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

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Tao Chen

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Dean: Professor Dr. B. J. Pichler
First reviewer: Professor Dr. A. Nüssler
Second reviewer: Privatdozent Dr. F. Medved

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To my parents

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List of abbreviations

AA	Ascorbic acid
AC	Articular cartilage
Ace	Acetaminophen
ACI	Autologous Chondrocyte Implantation
AP	Alkaline Phosphatase
BMD	Bone mineral density
CAD	Coronary Artery Disease
COPD	Chronic obstructive pulmonarydisease
CS	Cigarette smoke
CSE	Cigarette smoke extract
CSs	Corticosteroids
CVD	Cardiovascular disease
DCFH-DA	Dichlorfluorescein-diacetate
DEPC	Diethylpyrocarbonate
Dex	Dexamethasone
DFO	Distal femoral osteotomy
Dic	Diclofenac
DMMB	Dimethyl-Methylene Blue
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular matrix
EDTA	Ethylene Diamine Tetraacetatic Acid
FCS	Fetal Calf Serum
GAG	Glycosaminoglycan
GBD	Global Burden of Disease
H_2O_2	Hydrogen peroxide
HA	Hyaluronic acid
HCl	Hydrogen Chloride
HTO	High tibial osteotomy
IA	Intra-articular
IL	Interleukin
KL	Kellgren and Lawrence
MMPs	Matrix metalloproteinases
MRI	Magnetic Resonance Imaging
MSC	Mesenchymal stem cell
MSS	Mainstream smoke
NaOH	Sodium Hydroxide
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis

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).

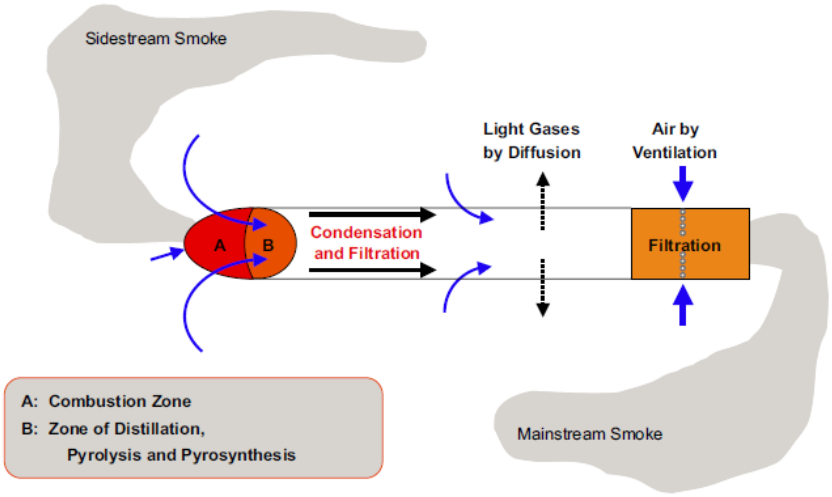


Figure 1.1 The sidestream smoke and mainstream smoke of a burning cigarette. **(**Reproduced with permission from (Thielen *et al*., 2008).

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.

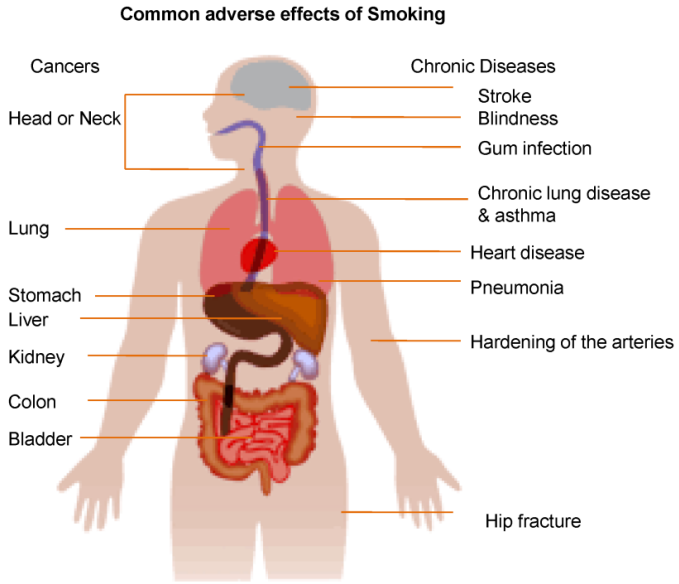


Figure 1.2 The effects of smoking on the human body. Smoking can damage every part of the body.

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).

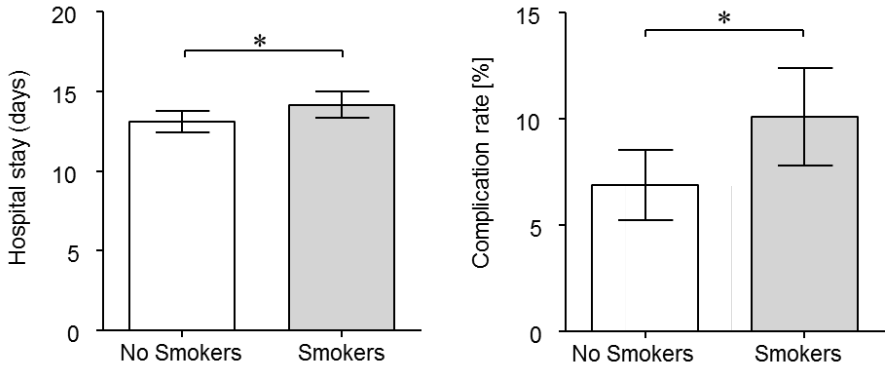


Figure 1.3 Effect of smoking on the hospital stay and surgical complication during fracture healing. Data from 1585 patients of the BG Unfallklinik Tübingen shows smokers encounter significantly more complications and longer hospital stays than non-smokers (*$p < 0.05$) **(**Reproduced with permission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

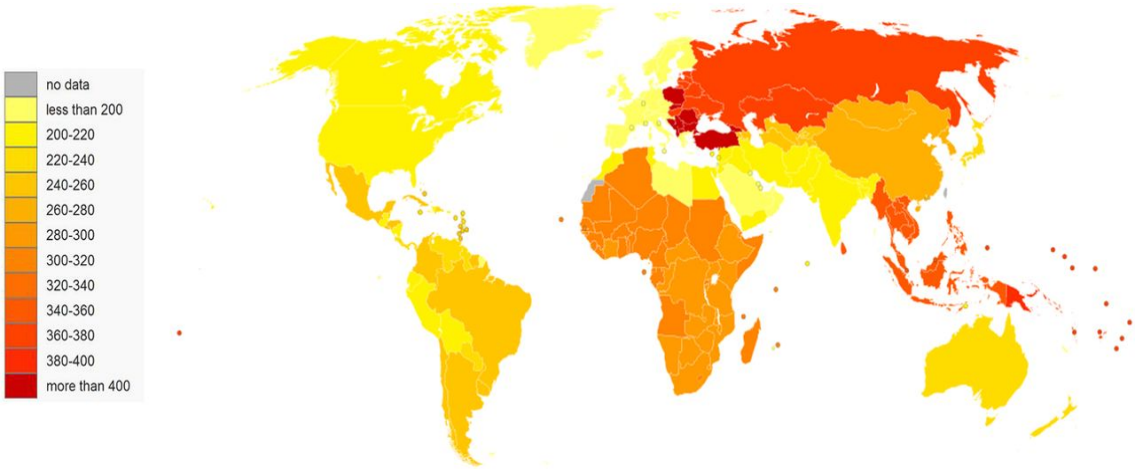


Figure 1.4 OA epidemiology and demographics. Age-standardized disability-adjusted life year rates for osteoarthritis by country (per 100,000 inhabitants). (Reproduced with permission from (El-Tawil *et al*., 2016).

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 aggrecan, 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).

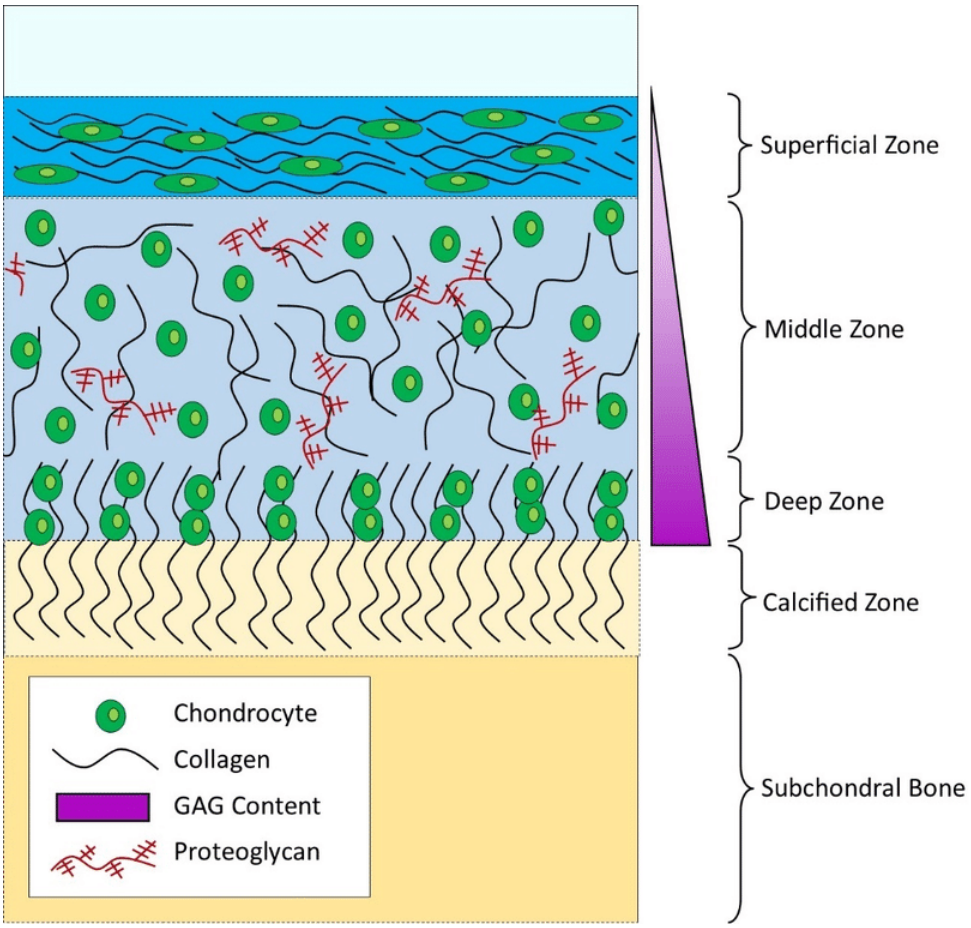


Figure 1.5 The structure of AC and underlying subchondral bone. There are four zones from the cartilage surface to the bone: the superficial, middle, deep and calcified zones. (Reproduced with permission, from (Davies and Kuiper, 2019).

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 (Troeberg 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).

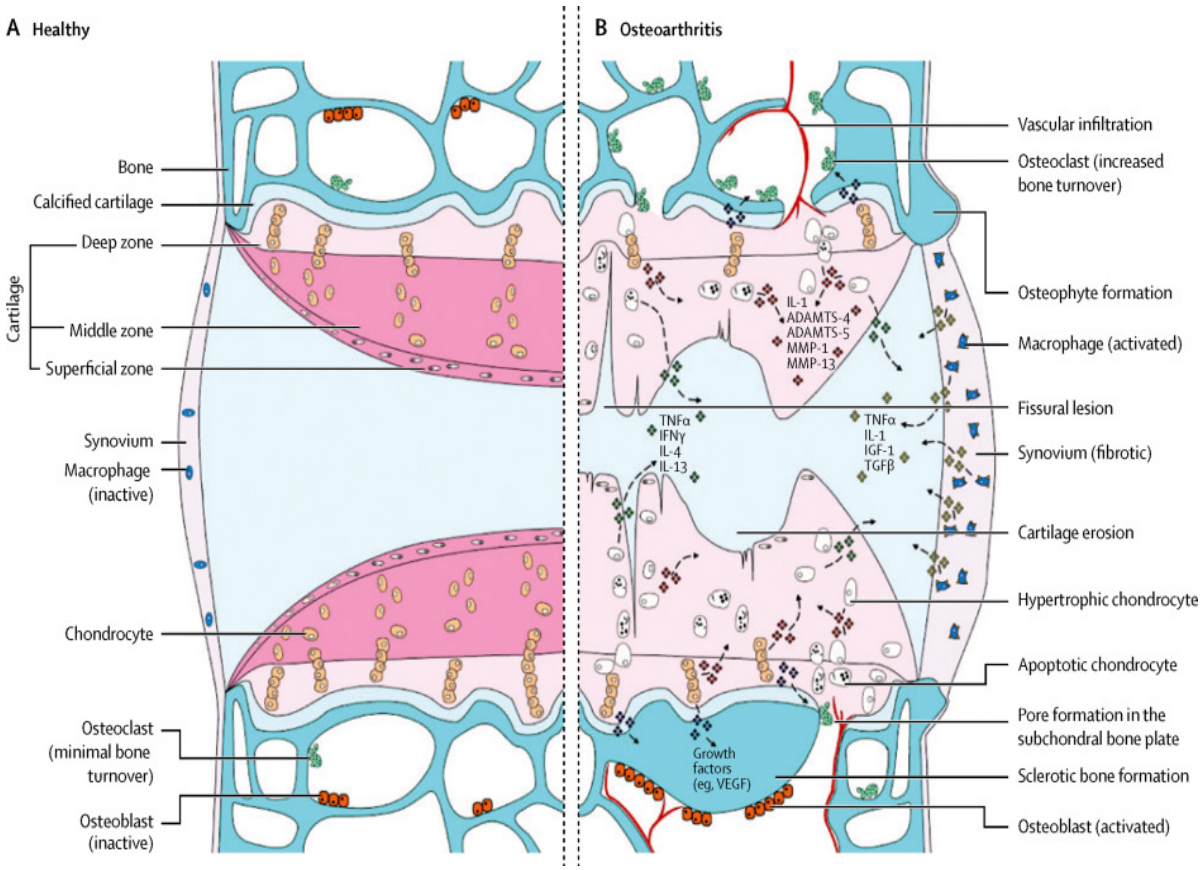


Figure 1.6 Signaling pathways and structural changes in the development of OA. **(**Reproduced with permission from (Hunter and Bierma-Zeinstra, 2019).

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).

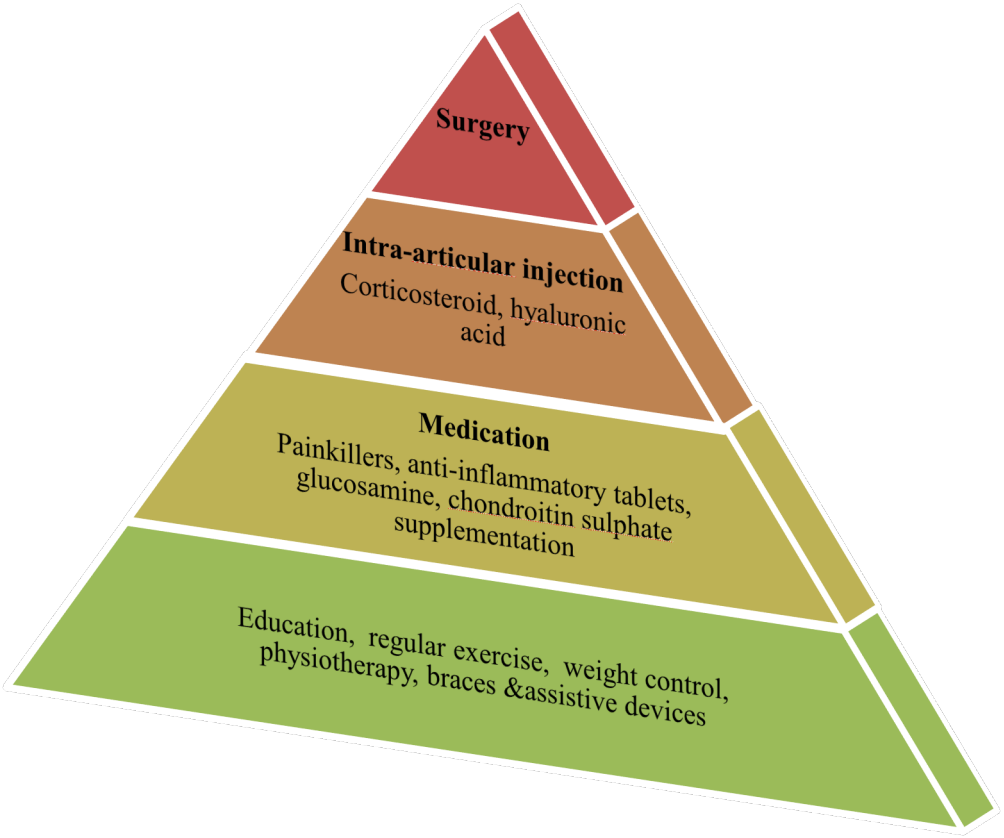


Figure 1.7 Ladder of treatment for knee OA. Treatment strategy varies from the mild (bottom), moderate to the advanced (top).

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 demolishment 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 cm^2 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 reimplanted 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).

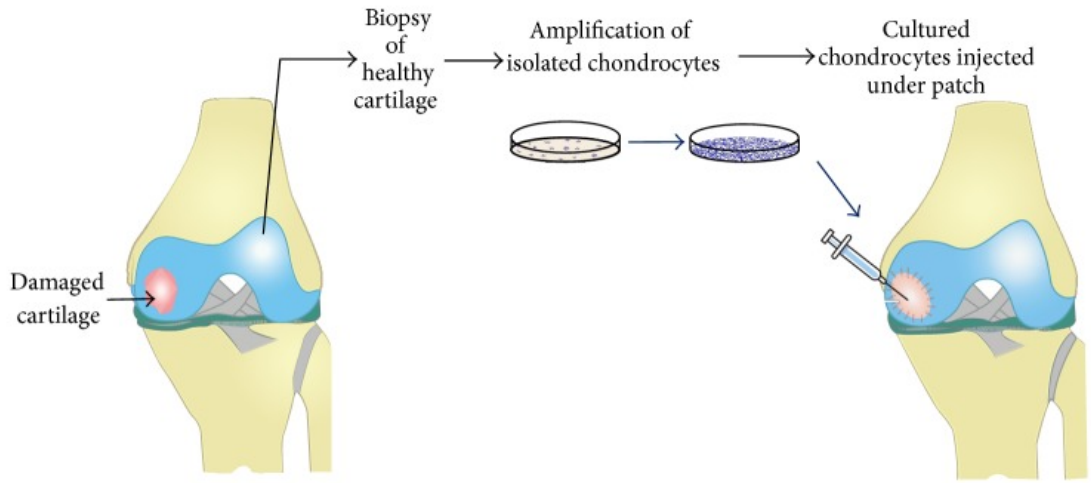


Figure 1.8 Schematic illustration of ACI. The procedure consists of two steps: (1) cartilage harvested from non-weight-loading regions and cultured in *in vitro* environment; and (2) reimplantation of the cells by injecting them into the lesion. **(**Reproduced with permission from (Bauge and Boumediene,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

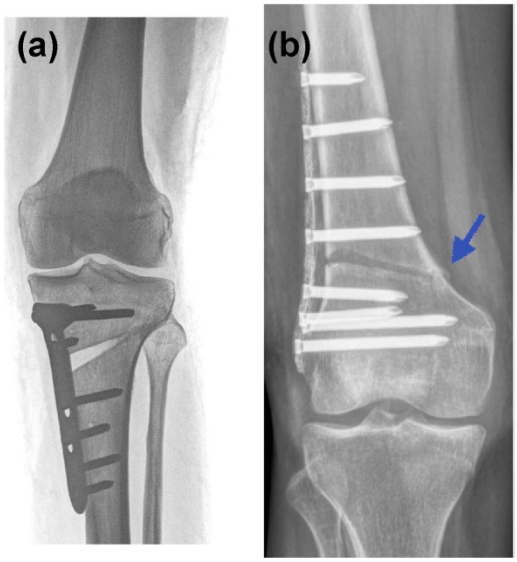


Figure 1.9 Postoperative X-ray images of HTO and DFO. (a) Open-wedge HTO in unicompartmental OA of the medial knee compartment (Lee and Byun, 2012) and (b) open-wedge DFO in unicompartmental OA of the lateral knee compartment. **(**Reproduced with permission from (Feucht *et al.*, 2017).

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).

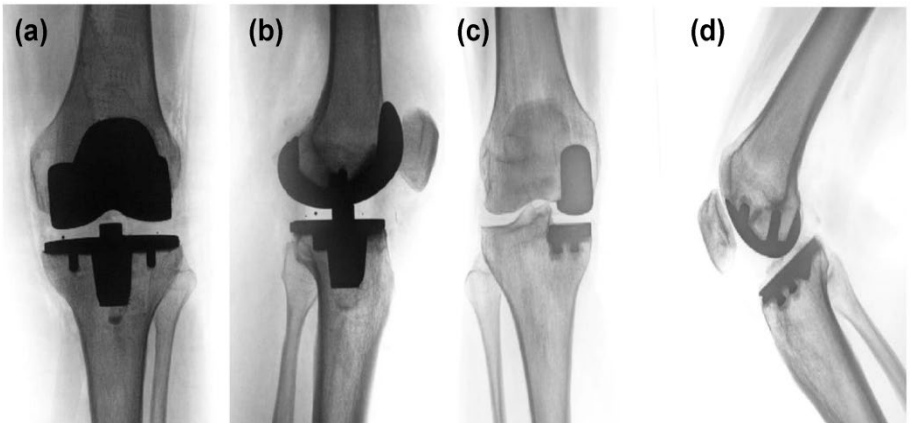


Figure 1.10 Plain radiographs of the TKA and UKA. (a) Anteroposterior (AP) view after the TKA surgery. (b) Lateral view after the TKA surgery. (c) AP view after the UKA surgery. (d) Lateral view after the UKA surgery(Reproduced with permission from (Ronn *et al*., 2011).

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.

Chapter 2

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and reagents

Table 2.1: List of used chemicals and reagents.

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

Glycine	3908.2	Carl Roth
Glycine, >99%, p.a.	A1067.5000	AppliChem
Guanidine Hydrochloride	G3272	Sigma
Guanidine Thiocyanate	0017.1	Carl Roth
Hoechst 33342	14533-100MG	Sigma
Hyaluronic Acid Sodium	53747	Sigma
Hydrogen Chloride (HCl)	4625.2	Roth
Isopropanol 100%	20842.33	VWR
Isopropanol 100%	33539	Honeywell
L-Ascorbate-2-Phosphate	A8960-5G	Sigma
Magnesium Chloride	KK36.2	Carl Roth
Methanol >99% (Met OH)	20847.307	VWR
N-Acetyl-L-Cyteine	4126.2	Carl Roth
Papain from papaya latex	P4762	Sigma
Paraformaldehyde	335.2	Carl Roth
Penicillin/Streptomycin (P/S)	P0781	Sigma
Resazurin Sodium Salt	199303-1G	Sigma
Roti Aqua Phenol	A980.3	Carl Roth
Safranin-O	T129.1	Carl Roth
Sodium Acetate	X891.2	Carl Roth
Sodium Chloride	S7653-1KG	Sigma
Sodium Chloride	S7653	Sigma
Sodium Ethylene Diamine Tetraacetatic Acid (Na-EDTA)	8043.2	Roth
Sodium Hydroxide (NaOH)	T135.1	Carl Roth
Sulforhodamine B Sodium Salt	S1402-1G	Sigma
Trisamine (Tris) Base, >99%, p.a.	T1503-1KG	Sigma
Trisaminomethan	AE15.1	Roth
TrizolQIAzol Lysis Reagent	79306	Qiagen
Trypan Blue	CN76.1	Roth
Trypsin/EDTA	T3924	Sigma

2.1.2. Buffers, medium and solutions

Table 2.2: List of buffers, medium and solutions.

Buffers/Mediums/Solutions	**Compounds and handling**
Calcein AM stock solution	502 µl DMSO 1 mg Calcein AM
Trypan blue solution	62.5 mg Trypan blue 50 ml Dulbecco's PBS
Alcian blue solution (1%, PH 2.5)	500 mg Alcian blue (8 GX) 50 ml Acetic Acid (3%)
Guanidine Hydrochloride (6 M)	28.65 mg Guanidine Hydrochloride 50 ml ddH_2O

Safranin-O solution (0.1%)	50 mg Safranin-O 50 ml ddH_2O
Formaldehyde (4%)	Formaldehyde (37%) in ddH_2O
AP Activity Assay buffer (PH 10.5)	2 g 4-nitrophenyl-phosphate (0.2%) 3.75 g Glycine (50 mM) 12.11 g Tris-Base (100 mM) 95.21 mg $MgCl_2$ (1 mM) 1 L ddH_2O
PCR loading buffer	25 mg Bromophenol blue 5 ml 10X TBE 5 ml Glycerol (20%)
10 X TBE buffer	108 g TRIS 55 g Boric acid 40 ml EDTA (0.5 M, PH 8) 1 L ddH_2O
Ethanol solution (70%)	99% Ethanol in ddH_2O
Sodium Acetate solution (3M, PH 5)	12.3 g Sodium Acetate in 50 ml ddH_2O
Acetic Acid Solution (1%)	100% acetic acid in ddH_2O
Acetic Acid Solution (3%)	100% acetic acid in ddH_2O
TRIS Solution (10 mM)	1.2g TRIS in 1L ddH_2O
SRB Solution	0.4% SRB in 1% acetic acid
Resazurin stock solution	0.025% in DPBS
Resazurin working solution	10% Resazurin stock solution in DPBS
PBE Buffer (PH 6.5)	6.5 mg N-Acetyl-L-Cyteine 138 mg Disodium hydrogen phosphate 14.9 mg EDTA Adjust PH to 6.5 with NaOH Adjust volume to 20 ml with ddH_2O
Papain stock solution (5 mg/ml)	5 mg papain from papaya latex 1 ml PBE buffer
Papain working solution (25 µg/ml)	5 mg/ml stock solution in PBE buffer
DMMB solution buffer (PH 3)	304 mg Glycine 160 mg sodium chloride 9.5 ml 0.1 M Acetic acid 90.5 ml ddH_2O
DMMB stock solution (8 mg/ml)	8 mg DMMB in 1 ml buffer
DMMB working solution (16 µg/ml)	8 mg/ml stock solution in DMMB buffer

Chondroitin Sulfate Standards stock solution (1 mg/ml)	10 mg Chondroitin Sulfate Standards in 10 mlPBE buffer
Chondroitin Sulfate Standards working solution (100 µg/ml)	1 mg/ml stock solution in PBE buffer
TE buffer (PH 7.5-8.0)	10 mM Tris base 1 mM EDTA
Calf Thymus DNA stock solution (1 mg/ml)	1 mg Calf Thymus DNA in 1 ml TE buffer
TNE buffer (PH 7.4)	121.1 mg Tris Base 37.2 mg EDTA 1.17 g sodium chloride Adjust PH to 7.4 with HCl Adjust volume to 100 ml with ddH_2O
Chondrocyte Cells Culture Medium	500 ml DMEM +500 ml Ham's F12 +50 ml FCS +10 ml Penicillin/Streptomycin +50 µl L-Ascorbic-2-Phosphate

2.1.3. Consumables

Table 2.3: List of consumables

Consumable	Manufacturer	Type	Serial number
Cell culture plate	Greiner bio-one	96-well,flat bottom	655180
Cell culture plate	Greiner bio-one	96-well, V bottom	651101
Cell culture plate	CorningInc.	48-well,flat bottom	3548
Cell culture plate	Greiner bio-one	24-well,flat bottom	662160
Cell culture plate	Corning Inc.	6-well, flat bottom	353046
Cell Star Tubes	Greiner bio-one	50 ml	227261
Cell Star Tubes	Greiner bio-one	15 ml	188271
Eppendorf tube	SARSTEDT AG	0.5 ml, white	72.699

Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, white	4182.1
Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, blue	4190.1
Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, green	4209.1
Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, red	4189.1
Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, yellow	4204.1
Eppendorf tube	Eppendorf	2.0 ml, white	2549
Pipette Tips	Sorenson BioScience, Inc.	0.1 - 10 µl	Colorless
Pipette Tips	Sarstedt AG & Co.	2 - 200 µl	Yellow
Pipette Tips	Ratiolab GmbH	100 - 1000 µl	Blue
Single-channel Pipette	Corning Inc.	10-100 µl	158240031
Single-channel Pipette	Corning Inc.	20-200 µl	158250088
Single-channel Pipette	Corning Inc.	100-1000 µl	058261237
Single-channel Pipette	Eppendorf	0.1-2.5 µl	P35434B
Spectrophotometer	BMG Labtech GmbH	Fluostar Omega	415-1264
Water-bath	Lauder Dr. R. Wobser GmbH	Al 25	LCB 0727-11-0094
Water-bath	Lauder Dr. R. Wobser GmbH	ECO ET 20	LY 06.1

2.1.4. Equipment

2.1.4. Equipment

Table 2.4: List of used equipment.

Equipment	Manufacturer	Type	Serial number
Agitator, magnetic stirrer	IKA-Werke GmbH	RH B2	06.050357
Agitator, magnetic stirrer	Heidolph Instruments GmbH	MR Hei-Mix L	040700340
Centrifuge	Dako Deutschland GmbH	Stat Spin	620E50000693
Centrifuge	Thermo Fisher Scientific	Megafuge 40 R	41307652
Centrifuge	Scientific Industries Inc.	SI DD 58	DD58-1001
Centrifuge (Mirco)	Labnet International	BN 08060235	C1301B
Centrifuge (Mirco)	HERAEUS Med GmbH	Fresco 17	41250019
Electrophoresispower supplies	Bio-Rad Laboratories GmbH	Power Pac 200	285BR05538
Freezer -20°C	BSH	IQ500	GS51NYW41 (01)

Freezer -20℃	Liebherr	Med Line	LGex3410-21K 001
Freezer -80 ℃	Thermo Fisher Scientific	905	827860-2521
Freezer -86 ℃	Revco	ULT1386-9-V17	R10G-333095-RG
Fridge +4 ℃	Liebherr	Comfort	3523-21L
Fridge +4 ℃	Cool Compact Kühlgeräte G	HKMT 040-01	CC00412514
Gas washing bottle	Lenz Laborglass GmbH & Co.KG	100 ml	5500537
Gas washing bottle	Lenz Laborglass GmbH & Co.KG	250 ml	5500549
Ice maker	Scotsmen	AF 80	DD 8837 11 X
Incubator	Thermo Fisher Scientific	Heratherm OMS 60	41296334
Incubator	Binder GmbH	9040-0078	11-22649
Incubator	Binder GmbH	9040-0081	11-22190
Laboratory pump (Bench)	Carl Roth GmbH + Co.KG	Cyclo 2	1109-065
Microscope	PeqlabBiotechnologie GmbH	EVOS-fl	91-AF-4301
Mixer	Corning Inc.	Vortex Mixer	804995
Mixer	Labinco BV	LD-76	76000
Multichannel Pipette	Corning Inc.	5-50 µl	151620022
Multichannel Pipette	Corning Inc.	20-200 µl	551630277
Multichannel Pipette	Thermo Electron Co.	0.5-10 µl	CH98998 4510
Multichannel Pipette	Corning Inc.	50-300 µl	151640033
PCR thermal cyclers	Thermo Fisher Scientific	Arktik	10040953
PCR thermal cyclers	Applied Biosystems GmbH	Forschungslabor	50132
pH meter	Mettler-Toledo GmbH	Five Easy FE 20	1232315296
Pipette controller	Integra GmbH	Pipetboyacu	629619
Pipette controller	Heathrow Scientific LLC	Rota-Filler 3000	HSA05119
Refrigerator	Cool Compact Kühlgeräte G	HKMT 040-01	CC 00412516
Refrigerator	Cool Compact Kühlgeräte G	HKMN 062-01	CC 00412513
Safety workbench	Thermo Fisher Scientific	Maxisave S20201.8	41293949
Safety workbench	Thermo Fisher Scientific	Maxisave S20201.8	41293948
Scale	Kern &Sohn GmbH	ABJ 120-4M	WB 1140084

Shaker, laboratory	LTF Labortechnik GmbH	DRS 12	11DE243
Shaker, laboratory	PeqlabBiotechnologie GmbH	ES-20	010111-1107-0119
Shaker, laboratory	LTF Labortechnik GmbH	DRS 12	11DE090
Shaker, Laboratory	Corning Inc.	LSE Vortex Mixer	1101260
Single-channel Pipette	Corning Inc.	0.5-10 µl	158220060
Single-channel Pipette	Corning Inc.	2-20 µl	158230441
Single-channel Pipette	Corning Inc.	10-100 µl	158240031
Single-channel Pipette	Corning Inc.	20-200 µl	158250088
Single-channel Pipette	Corning Inc.	100-1000 µl	058261237
Single-channel Pipette	Eppendorf	0.1-2.5 µl	P35434B
Spectrophotometer	BMG Labtech GmbH	Fluostar Omega	415-1264
Water-bath	Lauder Dr. R. Wobser GmbH	Al 25	LCB 0727-11-0094
Water-bath	Lauder Dr. R. Wobser GmbH	ECO ET 20	LY 06.1

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 λ = 320 nm (OD_{320}). An OD_{320} of 0.7 was regarded as 100% CSE. The fresh CSE was passed through a 0.22 µm 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 ℃ 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 λ = 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 λ = 530 nm and λ = 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 decribed 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% *V/V* formaldehyde for 30 min at RT followed by a washing step in PBS. Subsequently, cells were labeled with 1% *w/v* Alcian blue and 0.1% *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 / OD_{405}) 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.

Gene	Accession Number	Forward primer (5′–3′)	Reverse primer (3′–5′)	Product length (bp)	Annealing temperature (℃)	Cycles （N°）
Aggrecan	NM 001135.3	CTTGGACTTGGGCAAACTGC	CACTAAAGTCAGGCAGGCCA	143	60	35
Collagen II	NM_001844.4	TGGATGCCACACTCAAGTCC	GCTGCTCCACCAGTTCTTCT	254	60	35
SOX9	NM_000346.3	GAAGGACCACCCGGATTACA	GCCTTGAAGATGGCGTTGG	120	60	35
Collagen X	NM_000493.3	AAACCTGGACAACAGGGACC	CGACCAGGAGCACCATATCC	124	60	35
GAPDH	NM_002046.4	GTCAGTGGTGGACCTGACCT	AGGGGTCTACATGGCAACTG	420	56	30

Statistics

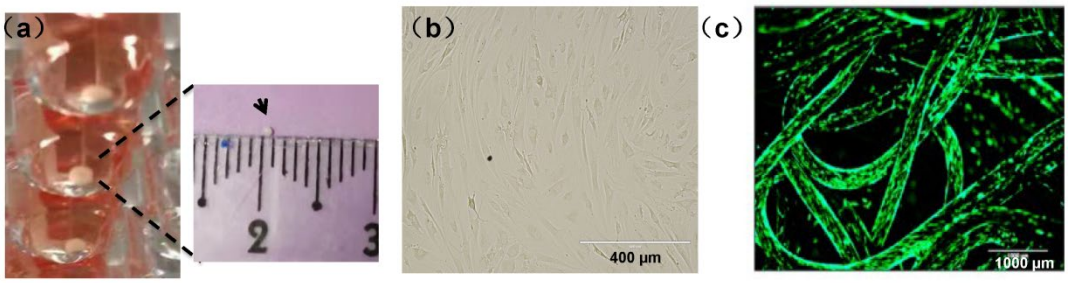
Graph Pad Prism software (El Camino Real, CA, USA) was used for statistical analyses. Results are expressed as bar or line diagrams (mean ± SEM). All the experiments were performed at least 3 times (N≥3) and measured as triplicates or more (n≥3). Data were analyzed by Mann–Whitney U-test or Kruskal–Wallis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



(d)

	Methods	Pellet	Monolayer	Scaffold
Viability	Resazurin	x	√	√
	SRB	x	√	x
	Live/Dead	x	√	√
Matrix formation	IHC	x	√	x
	Alcian blue	√	√	x
	Safranin-O	√	√	x
Function	GAG content	√	√	√
	DNA content	√	√	√
	PCR (COL II、X、SOX9、ACAN)	√	√	√

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).

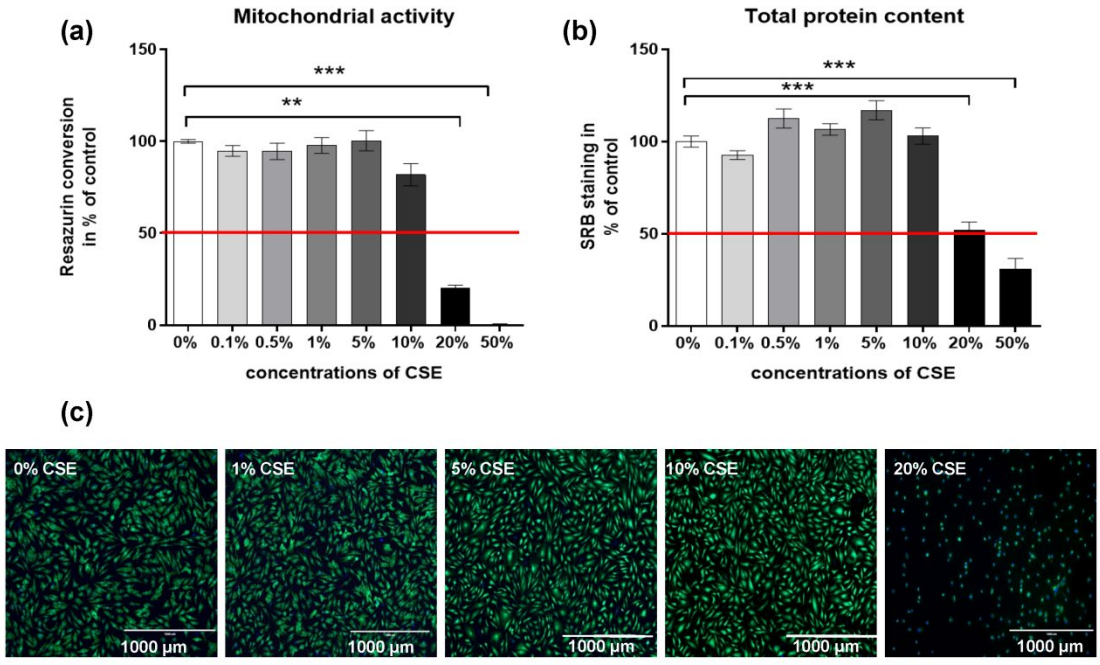


Figure 3.2 CSE exposure induces the reduction in viability of primary human chondrocytes after 24 h. After 24 h, **(a)** Resazurin conversion and **(b)** SRB staining were performed for cell viability. **(c).** Representative microscopic images for Calcein-AM staining. Cells were visualized with Calcein-AM (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.

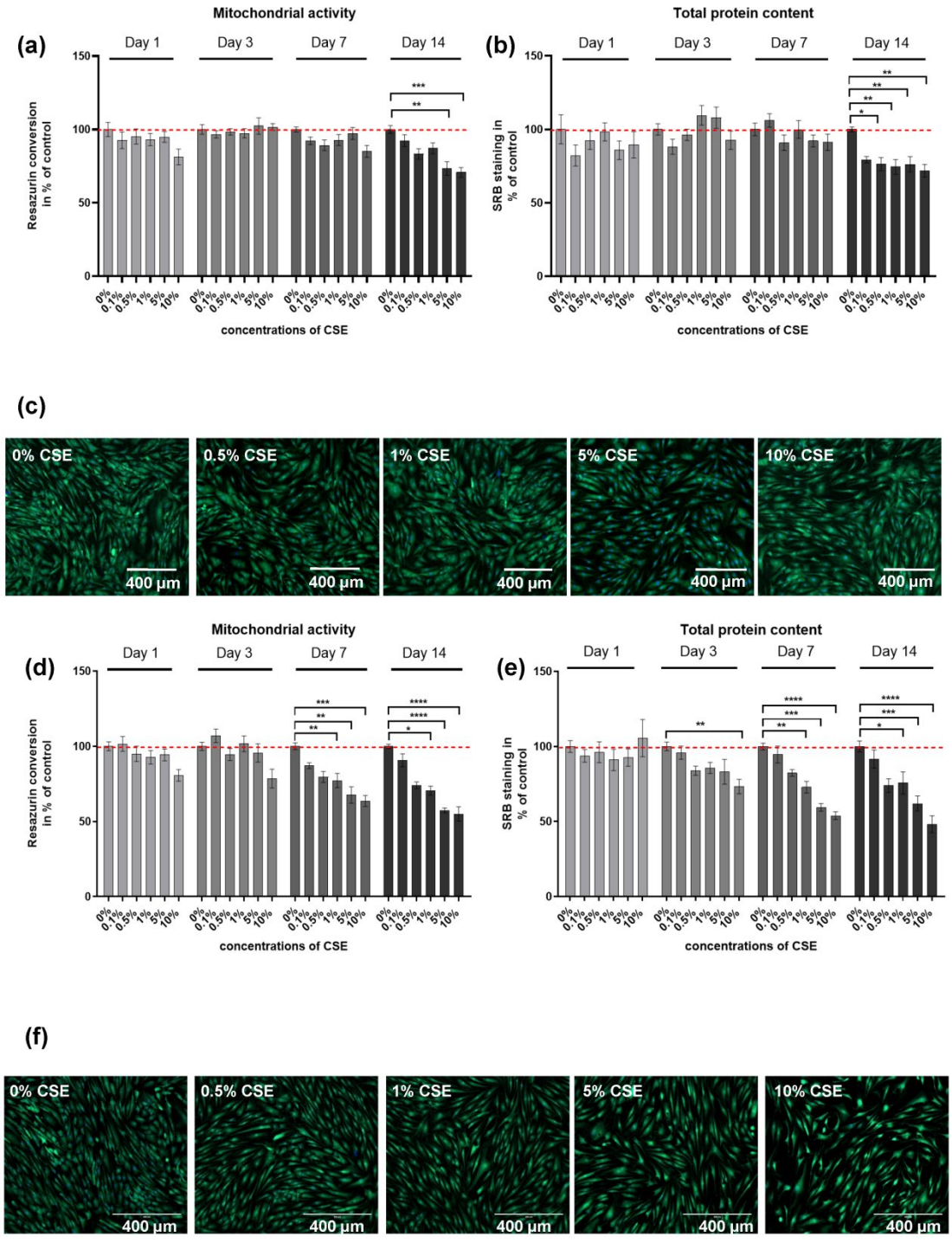


Figure 3.3 Comparison of the effect of CSE exposure twice a week and once a day on the primary human chondrocytes. On days 1, 3, 7 and 14, **(a)** Resazurin conversion and **(b)** SRB staining were performed for cell viability. **(c).** Representative microscopic images for Calcein-AM staining. Cells 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. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ 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%, ***$p \leq 0.001$) and day 14 (Fig. 3.4.b, 5% and 10%, **$p \leq 0.01$ and ***$p \leq 0.001$, 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.

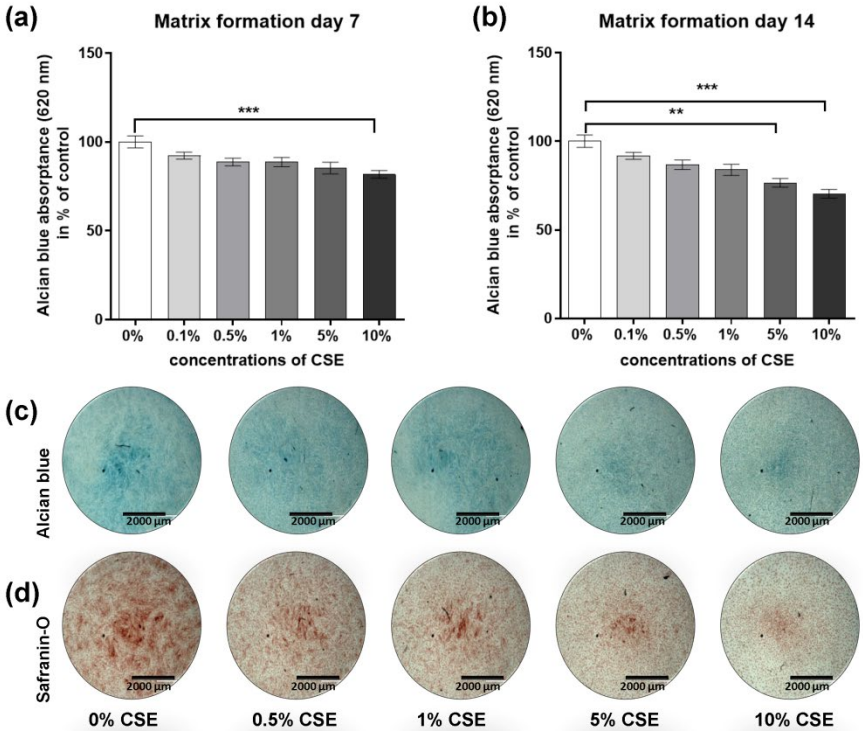


Figure 3.4 CSE exposure inhibits the accumulation of ECM in primary human chondrocytes. Matrix formation was quantified on day 7 **(a)** and day 14 **(b)** by Alcian blue staining. Representative microscopic images for Alcian blue **(c)** and Safranin-O **(d)** staining were performed on day 7. Data are presented as bar diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. **$p \leq 0.01$, ***$p \leq 0.001$ 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.

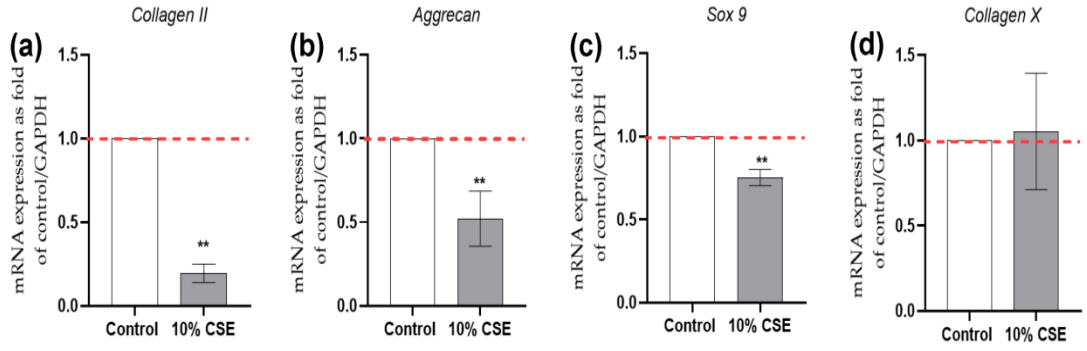


Figure 3.5 CSE exposure down-regulates the anabolic gene expression of primary human chondrocytes. Semi-quantitative RT-PCR was performed using primers for gene expression. The gene expression of (**a**) *Collagen II*, (**b**) *Aggrecan*, (**c**) *Sox9*, and (**d**) *Collagen X* was normalized to the *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, $^{**}p \leq 0.01$). 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.

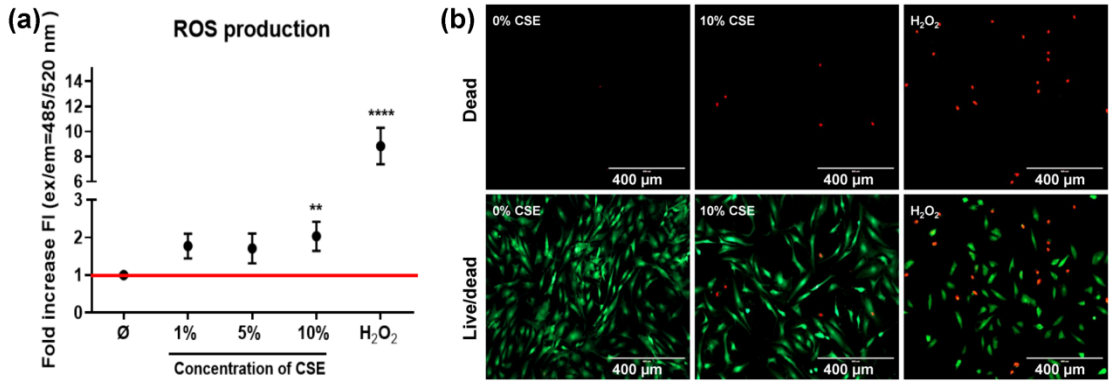


Figure 3.6 CSE exposure increases oxidative stress and accelerates the death of primary human chondrocytes. (a). ROS production was measured by DCFH-DA assay. **(b).** Living cells (green) and dead cells (red) were visualized by Calcein AM and ethidium homodimer, respectively (Representative figure for day 3. H_2O_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. $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$ 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, ****$p \leq 0.0001$) and total protein content (Fig. 3.7.b, 2000 µg/ml and 4000 µg/ml, **$p \leq 0.01$ and ***$p \leq 0.001$, respectively) showed a dose-dependent inhibition in Dex-treated chondrocytes, suggesting detrimental effects of Dex on chondrocytes (Chen *et al.*, 2020).

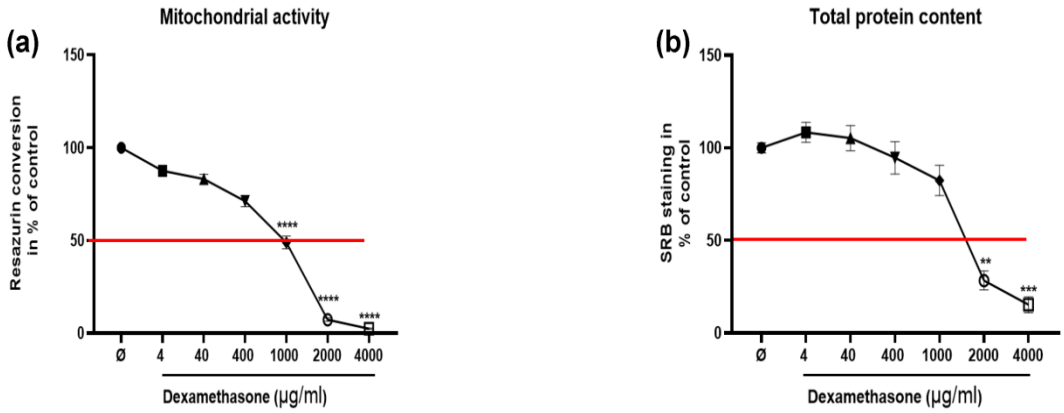


Figure 3.7 Clinical doses of Dex are toxic to the primary human chondrocytes.
After 24 h, **(a)** Resazurin conversion and **(b)** SRB staining were performed for cell viability. Data are presented as line diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ 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, $***p \leq 0.001$) 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.

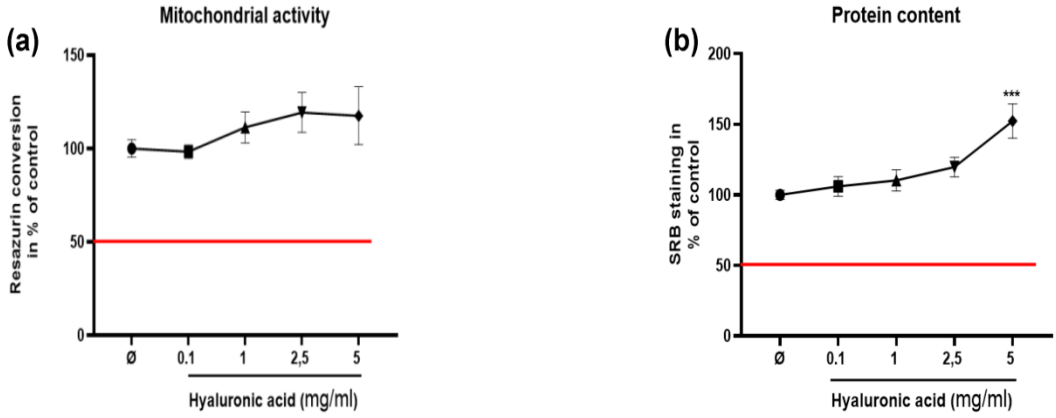


Figure 3.8 Clinical doses of HA increases the viability of primary human chondrocytes. After 24 h, **(a)** Resazurin conversion and **(b)** SRB staining were performed for cell viability. Data are presented as line diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. $***p \leq 0.001$ as compared 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, ****$p \leq 0.0001$) and total protein content (Fig. 3.9.b) showed that the concentrations of AA over 1 mM were toxic to the chondrocytes.

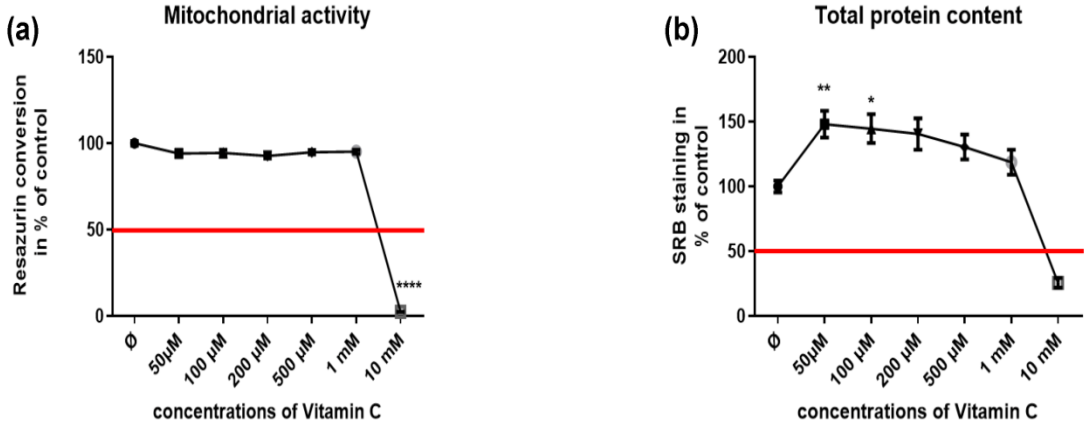


Figure 3.9 Cytotoxicity assessment of Vitamin C on the primary human chondrocytes. After 24 h, **(a)** Resazurin conversion and **(b)** SRB staining were preformed for cell viability. Data are presented as line diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. *$p \leq 0.05$, **$p \leq 0.01$, *****$p \leq 0.0001$ 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, ***$p \leq 0.001$), total protein content (Fig. 3.10.b, 50, 100, 200 µM, ****$p \leq 0.0001$), and matrix formation were increased (Fig 3.10.c, 50 µM, **$p \leq 0.01$ and 100, 200 µM, *$p \leq 0.05$) when the cells were solely incubated with AA on day 7. However, mitochondrial activity (Fig. 3.10.a, 200 µM, °°°°$p \leq 0.0001$), total protein content (Fig. 3.10.b, 200 µM, °°$p \leq 0.01$) and matrix formation showed a dose-dependent decrease (Fig. 3.10.c; 200 µM, °°$p \leq 0.01$) when the chondrocytes were co-incubated with CSE and AA on day 7.

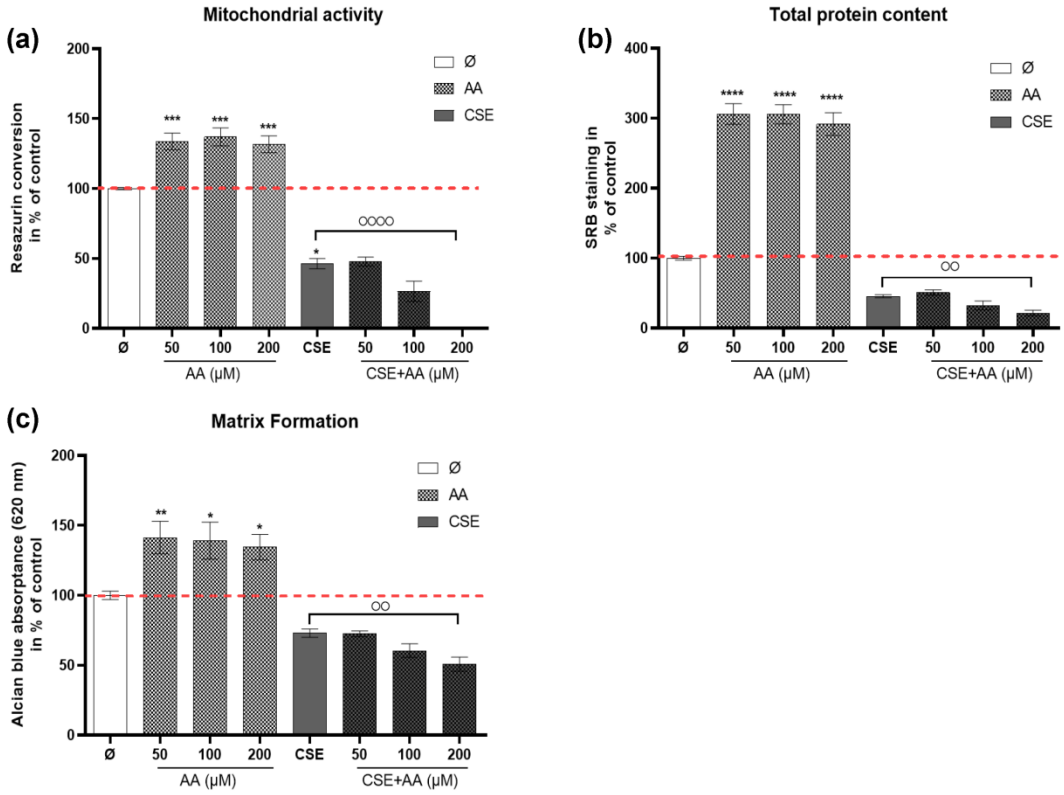
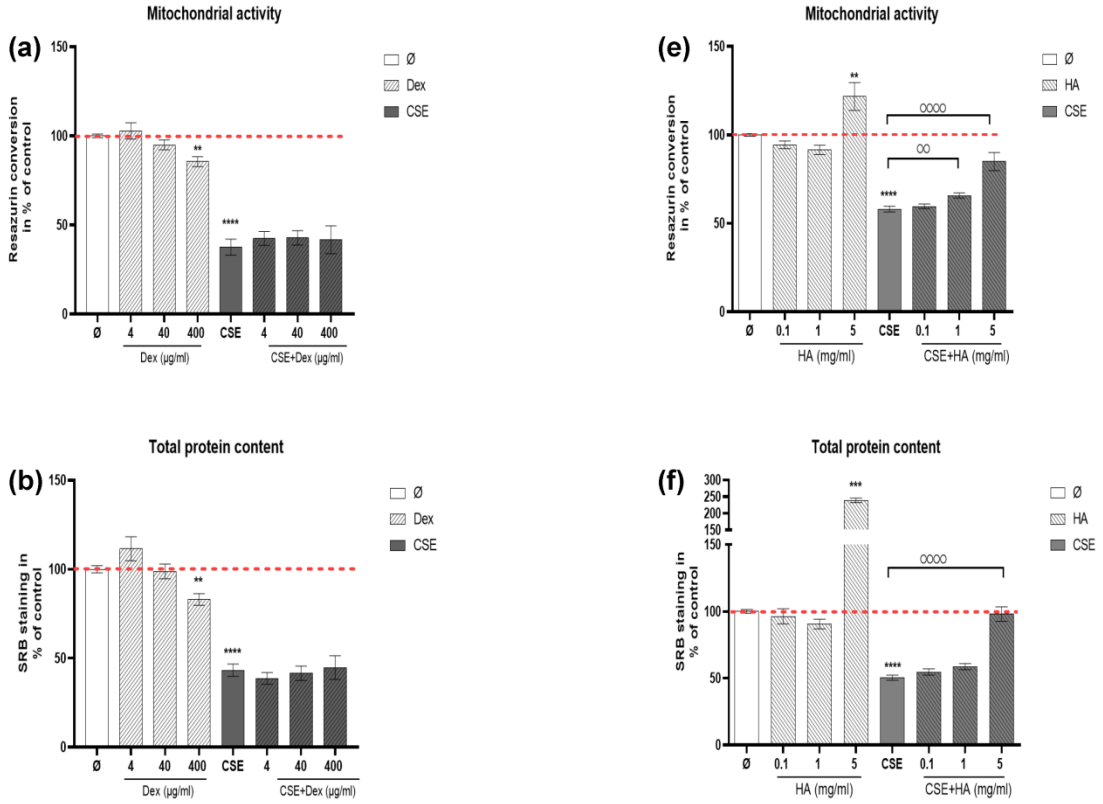


Figure 3.10 Effects of AA on the CSE-exposed primary human chondrocytes. On day 7 of treatment, **(a)** Resazurin conversion and **(b)** SRB staining were performed for cell viability**.** Matrix accumulation was quantified by **(c)** Alcian blue staining. Data are presented as bar diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ vs. the untreated group, °°$p \leq 0.01$, °°°°$p \leq 0.0001$ 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, $^{**}p \leq 0.01$), total protein content (Fig. 3.11.b, 400 µg/ml, $^{**}p \leq 0.01$) and matrix formation (Fig. 3.11.c, 400 µg/ml, $^{**}p \leq 0.01$) 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, $^{*}p \leq 0.05$, 40µg/ml, $^{**}p \leq 0.01$ and 400 µg/ml, $^{**}p \leq 0.01$) 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, $^{\circ\circ}p \leq 0.01$ and $^{\circ\circ\circ\circ}p \leq 0.0001$, respectively) and total protein content (Fig. 3.11.f, 5 mg/ml, $^{\circ\circ\circ\circ}p \leq 0.0001$) 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, $^{\circ}p \leq 0.05$ and 5 mg/ml, $^{\circ}p \leq 0.05$) on day 7.



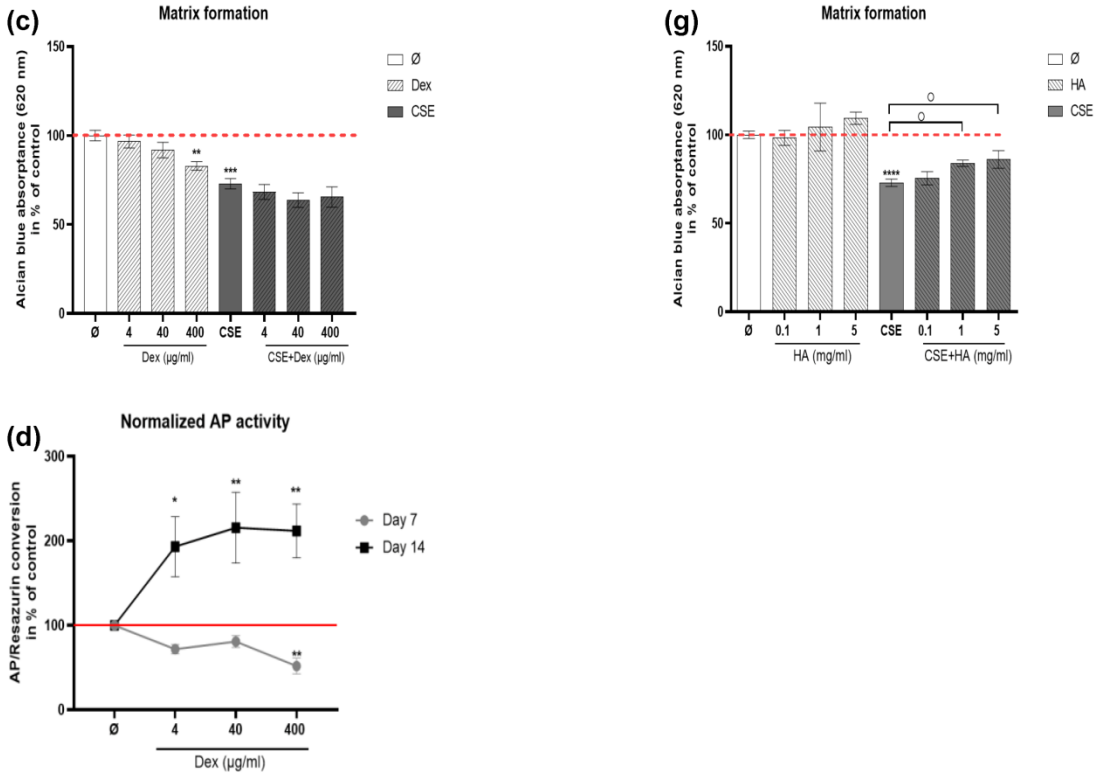


Figure 3.11 Evaluation of low doses of Dex and HA on the CSE-impaired primary human chondrocytes. On day 7 of treatment, **(a, e)** Resazurin conversion and **(b, f)** SRB staining were performed for cell viability**.** Matrix accumulation was quantified by **(c, g)** Alcian blue staining. **(d).** AP 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).

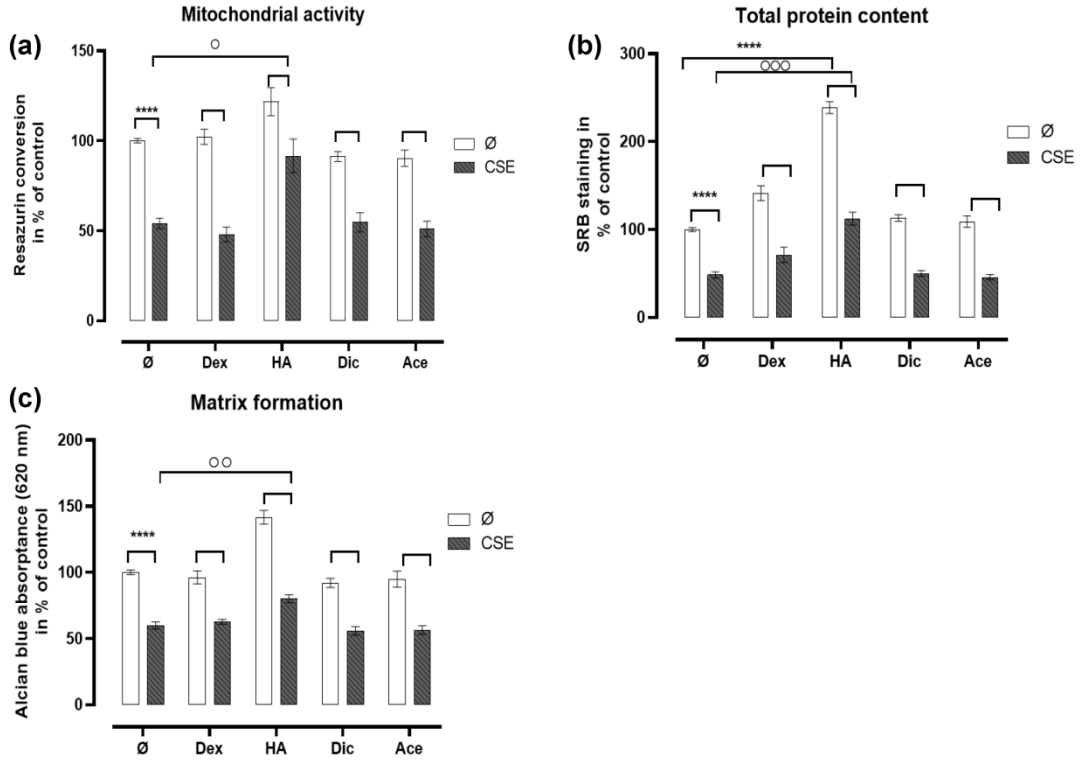


Figure 3.12 Effects of Ace and Dic on the CSE-impaired primary human chondrocytes. On day 7 of treatment, **(a)** Resazurin conversion and **(b)** SRB staining were performed for cell viability**.** Matrix accumulation was quantified by **(c)** Alcian blue staining. Data are presented as bar diagrams (mean ± SEM); analyzed by Mann Whitney test. ****$p \leq 0.0001$ vs. the untreated group, °$p \leq 0.05$, °°$p \leq 0.01$, °°°$p \leq 0.001$ 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, °°°$p \leq 0.001$), and matrix accumulation (Fig. 3.13.c, HA+Dic and HA+Ace, °$p \leq 0.05$, °°$p \leq 0.01$, 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, °°$p \leq 0.01$,

HA+Ace, °°°$p \leq 0.001$) 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+Dic, °°$p \leq 0.01$, HA+Ace, °°°$p \leq 0.001$), indicating that HA alone and HA combinatory treatments retrieved the chondrocyte impairment caused by CSE through suppressing and/or quenching the production of ROS.

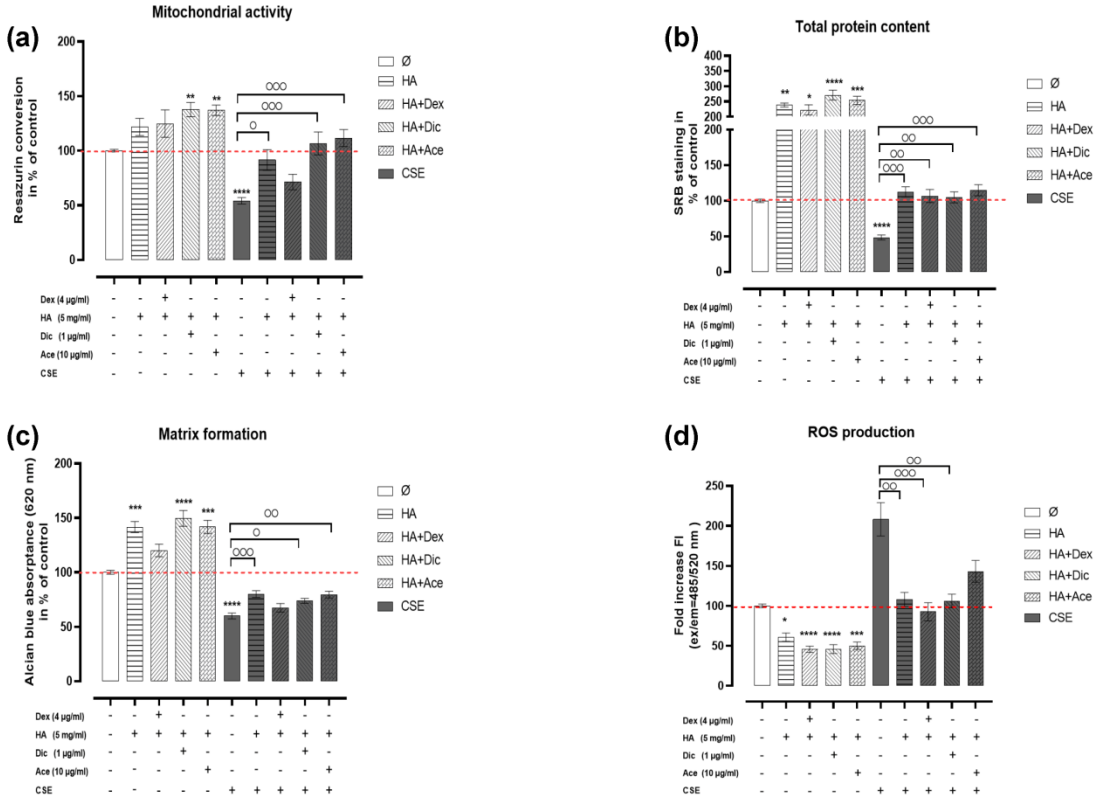


Figure 3.13 Evaluation of HA combined with anti-inflammatory drugs on the CSE-impaired chondrocytes. On day 7 of treatment, **(a)** Resazurin conversion and **(b)** SRB staining were performed for cell viability**.** Matrix accumulation was quantified by **(c)** Alcian blue staining. **(d).** ROS production was measured by DCFH-DA assay. H_2O_2 (0.01% $_{V/V}$) was performed as a positive control. Data are presented as bar diagrams (mean ± SEM); analyzed by Kruskal–Wallis H test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ vs. the untreated group, °$p \leq 0.05$, °°$p \leq 0.01$, °°°$p \leq 0.001$ 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-ß, 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_2O_2 and O_2^-) and H_2O_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 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) 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

Obwohl mehrere Forscher die schädlichen Auswirkungen des Rauchens auf den Bewegungsapparat bestätigt haben, ist der Zusammenhang zwischen Rauchen und dem Auftreten von Arthrose (OA) immer noch unklar. Hier untersuchten wir die Auswirkungen von Zigarettenrauchextrakt (CSE) auf die primäre Chondrozytenfunktion des Menschen. Darüber hinaus wurde getestet, ob die pharmakologische Behandlung von Dexamethason für durch CSE beeinträchtigte Chondrozyten von Vorteil ist oder ob sie durch andere Behandlungen wie Paracetamol und NSAR ersetzt werden kann. Zusätzlich untersuchten wir die Auswirkungen von Hyaluronsäure (HA)- und HA-Kombinationen (Dexamethason, Acetaminophen oder Diclofenac) auf die Funktion und Viabilität von CSE exponierten Chondrozyten, um eine mögliche therapeutische Alternative für die klinische Anwendung bei Rauchern mit symptomatischer OA zu ermitteln.

Humanes Knorpelgewebe, welches im Rahmen von Routineoperationen entnommen werden musste, wurde nach ethischer Genehmigung der Etikkomission 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

ungefähr dem Rauchen einer Packung pro Tag verbunden ist. Als entzündungshemmende Behandlungsstrategie wurde die traditionelle pharmakologische Therapie mit Dexamethason (Dex) evaluiert. Klinische Dex-Dosen waren für die Zellen toxisch, und eine Langzeitinkubation mit niedrigeren Dex-Dosen (4–400 µg / ml) würde zu einem hypertrophen Chondrozyten-Phänotyp führen. Um Dexamethason zu ersetzen, wurde eine klinische Dosierung von Diclofenac (Dic) und Paracetamol (Ace) an Chondrozyten getestet. Interessanterweise verstärkten therapeutische Dosen von Dic (1 µg / ml) und Ace (10 µg / ml) die schädlichen Auswirkungen von CSE auf den Gesamtstoffwechsel von Chondrozyten nicht. Zusätzlich hatte eine klinische Dosis von HA (5 mg / ml) und HA in Kombination mit Dic, Ace oder Dosen von Dex eine schützende Wirkung auf die CSE-exponierten Chondrozyten, da sie die Bildung freier Radikale signifikant hemmten und die Lebensfähigkeit förderten und ECM-Akkumulation von Zellen. Unsere Studie zeigt, dass Zigarettenrauch durch Erhöhung des oxidativen Stresses den Zelltod induziert und die Knorpelbildung zerstört. Eine intraartikuläre (IA) Injektion von HA in Kombination mit therapeutischen Dosen von Analgetika / entzündungshemmenden Mitteln (Ace oder Dic) könnte die schädlichen Wirkungen von CSE auf primäre menschliche Chondrozyten umkehren und somit potenzielle therapeutische Alternativen bei der Behandlung von Rauchern für symptomatische Patienten eröffnen OA.

Chapter 6

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.

Journal:

International journal of molecular sciences. 2020, Mar; 21(5): 1901.

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11. Curriculum vitae – Tao Chen

Education:

2010-2017 **Degree:** Seven-year program including B.S.M. & M.Sc.
University: Zhengzhou University (School of Medicine)
2015-2017 **Degree:** Master
University: Zhengzhou University (Clinical Medicine /
Sports Medicine and Joint Surgery)
Since 10/2017 Siegfried Weller Institute (SWI) for trauma research
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:

- **Chen T**, Ehnert S, Nüssler AK, *et al*. Primary Human Chondrocytes Affected by Cigarette Smoke- Therapeutic Challenges. Int J Mol Sci. 2020, 21 (5). IF 4.18
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- **Chen T**, Xu J, Li G, *et al.* A comparative study of the effect of arthroscopic tenodesis with mini-incision subpectoral tenodesis in the treatment of LHB

tendonitis. Chinese Journal of Bone and Joint Surgery [J] 2017, 10 (1): 44-47.

Research experience:

1. 2015.07-2015.09 Hip, knee replacement surgery multi -center data base by West China Hospital.
2. 2015.09-2016.06 A comparative study of the effect of arthroscopic tenodesis with mini-incision subpectoral tenodesis in the treatment of LHB tendonitis.
3. 2017.10-Now The effect of cigarette smoke extract (CSE) on the primary human chondrocytes.

Clinical experience:

2014.09-2015.07 Internship in the First Affiliated Hospital of Zhengzhou University
2015.09-2017.07 Internship specializing in Department of Sports Medicine and Joint Surgery, the First Affiliated Hospital of Zhengzhou University

Languages:

First language: Chinese (Mandarin)
English (fluent)