential therapeutic alternatives in treating smokers to suffering from symptomatic OA.
6. Zusammenfassung


Humanes Knorpelgewebe, welches im Rahmen von Routineoperationen entnommen werden musste, wurde nach ethischer Genehmigung der Etikkommission des Universitätsklinikums Tübingen und mit schriftlicher Zustimmung des Patienten für die Isolation der Chondrozyten genutzt. Humane primäre Chondrozyten wurden physiologisch erreichbaren CSE Konzentrationen (0%, 0,1%, 0,5%, 1%, 5%, 10%) ausgesetzt, was Nikotinkonzentrationen von 3,6 ng / ml bis 72 ng / ml sowie Teerkonzentrationen von 40 ng / ml bis 800 ng / ml entspricht. Die Viabilität der Zellen wurde durch die Messung des Resazurinumsatzes sowie durch eine SRB-Färbung analysiert, die Matrixbildung wurde unter Verwendung von Alcianblau- und Safranin-O-Färbung gefärbt. Die Produktion freier Radikale wurde mit einem DCFH-DA-Assay bewertet. Die Genanalyse wurde mittels semi-quantitativer RT-PCR durchgeführt.

Unsere vorliegende Studie zeigte, dass die mitochondriale Aktivität, der Gesamtproteingehalt und die Akkumulation von Matrix durch CSE in primären menschlichen Chondrozyten dosis- und zeitabhängig gehemmt wurden. Darüber hinaus führte erhöhter oxidativer Stress zu einem Zelltod von 10% CSE, was mit
7. Bibliography


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8. Declaration

This work was carried out in the Siegfried Weller Institute (SWI) for trauma research under the supervision of Professor Dr. A.K. Nüssler.

The conception of this work was proposed by Professor Dr. A.K. Nüssler and Dr. Sabrina Ehnert. The experiments were designed by Dr. Romina H. Aspera-Werz and me. All experiments were performed by myself and all data was analyzed by myself.

I declare that all the results are from my own research data, except for the quoted references and figures. Also, all the figures from the website or other papers have permission licenses.

I hereby declare that the submitted doctoral dissertation entitled: “Potential therapeutic alternatives for smokers with osteoarthritis – an in vitro study for preclinical application” was written by myself independently. I am aware that false declarations or plagiarism would be punished, so I declare that these statements are true and that I have concealed nothing.

_____________________________________________________

Place/date/signature of doctoral candidate
9. Publication

Results of this thesis were partially used for publication:

Title: Primary Human Chondrocytes Affected by Cigarette Smoke—Therapeutic Challenges

Author: Chen T, Ehnert S, Tendulkar G, Zhu S, Arnscheidt C, Aspera-Werz RH, Nüssler AK.

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11. Curriculum vitae – Tao Chen

Education:

2010-2017    **Degree:** Seven-year program including B.S.M. & M.Sc.
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2015-2017    **Degree:** Master
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**University:** Eberhard Karls University Tuebingen

Extracurricular activities:

- Poster presentation in BGU Doktorandentag, 2019.
- Oral presentation in International conference on biomaterial-based therapeutic engineering and regenerative medicine, India, 2019.

Publication:


Research experience:


Clinical experience:

2014.09-2015.07 Internship in the First Affiliated Hospital of Zhengzhou University
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