SHILAJIT, A NOVEL REGULATOR OF BONE/CARTILAGE HEALING

Nawaf Yousef Labban

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the School of Dentistry,
Indiana University

February 2013

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

_____

L. Jack Windsor, Ph.D., Chair

_____

Fengyu Song, D.D.S., M.S., Ph.D.

_____

Doctoral Committee

Ahmed Ghoneima, B.D.S., M.S.D., Ph.D.

_____

Angela Bruzzaniti, Ph.D.

_____

November 2, 2012

Matthew Allen, Ph.D.

_____

Jo Ann Cameron, Ph.D.

DEDICATION

Firstly, I would thank God, who without his blessing I wouldn't have completed this dissertation. I dedicate this dissertation to my late father, mother, brothers and sisters for their everlasting support and continuous prayers. Special dedication goes out to my wife (Nouf) and my children (Tala, Talal and Sultan) for their love, patience, and support.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my mentor (Dr. L. Jack Windsor) for his guidance, patience and most importantly his friendship during my Masters and Ph.D. studies. He has never failed me as a mentor or a friend during the ups and downs of my research project. Without his vision, none of this work would have been possible. He is and will remain my role model for a mentor, teacher and scientist. I would also like to extend my gratitude to my committee members for their help and encouragements: Dr. Fengyu Song and Dr. Ahmed Ghoneima for their continuous encouragement, scientific advice, knowledge and support; Dr. Matthew Allen and Dr. Jo Ann Cameron for their invaluable expertise, insightful discussions, and supervision of the different studies. I would like to especially express my gratitude to Dr. Angela Bruzzaniti for her constant encouragement and support throughout the different studies, especially in helping me in the study designs and writing up abstracts and the dissertation, as well as finalizing my presentations. Her technical and editorial advice, constructive criticism, and great insights were critical in the completion of a large part of this dissertation.

Many members of the faculty and staff from **Indiana University School of Dentistry (IUSD)** have assisted me in multiple ways during my time at IUSD. I am especially grateful to Drs. Richard Gregory (the Ph.D. Program Director), Tien-Min Gabriel Chu, Jeffrey Platt, Sean Shih-Yao Liu, Marco Bottino, and

William Babler for their help and guidance. Special thanks go out for my colleagues who made research entertaining through their humorous jokes and great attitudes (Drs. Weiping Zhang, Eman Allam, Jun Sun Ghaeth Yassen and Ghada Batarseh). I am also very thankful to Mr. George Eckert for his help in the statistical analyses of the studies. In addition, my research would not have come to a successful completion without the help from the Animal Facility Staff including Janice Warrick-Polakoff, Cheryl Kantmann, Judy Powers, Carl Lerner, and Ashley Marlow in conducting the animal studies.

From the **Indiana University School of Medicine**, I would like to thank Mohammad Aref for his help in conducting the bone histomorphometric analysis in the osteoporotic rat study, Drew Brown for harvesting the bone samples and conducting the micro-CT imaging, and Dr. David Burr for his help and guidance in the area of bone biology. I would like to thank Dr. **Stephen** Trippel and his student Albert Chan for their invaluable help in the area of chondrogenesis.

From the **Indiana University School of Science**, I would like to thank Drs. David Stocum and Xiaoping Chen for their help and expertise during the Xenopus study.

From the **Indiana University School of Health and Rehabilitation**, I would like to thank Dr. Stuart Warden for his help and assistance in the experimental designs of some of my studies.

From the **University of Illinois School of Molecular and Cellular Biology**, I am sincerely grateful to Dr. Derek Milner for his time and help in the experimental design of the Xenopus studies, histological sectioning, the analysis and interpretation of the results, and for writing part of the results section of these studies in this dissertation. Also, special thanks to Patrick Redwood for helping conduct the surgeries in the Xenopus studies, injections, and harvesting the Xenopus limbs.

From the **University of Wisconsin-Madison College of Engineering**, I would like to thank Dr. Wan-Ju Li and his student Andrew Handorf for sharing their knowledge in the area of chondrogenesis.

From **King Saud University College of Pharmacy**, I would like to thank Dr. Mohammed Al-Yahya for sharing his expertise in shilajit research.

Last but not least, I would like to thank my wife (Dr. Nouf Al-Shibani) for her support and love throughout our time at IUSD, my mother and late father who always encouraged me to pursue this career, my sisters and brothers for their everlasting love and prayers. Special thanks and love goes to my children (Tala, Talal and Sultan) to whom all this work is dedicated to.

ABSTRACT

Nawaf Yousef Labban

SHILAJIT, A NOVEL REGULATOR OF BONE/CARTILAGE HEALING

Enhancing osteoblast proliferation, survival, and extracellular matrix protein secretion are potential therapeutic approaches to treat bone fractures and diseases such as osteoporosis. Shilajit is a traditional medicine used in many countries such as India for thousands of years to treat many diseases including bone diseases. In this study, molecular, cell-based and *in vivo* approaches were utilized to investigate the effects of shilajit on bone and cartilage regeneration. An osteosarcoma cell line (MG63) was incubated in serum free media with and without 0.8 mg/ml of shilajit. Shilajit significantly increased cell survival up to 30 days and these cells retained their ability to proliferate in fresh media with serum. After adding shilajit, there were statistically significant decreases in the expression of both anti-apoptotic and pro-apoptotic proteins. An *in vivo* non-critical size segmental bone defect Xenopus system was used to evaluate the ability of shilajit to enhance cartilage formation. After a small segment of the anterior hemisection of the tarsus bone was excised, the frogs were divided into three groups and given subcutaneous injections of either phosphate-buffered saline or shilajit once daily for 30 days and then bone/cartilage formation evaluated. The total cartilage area/total section area was significantly increased (2.6 fold) in the shilajit treated samples. In an osteoporotic rat model, the anabolic properties of shilajit on bone mass were assessed by histomorphometric

analyses. Ovariectomized (OVX) rats received daily intraperitoneal injections for 4 weeks. Bone formation rates (BFRs) for the cortical periosteal bone surface of the midshaft tibia were 383.2, 223.9, 308.8, 304.9, and 370.9 $\mu m^3/\mu m^2$/year, and for the trabecular surface were 82.2, 113, 212.1, 157, and 165 $\mu m^3/\mu m^2$/year for the sham, OVX, PTH, 3 mg/kg shilajit, and 30 mg/kg shilajit groups, respectively. Shilajit increased both trabecular and cortical BFRs. It generated better results on cortical periosteal bone surface than did PTH. Taken together, these findings suggest that shilajit promotes osteoblast survival due to its effects on altering the balance between pro-apoptotic and anti-apoptotic proteins. In addition, *in vivo* studies revealed that shilajit enhanced cartilage formation in Xenopus and BFRs in rats. Therefore, shilajit may possess anabolic bone/cartilage properties.

L. Jack Windsor, Ph.D., Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

STZ	Streptozotocin
HMSCs	Human Mesenchymal Stem Cells
HFOs	Human Fetal Osteoblasts
ROS	Reactive Oxygen Species
ECM	Extracellular Matrix
MG63	Human-Derived Osteosarcoma cell line
TGF-β	Transforming Growth Factor-Beta
BMPs	Bone Morphogenetic Proteins
ALP	Alkaline Phosphatase
ERK	Extracellular Signal-Regulated Kinase
BDGF	Bone-Derived Growth Factors
bFGF	Basic Fibroblast Growth Factor
IGFs	Insulin-Like Growth Factor
Cbfa1	Core-Binding Factor-1
GDF-5	Growth Differentiation Factor-5
PTH	Parathyroid Hormone
DMEM	Dulbecco’s Modified Eagle’s Medium
PBS	Phosphate Buffer Solution
HGFs	Human Gingival Fibroblasts
IRB	Institutional Review Board
LDH	Lactate Dehydrogenase

WST-1	Water-Soluble Tetrazolium Salt-1
HRP	Horseradish Peroxidase
IACUC	Institutional Animal Care and Use Committee
OVX	Ovariectomy
IP	Intraperitoneal Injection
MAR	Mineral Apposition Rate
BV/TV	Bone Volume/Tissue Volume
Micro-CT	Microcomputed Tomography
MTS	Mechanical Testing Machine
SPSS	Statistical Package for Social Science
AVOVA	Analysis of Variance
Pos	Positive
Neg	Negative
Bad	Bcl2 Antagonist of Cell Death
Bax	Bcl-2-Associated X Protein
Bcl-2	B-Cell Lymphoma-2
BID	BH3-Interacting Domain Death Agonist
BIM	BCL2 Interacting Protein
Bcl-w	B Cell Lymphoma-w
IAP	Inhibitor of Apoptotic Protein
CytoC	Cytochrome c
DR	Death Receptor
Fas	Fatty Acid Synthetase

FasL	Fatty Acid Synthetase Ligand
HSP	Heat Shock Protein
HTRA2	High Temperature Requirement Protein A2
IGF	Insulin Like Growth Factor
IGFBP	Insulin Like Growth Factor Binding Protein
SMAC	Second Mitochondria-Derived Activator of Caspases
Caspase 8	Cysteinyl Aspartic Acid-Protease 8
TNF	Tissue Necrotizing Factor
TRAIL-R	TNF-Related Apoptosis-Inducing Ligand Receptor
XIAP	X-Linked Inhibitor of Apoptosis Protein
sTNF-R1	Serum Tumor Necrosis Factor Receptor 1
SE	Standard Error
SW1353	Chondrosarcoma Cell Line
COL1A1	Collagen, Type I, Alpha 1
COL3A1	Collagen, Type III, Alpha 1
COL1A2	Collagen, Type I, Alpha 2
FBN2	Collagen Fibrillin 2
COL11A1	Collagen, Type XI, Alpha 1
COL5A2	Collagen, Type V, Alpha 2
FNDC1	Fibronectin Type III Domain Containing 1
COL5A1	Collagen, Type V, Alpha 1
COL12A1	Collagen, Type XII, Alpha 1
SPARC	Secreted Protein, Acidic and Rich in Cysteine

COL23A1	Homo Sapiens Collagen, Type XXIII, Alpha 1
SD	Standard Deviation
MS/BS	Mineralizing Surface/Bone Surface
BFR	Bone Formation Rate
G0	G Zero (Resting Phase)

CHAPTER ONE

Introduction and Review of Literature

The overall objective of the different studies involved in this dissertation was to evaluate the effects of shilajit on bone and cartilage regeneration using molecular, cell-based, and *in vivo* approaches.

Shilajit

Many Asian and middle eastern countries such as India, Saudi Arabia, and Yemen consider traditional medicines essential parts of health care (Agarwal, Khanna et al. 2007). Although many of these traditional remedies have been used for thousands of years in these countries, they lack significant scientific evidence to document their mechanisms of actions (Agarwal, Khanna et al. 2007). It is essential that scientific evidence be obtained about the abilities of these remedies. Shilajit is a traditional remedy that has been utilized as a rejuvenator in Siddha and Ayurveda medicine for more than 3000 years (Agarwal, Khanna et al. 2007). Shilajit is a Sanskrit word, which means “Winner of rock” and “Conqueror of mountains and destroyer of weakness” (Meena, Pandey et al. 2010). Shilajit is also known as salajit, shilajeet, salajeet, shilajatu, shilajita, bitumen, Jew's pitch, mineral pitch, mineral wax, asphalt, mumie, mummiyo, dathusara, shiladhatu, dathuras, or ozokerite (Agarwal, Khanna et al. 2007, Biswas, Pandit et al. 2010, Meena, Pandey et al. 2010, Wilson, Rajamanickam et al. 2011, Gaikwad, Panat et al. 2012).

Shilajit is a multi-component natural occurring mineral exudate, which has a bitter taste and a pale-brown to blackish-brown color (Biswas, Pandit et al. 2010, Meena, Pandey et al. 2010, Wilson, Rajamanickam et al. 2011). Although this exudate oozes mainly from rocks in the Himalayas, it can be also found in Tibet, Russia, China, Pakistan, Afghanistan, Norway, and the north of Chile (known as *Andean Shilajit*) at altitudes between 1000 and 5000 meters (Biswas, Pandit et al. 2010, Cornejo, Jimenez et al. 2011, Carrasco-Gallardo, Guzman et al. 2012). Many have described it as paharki-khoon (mountain blood), shilaras (rock juice), or pahar-ki-pasina (sweat of mountains) (Biswas, Pandit et al. 2010). Although the chemical composition and physical properties of shilajit samples from the different countries are very comparable, numerous components are present at different ratios (Schepetkin, Xie et al. 2009). These ratios are affected by several factors such as temperature, altitude, humidity, plant-species, and the rock's geological nature where the samples are collected (Agarwal, Khanna et al. 2007). For instance, the percentage of fulvic acid in shilajit found in India is 21.4%, while it is 15.5% in Pakistan samples, and 19% in Russia samples. (Agarwal, Khanna et al. 2007). Therefore, shilajit therapeutic effects could vary depending on the region it comes from (Agarwal, Khanna et al. 2007, Carrasco-Gallardo, Guzman et al. 2012). Shilajit can be classified into 4 different types: a) lauha (iron containing shilajit), which is brownish-black in color and thought to have the most therapeutic value; b) savrana (gold shilajit), which is red in color; c) rajat (silver shilajit), which is white in color; and d) tamra (copper shilajit), which is blue in color (Agarwal, Khanna et al. 2007).

According to researchers and the ancient writings, the origin of shilajit is mainly vegetative although solid scientific evidence remains incomplete (Agarwal, Khanna et al. 2007). It has been hypothesized that shilajit is formed by the degradation of plant material from the species such as *Trifolium repens*, *Euphorbia royleana, Fissidens, Barbula, Thuidium, Minium, Asterella, Marchantia, Dumortiera, Plagiochasma, Pellia,* and/or *Stephenrencella-Anthoceros* (Agarwal, Khanna et al. 2007, Einhorn 2011, Carrasco-Gallardo, Guzman et al. 2012). Then due to the compression underneath many layers of rocks for hundreds of years at high temperature, this vegetation have gone through modifications before it oozes out as a gummy exudation from the rocks of the mountains (Agarwal, Khanna et al. 2007). This gummy exudate becomes less viscous and moves out within the layers of rocks during the warm summers (Agarwal, Khanna et al. 2007).

Early research on the chemical nature of shilajit in the 1980s has shown that approximately 60~80% of its composition is humus, as well as several other organic ingredients (Agarwal, Khanna et al. 2007, Yin, Yang et al. 2011). The most important ingredients identified in *shilajit* are believed to be humic acid, fulvic acid, dibenzo-alpha-pyrones, uronic acids, eldagic acid, gums, ichthyol, triterpenes, latex, resin, albuminoids, ellagic acid, albumins, fatty acids, benzoic acid, vegetable matter, vitamins, silica, sterols, phenolic lipids, hippuric acid, 3,4-benzocoumarins, sterol, aromatic carboxylic acid, triterpenes, phenolic lipids, and amino acids (Agarwal, Khanna et al. 2007, Kennel and Drake 2009, Miki and

Masaki 2009, 2010, Meena, Pandey et al. 2010, Arboleya, Alperi et al. 2011, Atik 2011, Carrasco-Gallardo, Guzman et al. 2012, Gaikwad, Panat et al. 2012, Kharazmi, Persson et al. 2012, Takeuchi and Amagase 2012). In addition, shilajit can contain more than 84 minerals such as silver, copper, lead, iron, and zinc (Meena, Pandey et al. 2010, Arboleya, Alperi et al. 2011). Dibenzo-alpha-pyrones along with fulvic acid and humic acids are hypothesized to be the major active components of shilajit (Yin, Yang et al. 2011, Kharazmi, Persson et al. 2012).

Based on water solubility, shilajit's ingredients are classified into humins, humic acid, and fulvic acid (Carrasco-Gallardo, Guzman et al. 2012). While humins are not water soluble at any pH level, humic acid is a water soluble component at high pH and fulvic acid can be dissolved in water at various pH levels (Carrasco-Gallardo, Guzman et al. 2012). Fulvic acid has many proposed benefits such as energy production, anti-stress agent, prevention of hypoxia, and stimulation of blood formation (Toussaint, Elder et al. 2009, Meena, Pandey et al. 2010, Barhanpurkar, Gupta et al. 2012). It has been utilized topically to treat conditions such as hematoma, osteochondrosis, and osteoarthritis. It has been used orally for diseases such as stomach ulcers and diabetes mellitus (Schepetkin, Khlebnikov et al. 2003, Agarwal, Khanna et al. 2007, Schepetkin, Xie et al. 2009). Unfortunately, scientific evidence supporting the use of fulvic acid is incomplete. Beside the above mentioned ingredients, analyses have revealed variable amounts of impurities contaminating the natural form of shilajit

such as: a) heavy metal ions that include lead, mercury, and arsenic; b) mycotoxins, which can be produced by fungi or mold; c) reactive free radicals, which can cause damage to different human tissues; and d) polymeric quinones, which can be found as the oxidation product of quinic acid, (Agarwal, Khanna et al. 2007). Research studies have shown that the consumption *of the native shilajit* without some processing might lead to risks of intoxication due to the above mentioned harmful impurities (Carrasco-Gallardo, Guzman et al. 2012). In one study, the antioxidant properties of processed shilajit were compared with the non-processed form. Processed shilajit significantly enhanced its antioxidant activities while the non-processed form gave inconsistent results (Agarwal, Khanna et al. 2007). Different studies have indicated that mixing herbal preparations such as shilajit with heavy metals and other minerals including gems might results in better synergic responses from the body, which is called *rasa-shastra* in traditional Indian medicine (Saper, Phillips et al. 2008, Carrasco-Gallardo, Guzman et al. 2012). A few animal toxicological studies have been conducted and they found that the LD50 (orally) was greater than 2 g/kg (Acharya, Frotan et al. 1988, Ghosal S. 1989, Carrasco-Gallardo, Guzman et al. 2012). In addition, it was shown that shilajit is safe at doses less than 1 g/kg when used chronically (Kelginbaev NS 1973, Anisimov VE 1982, Fortan MH 1984, Al-Hamaidi AR 2003, Biswas, Pandit et al. 2010).

Uses of Shilajit

Traditionally, people including adults and children from different countries such India and Nepal consume *shilajit* orally after mixing it with milk during breakfast (Carrasco-Gallardo, Guzman et al. 2012). Shilajit has been accredited with numerous healing properties and described as a cure for multiple diseases in early Ayurvedic texts from the Charaka Samhita, as well as a rejuvenator that might be able to increase longevity and arrest aging (Agarwal, Khanna et al. 2007, Biswas, Pandit et al. 2010, Gaikwad, Panat et al. 2012). Shilajit has many other traditional uses such as jaundice, genitourinary disorders, enlarged spleen, digestive disorders, nervous disorders, epilepsy, anemia, diabetes, chronic bronchitis, angina, and anorexia (Schepetkin I 2002, Yin, Yang et al. 2011, Carrasco-Gallardo, Guzman et al. 2012, Kharazmi, Persson et al. 2012). S*hilajit has been used* for the treatment of kidney stones, energy production, internal antiseptic, edema, spondylitis, hemorrhoids, injured muscles, bone fractures, and diseases such as osteoporosis (Agarwal, Khanna et al. 2007, Toussaint, Elder et al. 2009, Meena, Pandey et al. 2010). Researchers have shown that shilajit has potential uses such as anti-inflammatory, anti-fungal, anti-ulcerogenic, anxiolytic activity, anti-allergic, analgesic, anti-diabetic, memory enhancer, and an antioxidant (Acharya, Frotan et al. 1988, Ghosal S. 1988., Ghosal S. 1989, Goel, Banerjee et al. 1990, Jaiswal 1992, Bhattacharya 1995, Bhattacharya SK 1995, Ghosal S. 1995, Wang, Wang et al. 1996, Islam K. 2005, Schepetkin, Xie et al. 2009, Shalini 2009, Arboleya, Alperi et al. 2011, Cornejo, Jimenez et al. 2011, Barhanpurkar, Gupta et al. 2012, Carrasco-Gallardo, Guzman et al. 2012,

Gaikwad, Panat et al. 2012, Kharazmi, Persson et al. 2012). Unfortunately, these therapeutic properties lack significant scientific evidence (Wilson, Rajamanickam et al. 2011, Carrasco-Gallardo, Guzman et al. 2012).

There have been a limited number of well-designed scientific studies that have investigated the therapeutic effects of shilajit and its actions on different human tissues or cells. In a study by Agzamoy et al., the effects of shilajit on lymphocytes of the cortical thymus layer were evaluated (Agzamov RA 1988, Schepetkin, Xie et al. 2009). They found that the proliferation of lymphocytes was stimulated and the migration of these cells into thymus-dependent zones of the lymph nodes and spleen were enhanced as a result of treatment with shilajit (Agzamov RA 1988, Schepetkin, Xie et al. 2009). In another study, the effects of shilajit on anxiolytic and nanotropic activities were evaluated in albino rats using elevated a plus-maze technique and passive avoidance learning acquisition and retention technique, respectively (Agarwal, Khanna et al. 2007). It was found that shilajit had significant anxiolytic and nanotropic activities (Agarwal, Khanna et al. 2007).

The effects of shilajit on diabetes mellitus was assessed by Bhattacharya in 1995 (Agarwal, Khanna et al. 2007). Diabetes mellitus was induced in rats by the administration of streptozotocin (STZ) (Agarwal, Khanna et al. 2007). On days 7, 14, 21, and 28, the pancreatic islet cells' superoxide dismutase activities and hyperglycemia were measured for these animals after receiving two different

doses of shilajit (50 and 100 mg/kg p.o.) (Agarwal, Khanna et al. 2007). The two doses of shilajit diminished the STZ hyperglycemic response starting from day 14 onwards and were statistically significant at the higher dose, while both doses had no effects on the level of blood glucose in the normal animals. (Agarwal, Khanna et al. 2007). In addition, the STZ-induced decrease in superoxide dismutase activity was reduced by both doses starting from day 14 onwards and was statistically insignificant at the lower dose. It was concluded that shilajit could be a treatment option for treating diabetes mellitus in adults (Bhattacharya 1995, Agarwal, Khanna et al. 2007).

It was shown that shilajit helped in mineral transfer such as Mg2+, Ca2+ and phosphate into bone and muscle tissues (Shvetskii AG 1978, Jung CR 2002). In the Russian military, shilajit was used for almost four decades to increase muscle mass and strength. (Jung CR 2002). The effects of shilajit on bone fracture healing have been reported in a few Russian studies (Kelginbaev NS 1973, Tkachenko SS 1979, Jung CR 2002). In one study, the use of shilajit improved bone healing in children after fractures (Kelginbaev NS 1973). In another study, it was found that a daily dose of 0.1 g/kg of shilajit (orally) accelerated callus formation and phosphate uptake in bone fracture model systems (Shakirov 1965, VN. 1965, Jung CR 2002). In addition, Tkachenko et al. showed that the effects of shilajit on bone healing varied based on the dose and time of delivery after surgery (Tkachenko SS 1979, Jung CR 2002). The most favorable bone healing results were observed when shilajit was given at oral

doses between 260 to 300 mg/kg daily starting as early as possible after surgery and continuing for <7 days (Tkachenko SS 1979). In these groups, shilajit enhanced osteoid formation and mineralization around 2 fold (Jung CR 2002). The same study observed a reduction in bone mineralization when shilajit was given for 2-3 weeks following surgery using the same doses (Tkachenko SS 1979, Jung CR 2002). In a study by Jung et al, the effects of shilajit on osteoclastogenesis and osteoblastic differentiation in cell culture assays of human bone marrow mesenchymal stem cells (hMSCs) and human fetal osteoblasts (hFOs) were investigated (Jung CR 2002). Calcium deposition and the expression of some of the osteoblastic differentiation markers such as osteocalcin, alkaline phosphatase (ALP), Extracellular signal-regulated kinase (ERK), and core-binding factor-1 (Cbfa1) were evaluated after shilajit treatment (Jung CR 2002). It was found that there were a significant increase in ALP activity, osteocalcin, and calcium deposition expression by hMSCs and hFOs after 14 days of incubation with shilajit (3-5 μg/ml) (Jung CR 2002). In addition, the expression of ERK and Cbfa1 were increased in murine pluripotent mesenchymal precursor cell lines (C2C12) and hMSCs (Jung CR 2002). It was also reported that increasing the concentration of shilajit in the presence of RANKL (40 ng/ml) and a specific inhibitor of ERK activity produced a dose-dependent decrease in TRAP-positive multinucleated cell formation from macrophage-like cells (RAW 264.7) (Jung CR 2002). It was concluded that shilajit is an inhibitor of osteoclastogenesis and a potent stimulator of osteoblastic differentiation of mesenchymal stem cells (Jung CR 2002).

There are several patents (Carrasco-Gallardo, Guzman et al. 2012) related to *shilajit* from Nepal and India that are outlined below.

1) No.5405613 (US Patent, vitamin/mineral composition)

2) No.20030198695 (US Patent, Herbo-mineral composition)

3) No.6440436 (US Patent, Process for preparing purified shilajit composition from native shilajit)

4) No.6558712 (US Patent, nutritional, delivery system for pharmaceutical, nutritional and cosmetic ingredients)

5) No.WO 2011/041920 (about phytocomplex with vitamins added)

Data about how shilajit affects the human body based on molecular and cellular mechanisms is limited (Jung CR 2002). Most of the published studies investigating the effects of shilajit were conducted in India since it is mainly extracted and marketed in this region of the world (Carrasco-Gallardo, Guzman et al. 2012). It is very important that more well-designed scientific studies be conducted to extent the scientific evidence about the different effects that shilajit may have in humans. Therefore, the overall goal of the studies described in this dissertation is to evaluate the effects of shilajit on bone and cartilage regeneration/repair during bone fracture and osteoporosis. Findings from these studies will provide the evidence needed to advance treatment approaches to accelerate bone/connective tissue healing, that has few side effects. Given its human use for thousands of years and its apparent ability to promote regeneration/repair, it should be easily translated into the market place and could

become the standard of care for bone/connective tissue pathologies, as well as may have other applications. Other applications could include bone and soft tissue grafting, periodontal disease and bone-implant interface healing. Great discoveries have been made by scientifically examining remedies/treatments that people have used for years based only on the fact that they work especially by identifying the active agent and translating that into practice.

Study 1: Effects of shilajit on osteoblast proliferation and survival

Introduction

Bone is the main component of the musculoskeletal system of vertebrates, which protects and supports the other organs of the body, and acts as a metabolic mineral reserve. Bone consists of extracellular matrix (ECM) and cells (Singh, Mehdi et al. 2012). The ECM has inorganic and organic components. The inorganic components are composed mainly of phosphorus and calcium in the form of hydroxyapatite crystals, while the main constituent of the organic component (approximately 95%) is type I collagen (Singh, Mehdi et al. 2012). There are three major types of cells in bone: 1) osteoclasts, 2) osteoblasts, and 3) osteocytes. Osteoclasts are hematopoietic in origin and are derived from the monocyte-macrophage cell lineage (Roodman 1999). Osteoclasts are multi-nucleated cells that are responsible for bone resorption (Huang, Yang et al. 2007, Soltanoff, Yang et al. 2009, Chen, Deng et al. 2012). Osteoblasts are derived from bone marrow pluripotent mesenchymal stem cells, which can also differentiate into chondrocytes, adipocytes, muscle cells, or fibroblasts (Pittenger, Mackay et al. 1999, Jung CR 2001, Jung CR 2002). Osteoblasts are mono-nucleated cells and synthesize bone, as well as mineralize its matrix. When osteoblast cells get surrounded and trapped by the ECM, they become osteocytes and act as mechanosensors to help in regulating bone structure and mass (Cowin 2002, Power, Loveridge et al. 2002, Singh, Mehdi et al. 2012). The initial bone formation by osteoblasts consists of a randomly oriented collagen

fiber pattern (woven bone), which is replaced by more organized collagen orientations (lamellar bone) (Shapiro 2008). Woven bone is characterized by a weak structure, while lamellar bone is mechanically stronger (Shapiro 2008). The process of bone formation and development is called osteogenesis. The ability of osteoblasts to proliferate and survive increases their cell number and extends the length of time that these cells are forming bone. Therefore, enhancement of osteoblastic activities such as proliferation and survival is one of the therapeutic approaches to treat bone diseases (Jung CR 2002).

The life span of osteoblasts is regulated by many pathways such as apoptosis. Apoptosis is a programmed, energy dependent cell death with no loss of the membrane integrity. The two major pathways for apoptosis are the intrinsic and extrinsic pathways (Figure 1.1.1). The extrinsic pathway can be initiated via one of the cell surface death receptors such as CD40 and sTNF-r1 after binding to its specific ligand. The activation of these receptors can be followed by the activation of caspase 8 and then caspase 3, which induces apoptosis (Fulda and Debatin 2006). The intrinsic pathway is initiated upon the activation of internal signals within the cells as a result of DNA damage, reactive oxygen species (ROS) production, and severe growth factor deprivation (Ashkenazi 2002). There is a balance between anti-apoptotic and pro-apototic proteins within cells. Any alterations in this balance due to one of the above mentioned reasons causes changing in the mitochondrial permeabilty and determines if the cell dies or lives (Yang, Zhu et al., Tsujimoto 1998, Coultas and Strasser 2003). Changing

mitochondrial permeability allows some proteins such as second mitochondria-derived activator of caspases (SMAC), high temperature requirement protein A (HTRA), and cytochrome c to be trans-located from the mitochondria to the cytosol. This leads to the activation of caspase 9 and then caspase 3, which induces the apoptotic process.

Aims

- To determine the cytotoxicity level of shilajit on human-derived osteosarcoma cell line (osteoblast like cells, MG63, ATCC**,** Manassas, VA) by measuring the proliferation and membrane damage of the treated cells.
- To evaluate the effects of shilajit on MG63 proliferation and survival/viability in long term cell cultures using serum free media.

Study 2: Effects of shilajit on the expression of osteogenic and connective tissue biomarkers

Introduction

Bone and connective tissue healing/regeneration can be enhanced by increasing the protein expression of several osteogenic and connective tissue biomarkers such as the collagens. Type I collagen is the most abundant collagen type in practically all connective tissues (Viguet-Carrin, Garnero et al. 2006). It represents around 95% of the total collagen in bone and approximately 80% of all the proteins formed in bone (Niyibizi and Eyre 1994, Viguet-Carrin, Garnero et al. 2006). Collagen type 1 provides elasticity and enhances tissue structure in all connective tissues and enhances the mechanical properties of bone (Viguet-Carrin, Garnero et al. 2006). It has been shown by many studies that the collagen matrix is very important key factor for bone toughness (Bailey, Wotton et al. 1992, Boskey, Wright et al. 1999, Zioupos, Currey et al. 1999, Thompson, Kindt et al. 2001, Wang, Bank et al. 2001, Zioupos 2001, Viguet-Carrin, Garnero et al. 2006). In addition to collagen type 1, osteocalcin, osteonectin, ALP, ERK and Cbfa1 are other osteoblastic differentiation markers, which can be used to evaluate the process of bone formation and healing (Jung CR 2002).

Similar to other human tissues, bone can respond to both bone-specific and bone non-specific growth factors (Long 2001). Shilajit contains inorganic and organic agents, which might regulate bone healing in a similar way to these

growth factors. Therefore, understanding the different mechanisms by which growth factors regulate bone growth and repair is very important to understanding how shilajit affects bone formation. The effects of several growth factors and cytokines such as transforming growth factor-beta (TGF-β) superfamily on bone formation during mammalian growth are well documented (Urist 1965, Katagiri and Takahashi 2002, Guo and Wang 2009, Chen, Deng et al. 2012). Disruptions in the functions of some of the members of the TGF-β superfamily results in bone diseases such as osteoarthritis (Siegel and Massague 2003, Miyazono, Maeda et al. 2005, Papachroni, Karatzas et al. 2009, Chen, Deng et al. 2012). The TGF-β superfamily contains more than forty members, which include Nodal, TGF-βs, Activin, and bone morphogenetic proteins (BMPs). They have the ability to regulate cell growth and differentiation during the processes of development and repair (Sporn and Roberts 1985, Long 2001, Guo and Wang 2009, Chen, Deng et al. 2012). TGF- β has positive effects on bone growth by promoting early osteoprogenitor differentiation and proliferation (Chen, Deng et al. 2012). Osteoblastic differentiation can also be enhanced by cooperation between the TGF-β and the Wnt signaling pathways (Chen, Deng et al. 2012). In addition to intramembranous bone formation, it has been documented that TGF-β isoforms and their receptors (type I and II) play very important roles in endochondral bone formation by enhancing the deposition of matrix components during chondrogenesis (Ignotz and Massague 1986, Massague 1987, Long 2001, Chen, Deng et al. 2012). Chondrocyte maturation and proliferation can be induced by BMP-2 during endochondral ossification

(Shu, Zhang et al. 2011, Chen, Deng et al. 2012). The expression of osteocalcin is greatly enhanced by BMP-2 (Huang, Ren et al. 2010, Chen, Deng et al. 2012). The bone volume of an ectopic bone formation stimulated by TGF-β1 and BMP-2 together was five-fold more than the one stimulated by BMP-2 alone (Shu, Zhang et al. 2011, Tachi, Takami et al. 2011, Chen, Deng et al. 2012). In addition to BMP-2, other BMPs (BMP-4, -5, -6, and -7) also have important roles in osteogenesis (Chen, Deng et al. 2012). The expression of multiple osteoblastic differentiation markers such as ALP activity and osteoblast mineralization are stimulated by BMP-7 (Gu, Zhang et al. 2004, Shen, Wei et al. 2010, Chen, Deng et al. 2012). While osteogenesis can be impaired by the absence of BMP-2, it has been shown that the loss of BMP-7 had no effect on bone mass preservation and post-natal limb growth in studies *in vivo*. This suggested that the other BMPs were compensating for BMP-7 (Bandyopadhyay, Tsuji et al. 2006, Tsuji, Cox et al. 2010, Chen, Deng et al. 2012).

Besides the BMPs, there are additional growth factors that are capable of regulating bone formation such as bone-derived growth factor (BDGF), which induces bone cell proliferation in serum-free media (Hanamura, Higuchi et al. 1980, Urist, DeLange et al. 1983, Linkhart, Jennings et al. 1986, Long 2001). Another example is basic fibroblast growth factor (bFGF), which plays a role in stimulating osteoprogenitor cell proliferation (Long, Robinson et al. 1995) and enhancing fracture-repair in an *in vivo* rat model system (Noda and Vogel 1989, Mayahara, Ito et al. 1993, Kawaguchi, Kurokawa et al. 1994, Long 2001). Insulin-

like growth factors (IGFs) play major roles in bone growth and have been implicated in osteogenesis (Long 2001). IGFs can be synthesized by osteoblasts and are the most abundant mitogens in the ECM of human bone (Mohan 1993, Long 2001). They activate MAP kinases such as ERK-1 and ERK-2 (Chaudhary and Avioli 1998) and stimulate osteoblast proliferation (Mohan 1993, Thomas, Gori et al. 1999, Zambonin, Grano et al. 1999, Farley, Dimai et al. 2000, Long 2001). In a study by Komori et al., IGF levels were found to be dysregulated in postmenopausal osteoporosis (Komori, Yagi et al. 1997, Long 2001). Multiple differentiation markers have been used repeatedly in the literature to evaluate osteoblast differentiation and bone growth, and include ALP, osteocalcin, and osteonectin. ALP is one of the early bone differentiation markers, which is secreted by osteoblasts during the process of osteogenesis and osteocalcin is a bone differentiation marker that is present later in the process (Weinreb, Shinar et al. 1990, Jung CR 2002). Osteocalcin is a calcium binding bone protein (57,000 Da) and is vitamin K-dependent. Its functions are not clear, but it is believed that it plays important roles in regulating mineralization during bone formation (Price, Otsuka et al. 1976, Termine, Kleinman et al. 1981, Ducy, Desbois et al. 1996, Long 2001). Osteonectin is a protein (43,000 Da) that is secreted by osteoblasts to promote their differentiation (Barbara Obermayer-Pietsch 2011). It has the ability to bind to hydroxyapatite, calcium, and collagen. It forms a connection between the organic phase and mineral in bone (Kelm, Swords et al. 1994, Long 2001). Therefore, it is believed to initiates mineralization and support mineral crystal formation. In addition, it has an

important role in collagen binding, collagen matrix formation and cell matrix interactions (Barbara Obermayer-Pietsch 2011). The gene expression of the above mentioned differentiation markers, such as type I collagen, bone sialoprotein, and osteopontin, can be up-regulated by Cbfa1 (Harada, Tagashira et al. 1999, Jung CR 2002). Cbfa1 is the first osteoblast-specific transcription factor to be recognized and is present in mesenchymal cells that differentiate into osteoblasts or chondrocytes and it plays a crucial role in maintaining the osteoblast phenotype (Handagama, Bainton et al. 1993, Ducy, Desbois et al. 1996, Kadiyala, Young et al. 1997, Lian, Stein et al. 1998, Franceschi 1999, Long 2001).

Recently, there has been an increase in the number of therapeutic strategies to improve bone formation and healing. Bone regeneration can be stimulated by multiple growth factors, which are capable of activating many cellular activities via signaling cell-surface receptors. Following the first description of the BMPs by Marshall Urist in 1965 (Urist 1965), many clinical applications of the BMP proteins have improved conditions such as segmental bone defects, open fractures, pseudarthrosis, spinal fusion, nonunion, and delayed union of fractures (Johnson, Urist et al. 1990, Chen, Zhao et al. 2004, Carlisle and Fischgrund 2005, Kanakaris and Giannoudis 2008, Mulconrey, Bridwell et al. 2008). For example, it has been shown that the rehabilitation of a critical-size bone defect in a mice model is enhanced after the use of BMPs (Chen, Deng et al. 2012). Although recombinant human BMP-2 is effective in

inducing osteogenesis, several drawbacks limit its clinical uses such as high cost, unwanted ectopic bone formation, and potentially life-threatening inflammation (Miyamoto, Takaoka et al. 1992, Benglis, Wang et al. 2008, Cahill, Chi et al. 2009). Therefore, studies are ongoing to find alternative osteoinductive agents other than members of the BMP family.

Aims

- To analyze the gene expression of MG63 and human gingival fibroblasts (HGFs) using Microarray illumina technology to identify the significantly up-regulated/down-regulated genes after shilajit treatment.
- To evaluate the effects of shilajit on ECM protein secretion in long term cell cultures using serum free media.

Study 3: Roles of shilajit on chondrogenesis

Introduction

Bone regeneration and growth are achieved by two main processes: a) intramembranous ossification, in which bones are formed due to the condensations of mesenchymal cells without the need for cartilage formation, and b) endochondral ossification, in which a cartilage formed earlier is replaced and mineralized to form bone (Soltanoff, Yang et al. 2009, Chen, Deng et al. 2012). These processes are also utilized for bone regeneration during fracture and repair (Shapiro 2008). Bone repair can be classified into four histological categories. Category 1 is endochondral bone repair, which is characterized by callus formation in which cartilage is first formed, followed by woven and lamellar bone formation. Category 2 is primary bone repair (direct contact repair without a cartilage phase) where necrotic bone at both fracture sites is resorbed by osteoclasts, and is followed by lamellar bone synthesis by osteoblasts in a parallel manner to the longitudinal axis of the bone. Therefore, no bone re-modeling is required for this process. This type of bone repair occurs in an environment of rigid stability and lacks an interfragmentary space. Category 3 is direct bone repair (gap repair without a cartilage phase), which involves bone formation (woven and lamellar) perpendicular to the long axis of the bone in an environment of rigid stability but with an interfragmentary space > 0.1 mm. For this reason, bone remodeling is needed in this scenario. Category 4 is distraction osteogenesis (callotasis) where woven and then lamellar bone in the gap are

formed parallel to the long axis of the bone in an environment with slow distraction and less stability (Shapiro 2008). Following bone fracture, bone repair cells can originate from: a) the periosteum inner layer, which is the main source for endochondral bone repair, b) the lining cells of the inner cortex (endosteal cells), c) the osteoprogenitor cells derived from blood vessels of the Haversian systems within the cortical bone, and/or d) the bone marrow's undifferentiated mesenchymal cells (Shapiro 2008). Others have claimed that the undifferentiated cells of the surrounding connective tissue and muscles could also be a source for bone repair cells (Shapiro 2008).

Cartilage is composed mainly of chondroblasts, cells specialized in producing large amounts of extracellular matrix (Cole 2011). Chondroblasts can become entrapped in the cartilage they produce and are then called chondrocytes. They are responsible for maintaining the matrix, as well as for its synthesis (Bhosale and Richardson 2008, Lubis and Lubis 2012). The extracellular matrix consists of collagen type II fibers, in addition to lesser amounts of collagen type IX, X, and XI (Cole 2011). The process of cartilage formation and growth is called chondrogenesis. In addition to the role of cartilage in endochondral bone formation, cartilage is a very important tissue structure involved in most of the joints in human body. Cartilage has been classified histologically based on the distribution of fibers within the extracellular matrix into elastic, fibrous, and hyaline cartilage (Cole 2011). Elastic cartilage contains extra elastin fibers, while fibrous cartilage contains areas of organized fibrous tissue

found in the extracellular matrix. Hyaline cartilage is the most abundant cartilage found in the body such as in the knee joints (Cole 2011).

Cartilage damage can occur as a result of direct trauma, chronic degeneration, or subchondral bone abnormalities. Unlike most human connective tissues, cartilage involved in the joints does not contain blood vessels and it heals very slowly. Cartilage damage can be classified into partial thickness defects and full thickness defects (Cole 2011). The partial thickness defects does not reach the subchondral zone leading to poor healing of the defects, while the full thickness defects reach the subchondral bone and have access to the progenitor cells of the bone marrow making them accessible to spontaneous repair.

The death of chondrocytic cells is an early result of cartilage injury that is followed at a later stage by extracellular matrix degradation and results in cartilage diseases such as osteoarthritis (Jeffrey, Gregory et al. 1995, Hashimoto, Takahashi et al. 1998, Quinn, Allen et al. 2001, Lo and Kim 2004, Morel and Quinn 2004, McKinley, Borrelli et al. 2010). Osteoarthritis is the most common type of arthritis characterized by wearing out of cartilage surface of a joint. It is believed that the inhibition of chondrocyte cell death could be the first step in preventing cartilage injuries from progressing to osteoarthritis. The treatment of osteoarthritis remains one of the most difficult challenges in medicine. Most of the management of osteoarthritis is non-surgical (Walker-

Bone, Javaid et al. 2000), although mesenchymal stem cells regeneration has been successfully used (Gupta, Das et al. 2012). TGF-β1 has been reported to stimulate chondrocyte activity (Blaney Davidson, van der Kraan et al. 2007), while growth differentiation factor-5 (GDF-5) has been shown to stimulate cartilage matrix synthesis (Fortier, Barker et al. 2011). Studies on cartilage regeneration have demonstrated promising results but more pre-clinical and clinical studies are needed to establish appropriate techniques for regeneration applications in humans (Lubis and Lubis 2012).

Aim

- To evaluate the effects of shilajit on chondrogenesis using a Xenopus model system.

Study 4: Effects of shilajit on osteoporosis

Introduction

Bone remodeling is a continuous process that occurs throughout life and the integrity of this process is maintained by a functional balance between osteoblasts and osteoclasts, which are two major types of bone cells involved in bone remodeling. Imbalance between the functions of osteoblasts and osteoclasts can lead to bone diseases such as osteoporosis (Chen, Deng et al. 2012). Osteoporosis is the most common bone disease in the United States. It affects approximately 10 million Americans (Orsini, Rousculp et al. 2005). It is characterized by low bone mass and structural deterioration of the bones, which reduce bone strength and increases the risk of fractures (Holroyd, Cooper et al. 2008, Silva and Bilezikian 2010). Each year, more than 1.5 million osteoporosis-related fractures occur (Orsini, Rousculp et al. 2005). In 2000, around 9.0 million clinical osteoporotic fractures worldwide were estimated to occur. Among these, 1.6 million were at the hip, 1.7 million at the forearm, and 1.4 million at vertebral sites (Johnell and Kanis 2006). Significant morbidity, mortality, and health care costs are all associated with osteoporosis-related fractures (Johnell and Kanis 2006, Holroyd, Cooper et al. 2008).

There are two main therapuetic approaches for the treatment of osteoporosis: 1) anti-resorptive agents such as calcitonin, raloxifene, bisphosphonates, and estrogen, and 2) anabolic agents such as parathyroid

hormone (PTH) or its analogs (Silva and Bilezikian 2010). Teriparatide (PTH 1-34) is the human recombinant peptide representing the first 34 amino acids of the PTH and is an approved treatment for patients with osteoporosis (Datta 2011). Intermittent exposure to PTH 1-34 stimulates the differentiation and activities of osteoblast cells and lead to an increase in trabecular bone mass and to a lesser extent cortical bone thickness (Hock and Gera 1992, Kulkarni, Wei et al. 2007, Datta 2011). The increase effects of PTH 1-34 on trabecular bone volume is due to enhancing the modeling of trabecular bone surface and changing basic multicellular unit (BMU) bone balance in favor of bone formation (Allen 2006). PTH 1-34 also enhances bone mass over time by increasing the bone remodeling rate of the trabecular surfaces (Allen 2006). Although the effects of PTH 1-34 on enhancing periosteal bone formation in humans is controversial, it stimulates both endocortical and periosteal bone formation in animals (Hirano, Burr et al. 1999, Jerome, Johnson et al. 1999, Hirano, Burr et al. 2000, Burr, Hirano et al. 2001, Mashiba, Burr et al. 2001). Periosteal expansion of the cortical bone significantly enhances bone strength and quality (Ahlborg, Johnell et al. 2003, Orwoll 2003, Allen, Hock et al. 2004). Nevertheless, prolonged use of PTH 1-34 can lead to osteosarcoma, hypercalcemia, and hypercalciurea (Alves de Oliveira, Szejnfeld et al. 2010, Datta 2011).

Recently, strontium has been introduced as a new therapeutic treatment for osteoporosis (Meunier, Roux et al. 2004, Reginster, Seeman et al. 2005). Strontium is an element, which has a high affinity for bone (Dahl, Allain et al.

2001). Unlike most pharmacological approaches, it has been suggested that strontium promotes bone formation and inhibits bone resorption simultaneously (Marie 2006). The molecular mechanisms for the effects of strontium on bone synthesis are not fully understood (Marie 2010).

Although many well-controlled clinical trials documented that anti-resorptive and anabolic agents reduce the risk of osteoporosis-related fractures, several side effects limit their widespread clinical applications such as osteonecrosis of the jaws and upper gastrointestinal disorders including esophagitis, dysphagia and gastric and esophageal ulcer (2010, Abrahamsen 2010). Therefore, investigators continue to search for better alternatives with no side effects and these include traditional medicines (Black, Cummings et al. 1996, Cummings, Black et al. 1998, Harris, Watts et al. 1999).

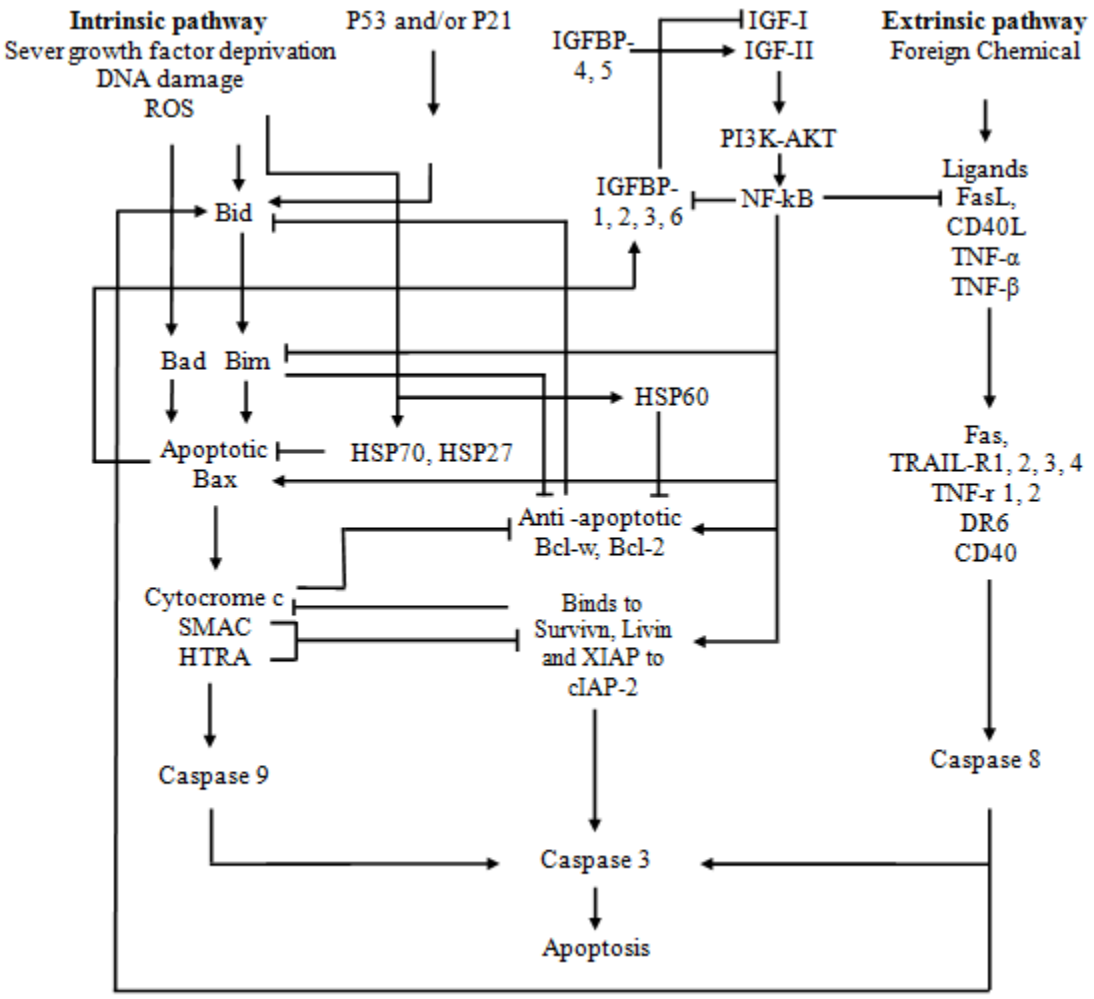
Aim

- To assess the anabolic properties of shilajit on bone mass using an osteoporotic rat model.

Dissertation Outline

This dissertation includes five chapters. The first chapter is a general introduction and review of literature about shilajit, bone structure, formation, repair, growth factors, types and structure of cartilage, and osteoporosis. The second chapter describes the different materials and methods of the three main studies evaluating the effects of shilajit on bone and cartilage regeneration using both *in vitro* and *in vivo* approaches. Chapter three reports the results of these studies and chapter four discusses the results in relationship to each other and other similar studies in the literature. Finally, chapter five is the conclusions and summary of this dissertation including future directions and studies.

Figure 1.1.1. Extrinsic and intrinsic apoptotic pathways.



Batarseh, Ghada thesis 2011. TEGDMA INDUCTION OF APOPTOTIC PROTEINS IN PULP FIBROBLASTS

CHAPTER TWO

Materials and Methods

Agent preparation

Crude shilajit was purchased from an Al Hosaini store located in Riyadh, Saudi Arabia and stored at room temperature. Eight milligram per milliliter of fresh aqueous solution of the agent was prepared by dissolving 400 mg of the agent in 50 ml of serum free Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, UT). Another shilajit stock solution was prepared in either saline or phosphate buffer solution (PBS) and used in the *in vivo* experiments. All the preparations were filtered through 0.22 µm filters and stored in 4°C until utilized.

Study 1: Effects of shilajit on osteoblast proliferation and survival

1.1: Effects of shilajit on cytotoxicity

Cell culture

MG63 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) (Figure 2.1.1.1). They were cultured in 10 ml cell culture dishes and incubated in low glucose (1 g/L) DMEM at 37°C, and 5% CO_2. DMEM was supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 U/ml

penicillin (Invitrogen, Carlsbad, CA), 4 mM L-glutamine (Hyclone), 2.5 µg/ml fungizone (Invitrogen) and 50 µg/ml gentamicin (Invitrogen).

WST-1 assays

Cell toxicity was determined by measuring the mitochondrial dehydrogenase activities during proliferation and Lactate dehydrogenase (LDH) activity after membrane damage utilizing dose responses. Water-soluble tetrazolium salt (WST-1) assays (Roche Applied Science, Penzberg, Germany) were utilized to measure mitochondrial dehydrogenase activities for MG63 cells with and without shilajit at different concentrations after 24 hours of incubation. Subconfluent MG63 cells were seeded into 6-well plate (200,000 cells/well) and incubated for 24 hours in DMEM with serum. After 24 hours, the cells were incubated with serum free DMEM in the presence of shilajit at concentrations ranging between 0.4-8.0 mg/ml. A negative control was utilized in the experiments and consisted of cells incubated without shilajit in serum free DMEM. After 24 hours, the media was collected and utilized for the LDH assays, while the cells were exposed to cell proliferation reagent WST-1 (100 µl WST-1 and 900 µl serum-free DMEM) after washing 3 times with 2 ml of serum-free DMEM. The cells were then incubated for one and half hour in a humidified atmosphere at 37°C and 5% CO_2. After incubation, a 100 µl from each well of the 6-well plates were placed into a 96-well plate. Blank controls were used in the experiments and consisted of 100 µl WST-1 and 900 µl serum-free DMEM only. Finally, the absorbance values of the treated samples, negative control and the

blank were measured using a microplate reader (Titertek, Multiskan MCC, Flow Laboratories, McLean, VA) at 450 nm absorbance. The experiments were repeated three times and their mean values were calculated. The following equation was used to calculate the percentage of the absorbance value of the treated cells at each concentration in comparison to the absorbance value of the untreated cell. The percentage of cell proliferation (%)=(absorbance value of the treated cells /absorbance value of untreated cell) ×100%**.** The percentage cell proliferation (%) of the untreated cell was considered 100%.

LDH assays

LDH activity was measured by a coupled enzymatic reaction, in which tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride is reduced to formazan. Membrane damage was assessed using a cytotoxicity detection kit plus (Roche Applied Science, Indianapolis, IN) to evaluate the levels of LDH released by the cells into the media. A 100 µl from the collected media of each well of the 6 well-plate as described previously earlier was transferred into a 96-well plate. A 100 µl of reconstituted mix prepared as described by the manufacturer was then added to each well and the plate incubated for 15 minutes at room temperature. A microplate reader (Titertek, Multiskan MCC, Flow Laboratories, Mclean,VA) was used to evaluate the colorimetric change quantitatively at 490 nm absorbance to determine the concentrations of shilajit that were toxic to the cells. The experiments were repeated three times for each group and their mean values calculated. The positive control (total cell death)

consisted of cells incubated without shilajit and then treated with lysis solution (1.9 ml of serum-free DMEM and 100 µl lysis solution provided by manufacturer) for 30 minutes before measuring the LDH activity as described by the manufacturer and gives the maximum release of LDH. The following equation was used to calculate the percentage cytotoxicity as determined by LDH release from the treated cells relative to the low control (minimum release of LDH):

Cytotoxicity (%)=(absorbance value of the treated cells - absorbance value of untreated cell) / (absorbance value of positive control - absorbance value of untreated cell) ×100%.

The percentage cytotoxicity (%) of negative control was considered 0% and the positive control percentage cytotoxicity (%) was considered 100%.

Statistical analyses

Statistical analyses were performed using the statistical package for social science (SPSS) 11.5 (SPSS Inc., Chicago, IL). The differences between the means of the different concentrations and the mean of control were evaluated using independent multiple T tests. For all comparisons, differences were considered significant at $p<0.05$.

1.2: MG63 Viability/Proliferation and LDH activity up to 33 Days

WST-1 assays were used to measure mitochondrial dehydrogenase activities for MG63 cells with and without shilajit at different time points up to 33 days of incubation and without media exchange. Subconfluent MG63 cells were seeded into 6-well plates (200,000 cells/well) and incubated for 24 hours in DMEM with serum. After 24 hours, the cells were incubated with serum free DMEM in the presence of shilajit at a final concentration of 0.8 mg/ml for 33 days. A negative control consisted of cells incubated without shilajit in serum free DMEM. On days 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 33, media was collected and utilized for the LDH assays, while the cells were exposed to cell proliferation reagent WST-1 as described by the manufacturer. The experiments were repeated three times for each time point and their mean values were calculated. The following equations were used to calculate the percentage of the absorbance value of the treated cells and untreated cell control at each time point in comparison to the absorbance value of the untreated cell at day 3. For the WST-1 assays, the percentage of cell viability/proliferation (%)=(absorbance value of the treated cells or untreated cell control /absorbance value of untreated cell at day 3) ×100%**.** The percentage cell viability/proliferation (%) of the untreated cell at day 3 was considered 100%. For LDH assays, the percentage cytotoxicity (%) = (absorbance value of the treated cells or untreated cell control - absorbance value of untreated cell at day 3) / (absorbance value of positive control at 3 days - absorbance value of untreated cell at day 3) ×100%. The percentage

cytotoxicity (%) of negative control at 3 days was considered 0% and the positive control percentage cytotoxicity (%) at 3 days was considered 100%.

Statistical analyses

The differences between the groups were evaluated using one-way analysis of variance (ANOVA). When a significant overall F value ($F<0.05$) was present, differences between the individual group means were tested using Dunnett's T3 post hoc tests. For all the tests, differences were considered significant at $p<0.05$.

1.3: Evaluation of apoptotic protein expression

Preparation of cell lysates

Subconfluent MG63 cells were seeded in 6-well plate (200,000 cells/well) and incubated for 24 hours in DMEM with serum. After 24 hours, the cells were incubated with serum free DMEM in the presence of shilajit at a final concentration of 0.8 mg/ml for 7 days. A negative control consisted of cells incubated without shilajit in serum free DMEM. On day 7, cell lysates were prepared for the membrane array according to the manufacturer's protocol (Ray Biotech, Norcross, GA). Briefly, 1 ml of PBS was added to each well after rinsing twice with PBS. The cells were scraped from the bottom of the plates. The cell suspension was collected from each well and transferred to microfuge tubes and centrifuged for 10 minutes to obtain the cell pellets. The PBS was then removed before adding the lysis buffer containing a protease inhibitor cocktail as described by the manufacturer. The cell pellets were solubilized by pipetting up and down followed by gentle shaking at 4°C for 20 minutes. The cell lysates were then centrifuged at 14,000 x g for 10 minutes before collecting the supernatant. Finally, the protein concentration of the supernatant for each group was measured using the Bio-Rad Protein Assay kit (Hercules, CA) and then stored at -80°C until utilized.

RayBio apoptosis array

The RayBio® Human Apoptosis Antibody Array kit (Ray Biotech, Norcross, GA) has the ability to simultaneously detect the relative level of 43 apoptosis related proteins (Table 2.1.3.1). Comparisons between the different groups can be achieved by monitoring the changes in the sizes and densities of the protein dots from the membrane exposed X-ray films. The experiment was repeated four times according to the manufacturer's protocol. Briefly, the cell lysates from the different groups were incubated with the antibody array membranes after they had been incubated with blocking buffer for 30 minutes. The membranes were washed several times with washing buffer I and II before being incubated with a cocktail of biotin-conjugated anti-apoptotic protein antibodies for 2 hours. The membranes were then incubated with horseradish peroxidase (HRP)-streptavidin before visualizing the signals by chemiluminescence on the X-ray films. Finally, quantity one analysis software (Bio-Rad, Hercules, CA) was utilized to analyze the images obtained on the X-ray films. The experiments were repeated four times and their mean values calculated.

Statistical analyses

The ratio of treated to control of each protein was calculated for each of the four experiments. One-sample t-tests were used to determine if the ratio was significantly different from 1, which would indicate a significant difference

between treated and control. The differences were considered significant at $p<0.05$ on a two tailed test.

Study 2: Effects of shilajit on the expression of osteogenic and connective tissue biomarkers

2.1: Gene expression analysis of MG63 and HGF cells

Cell culture

Human gingival fibroblast cells (HGFs) harvested from healthy human gingival tissues as previously described by Zhou and Windsor at Indiana University School of Dentistry were utilized in the following experiments (Zhou and Windsor 2006). Briefly, the healthy human gingival tissues were kept in PBS after crown lengthening surgeries until they were transferred to the lab. They were then washed with 70% ethanol followed by rinsing with PBS to remove the ethanol. After that, the tissues were cut into small pieces (1 to 3 mm), placed in 10 ml cell culture dishes, air dried and incubated in low glucose (1 g/L) DMEM for 7 days at 37°C, and 5% CO_2. DMEM was supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), 4 mM L-glutamine (Hyclone), 2.5 µg/ml fungizone (Invitrogen) and 50 µg/ml gentamicin (Invitrogen). The cells that grew out of the tissue fragments were subcultured and utilized later in the experiments at passages between 4 and 8 (Figure 2.2.1.1). Institutional Review Board (IRB) approval (0609-60) and Informed Patient Consent for obtaining the HGFs were completed before starting the study. MG63 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). They were cultured as described for the HGFs.

Microarray illumina technology

This experiment was conducted in order to determine what genes were being up-regulated and down-regulated in the treated and untreated cells, and to compare these between the groups. The mRNA gene expression was evaluated using Microarray Illumina Technology (SABiosciences, a QIAGEN company, Frederick, MD). Based on this technology, the mRNA expression of more than 47,000 human genes was compared between the groups. Subconfluent cells were seeded into 10 cm cell culture plates (1,000,000 cells/plate) and incubated for 24 hours in DMEM. After 24 hours, the cells were incubated with serum free DMEM in the presence of shilajit at a final concentration of 0.8 mg/ml. A negative control consisted of cells incubated without shilajit in serum free DMEM. Treated and untreated cells at different time points: 0 time point (before adding shilajit), 5, 10, and 15 days after adding shilajit; were washed 3 times with PBS, trypsinized and centrifuged to obtain the cell pellets. The cell pellets were washed with PBS. The supernatant was discarded and cell pellets were stored at -80°C until being shipped to SABiosciences on dry ice.

Statistical analyses

Due to the high cost of microarray Illumina technology, this preliminary study involved only one sample for each group to compare the effects of shilajit on MG63 and HGFs.

2.2: ECM proteins

MG63 cells (200,000 cells per well) were seeded into 6-well plate and then incubated with serum free DMEM with and without shilajit at a final concentration of 0.8 mg/ml for 33 days. On day 33, treated and untreated cells were washed 3 times with PBS, trypsinized, discarded and the plates stained with Coomassie blue to quantify and compare the ECM proteins between the groups. Quantity one analysis software (Bio-Rad, Hercules, CA) was utilized to measure the density of the images obtained from the stained wells. In addition, the ECM proteins of each well were dissolved by 6 M urea and their concentrations were measured using a Bio-Rad Protein Assay kit (Hercules, CA, USA) to further confirm the results obtained by the Coomassie blue stain analysis. The experiments were repeated three times and their mean values calculated.

Statistical analyses

The data were expressed as mean and standard deviation. Comparison between the two groups was made using Student's t-test. The difference was considered significant at $p<0.05$.

Study 3: Role of shilajit on chondrogenesis

3.1: Non-critical size defect Xenopus model system

The Institutional Animal Care and Use Committee (IACUC) approval (10111) was obtained prior to starting the study. Young adult Xenopus were utilized in this study. Each frog had a small segment of the anterior hemisection of the tarsus bone excised to serve as a fracture model. The segments removed were ~10% or less of the total tarsus bone length (Figure 2.3.1.1). According to Feng et al., the critical size defect for this animal model was calculated to be 35.2% of the bone (Feng, Milner et al. 2011). After allowing the incisions a few days to heal, the frogs were divided randomly into 3 groups:

1) Subcutaneous injection of PBS for 4 weeks (negative control),

2) Subcutaneous injection of 3 mg/kg of shilajit for 4 weeks (low-dose treated group), and

3) Subcutaneous injection of 6 mg/kg of shilajit for 4 weeks (high-dose treated group).

Each group had a total of at least 3 animals. Daily subcutaneous injections (300 µl) were administered to the animals in the tarsus limb segment area for 4 weeks. The frogs were then euthanized and the hindlimbs were processed, cryosectioned and stained with hematoxylin/eosin.

Sample collection and fixation

Three to four samples from each group were collected, fixed and prepared for cryosectioning after 4 weeks as previously described by Feng et al. (Feng, Milner et al. 2011). Simply, the frogs were euthanized by over-anesthetization with a 0.3% Tricaine Methane Sulphonate (MS222) solution in osmotically balanced dechlorinated water. Collection of the tarsus limb segments was done using a razor blade to cut just proximal to the ankle joint and just distal to the tarsal-metatarsal joint (Feng, Milner et al. 2011). Washing of the tarsus samples was done three times for 10 minutes in PBS and then they were fixed in 4.0% paraformaldehyde in PBS overnight.

Histology

The fixed samples were prepared for cryosectioning as described by Feng et al. (Feng, Milner et al. 2011). Decalcification of the samples was done using Calci-Clear Rapid (National Diagnostics, Atlanta, GA) solution for 6 hours, then washed with PBS and later cryoprotected by continuous incubation in sterile solutions of 10%, 20%, and 30% sucrose in PBS. Incubation of the samples was done in each sucrose solution for at least 24 hours. The Samples were then transferred into molds containing a 50:50 solution of 30% sucrose in PBS and Neg 50 frozen section medium (Richard-Allan Scientific, Lafayette, CO) and frozen in isopentane cooled by liquid nitrogen. Cryosectioning of the embedded samples was performed using a Leica a CM 1900 cryostat (Buffalo Grove, IL). The samples were cut completely through the defect site at a thickness of 20-30

mm. The sections were then stained with hematoxylin and eosin using standard methods. Nanozoomer digital pathology system (Hamamatsu Photonics, Baltimore, MD) was used to capture the images. NDP.view software (Hamamatsu Photonics, Baltimore, MD) was used to obtain the total cartilage area/total section area ratios by measuring the total section area and the total cartilage area of both the cut and the uncut tarsus bones as they appeared in the histological sections of each group. At least three measurements for the ratios of the total cartilage area /total section area were calculated from each animal at three different regions within the defect. The regions were selected at similar distances within all of the animals proximal to cut site, at the cut site, and in the center of the defect. The mean value for each group was calculated and comparisons were made between the groups.

Statistical analyses

The differences between the groups were evaluated using one-way analysis of variance (ANOVA). When a significant overall F value ($F<0.05$) was present, differences between individual group means were tested using Tukey's post hoc tests. For all the tests, differences were considered significant at $p<0.05$.

3.2: Non-injured Xenopus model system

The Institutional Animal Care and Use Committee (IACUC) approval (10111) was obtained prior to starting the study. Young adult Xenopus were utilized in this study. No surgeries were performed on the animals in this experiment. The animals were randomly divided into three groups:

1) Subcutaneous injection of PBS for two weeks (negative control),

2) Subcutaneous injection of 3 mg/kg of shilajit for 4 weeks (low-dose treated group), and

3) Subcutaneous injection of 6 mg/kg of shilajit for four weeks (high-dose treated group).

Each group had a total of at least 3 animals. Daily subcutaneous injections (300 µl) were administered to the animals in the tarsus limb segment area for 4 weeks. The frogs were then euthanized at the end of the injection cycle and the hindlimbs were processed, cryosectioned and stained with hematoxylin/eosin.

Sample collection, fixation and histology

The frogs were euthanized at the end of the injection cycle and the tarsus limb segments were collected, fixed, prepared for cryosectioning, and stained for histology using the same protocol as described previously for the non-critical size defect model. NDP view software (Hamamatsu Photonics, Baltimore, MD) was used to evaluate any histological changes for the bone tissues.

Study 4: Effects of shilajit on osteoporosis

4.1: Osteoporotic rat model

Indiana University Animal Care and Use Committee (IACUC) approval (DS0000926R) was obtained before starting the study. The total sample size for the study was 72 rats. For handling issues, the study was divided into 2 cohorts and only half of these animals were utilized in the 1st cohort. Therefore, a total of 36 six months old female Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). The rats had ovariectomy (OVX) or sham operations at Harlan Laboratories. One week after the surgeries, the rats were shipped to Indiana University's Laboratory Animal Resource Center. The rats were pair-housed in plastic cages under standard environmental conditions and received Harlan Teklad 2018 rat chow and water ad libitum. Teriparatide parathyroid hormone (PTH 1-34, Bachem Americas Inc., Torrance, California) was used as a positive control and is approved for the treatment of osteoporosis although it has a 2 year maximum treatment limit (Kim and Jahng 1999). PTH was selected since it is the only current anabolic agent used in osteoporosis treatment. Four weeks after the surgeries, the rats were randomized into 6 groups (Figure 2.4.1.1):

1) Saline-treated control sham group (n=6),

2) Saline-treated control OVX group (n=6),

3) PTH-treated control OVX group (n=6, 10 µg/kg/d),

4) Low-dose shilajit treated OVX group (n=6, 3 mg/kg/day),

5) High-dose shilajit treated OVX group (n=6, 30 mg/kg/day),

6) Six rats were used as baseline OVX controls and were euthanized at the start of the treatment period.

The rats for groups 1-5 were treated 1 time/day by intraperitoneal injections (IP, 0.5 ml total volume) for 4 weeks.

Processing and preparation of bone samples

Ten mg/kg calcein was injected into the rats 4 and 14 days before the end of the experiment. Calcein was used to label the active bone formation surfaces. After sacrificing the animals, the bones were collected and stored for analysis (Table 2.4.1.1).

Microcomputed tomography (Micro-CT)

On the proximal tibia metaphysic, trabecular bone volume (BV/TV,%) was assessed using Micro-CT. A resolution of 8 µm for all scans was used with the bones being wrapped in parafilm to prevent drying during the scan.

Bone histomorphometry

Using standard methods, methyl methacrylate was used to embed the tissues for histology. The midshaft of the right tibia was used to collect the dynamic histomorphometric measurements for the cortical bone analyses. Cross-sections (80-100 µm) were taken at 3 mm proximal to the tibia-fibula junction of

the tibia by a diamond wire saw. For histomorphometric analyses of the cancellous bone, mid-sagittal (8 µm) sections from the proximal tibia was cut using a Reichert-Jung 2050 microtome (Recycledgoods, Ventura, CA). A specific region of interest (~0.8 mm from the growth plate and encompassing 7-8 mm^2) was used. All the sections of the cortical and cancellous bones were left unstained for the dynamic histomorphometric measurements.

All the histomorphometric measurements were obtained using a semiautomatic analysis system (Bioquant OSTEO 7.20.10, Nashville, TN) that was attached to a microscope equipped with an ultraviolet light source (Nikon Optiphot 2 microscope, Melville, NY). The total bone surface, single-labeled surface, double-labeled surface, mineral apposition rate (MAR, distance between calcein labels divided by the time between labels), and mineralizing surface per unit bone surface (MS/BS=([(single labeled surface/2) + double label surface]/surface perimeter) × 100) were measured on all the sections. From these primary measurements, the bone formation rate (BFR=MAR x MS/BS) was calculated for the cortical (endocortical and periosteal surfaces) and trabecular bones. The histomorphometric measurements were performed according to the guidelines of the American Society for Bone and Mineral Research (Parfitt, Drezner et al. 1987).

Statistical analyses

The differences between the groups were evaluated using one-way analysis of variance (ANOVA). When a significant overall F value ($F<0.05$) was present, differences between individual group means were tested using Dunnett's T3 post hoc tests. For all the tests, differences were considered significant at $p<0.05$.

Figure 2.1.1.1. Human-derived osteosarcoma cell line (osteoblast like cells, MG63).

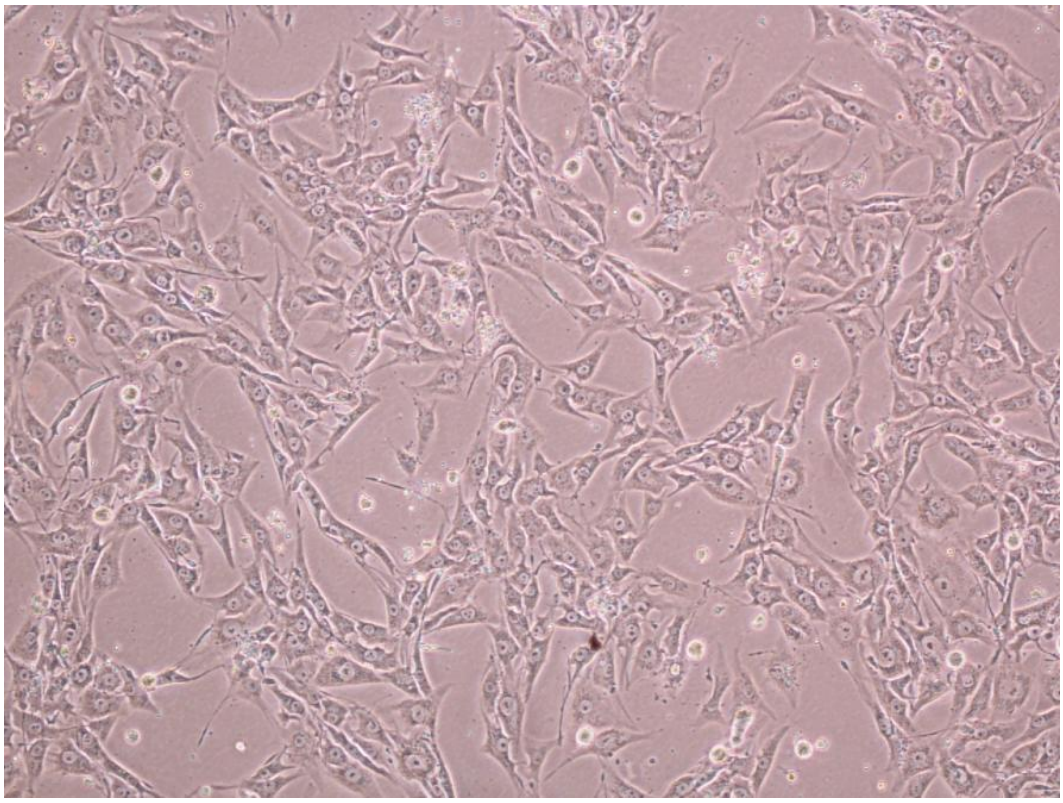


Figure 2.2.1.1. Primary human gingival fibroblast cells (HGFs).

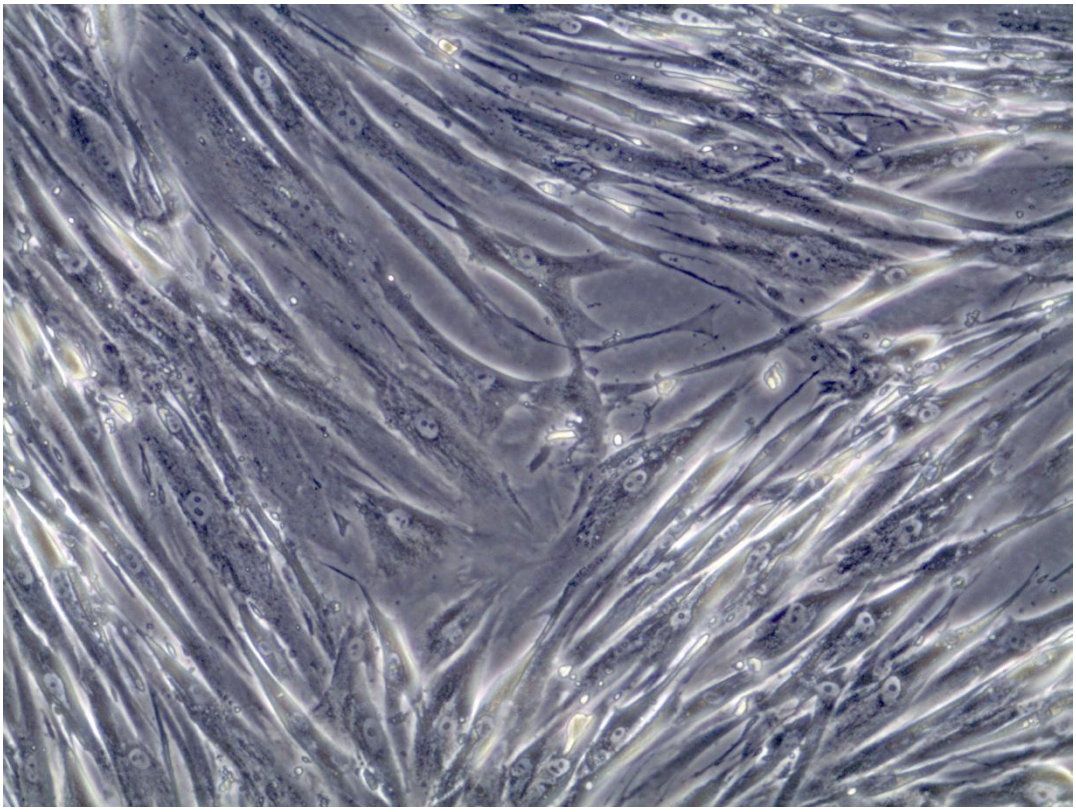


Figure 2.3.1.1. Schematic of non-critical size segmental defect performed on Xenopus' hind limbs.

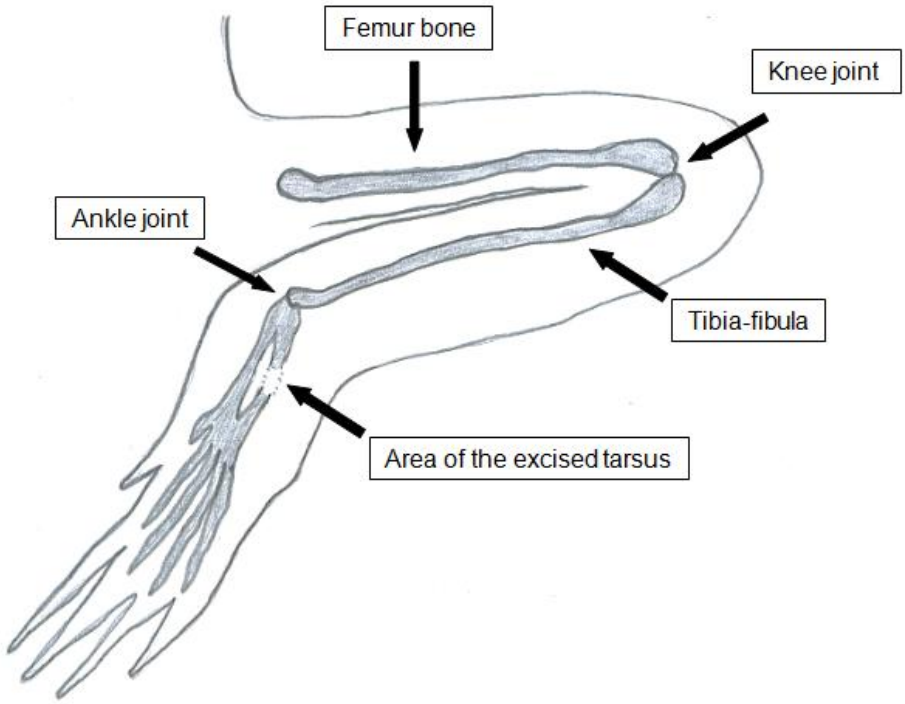


Figure 2.4.1.1. Osteoporotic rat study design: Six-month old female Sprague-Dawley rats (n=36) were ovariectomized (OVX) or Sham-operated. Four weeks after the surgeries, OVX and sham rats were randomized into six groups:, OVX Saline, OVX PTH 10 µg/kg, OVX Shilajit 3 mg/kg, OVX Shilajit 30 mg/kg, Sham Saline, and OVX Baseline. The rats for the first five groups were treated 1 time/day by IP injections for 4 weeks while the rats for the baseline group were euthanized at the start of the treatment period.

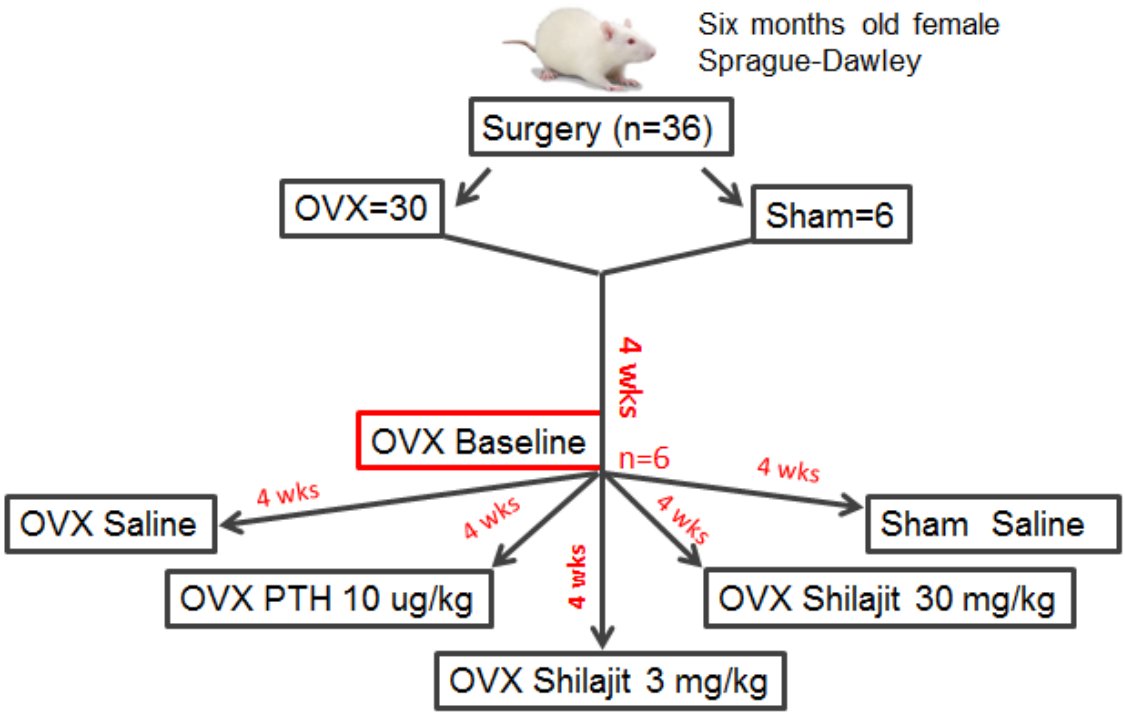


Table 2.1.3.1. RayBio human apoptosis antibody array (www.raybiotech.com).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Pos	Pos	Neg	Neg	Blank	Blank	Bad	Bax	Bcl-2	Bcl-w	BID	BIM	Caspase 3	Caspase 8
2	Pos	Pos	Neg	Neg	Blank	Blank	Bad	Bax	Bcl-2	Bcl-w	BID	BIM	Caspase 3	Caspase 8
3	CD40	CD40L	cIAP-2	CytoC	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
4	CD40	CD40L	cIAP-2	CytoC	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
5	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	Blank	Livin	P21	P27	P53	SMAC	Survivin
6	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	Blank	Livin	P21	P27	P53	SMAC	Survivin
7	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Neg	Neg	Neg	Pos
8	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Neg	Neg	Neg	Pos

Bad: Bcl2 Antagonist of Cell Death; Bax: Bcl-2-Associated X Protein; Bcl-2: B-cell Lymphoma 2; BID: BH3-Interacting Domain Death Agonist; BIM: Bcl-2 Interacting Protein BIM; Bcl-w: Apoptosis Regulator Bcl-w; IAP: Inhibitor of Apoptotic Protein; CytoC: Cytochrome c; DR: Death Receptor; Fas: Fatty Acid Synthetase; FasL: Fatty Acid Synthetase Ligand; HSP: Heat Shock Protein; HTRA2: High Temperature Requirement Protein A2; IGF: Insulin Like Growth Factor; IGFBP: Insulin Like Growth Factor Binding Protein; SMAC: Second Mitochondria-Derived Activator of Caspases; Caspase 8: Cysteinyl Aspartic Acid-protease 8; TNF: Tissue Necrotizing Factor; TRAIL-R: TNF-related Apoptosis-Inducing Ligand Receptor; XIAP: X-linked Inhibitor of Apoptosis Protein; and sTNF-R1: Serum Tumor Necrosis Factor Receptor 1.

Table 2.4.1.1. The storage specifications and the types of analyses utilized for the different bone samples.

Specimen	Storage	Analyses
Right Tibia	10% Neutral buffered formalin	Proximal metaphysic/midshaft: histomorphometry
Left Tibia	PBS-soaked gauze @ -20°C	Stored for future experiments

CHAPTER THREE

Results

Study 1: Effects of shilajit on osteoblast proliferation and survival

1.1: Effects of shilajit on cytotoxicity

WST-1 assays

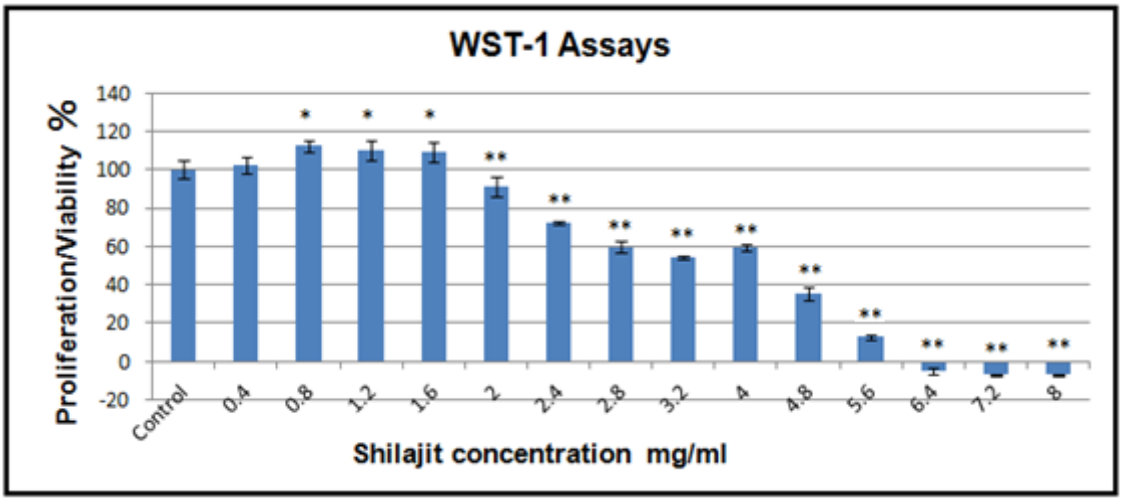
The cell toxicity of shilajit was determined by measuring the mitochondrial dehydrogenase activities during proliferation and LDH activity after membrane damage utilizing dose responses. WST-1 assays (Roche Applied Science, Penzberg, Germany) were utilized to measure mitochondrial dehydrogenase activities for MG63 cells treated with and without shilajit at different concentrations. The percentages of cell proliferation for the MG63 cells after incubation with the different concentrations of shilajit for 24 hours were calculated and compared relative to the controls (Figure 3.1.1.1 and Table 3.1.1.1). The percentage of cell proliferation for the controls was considered as 100%. Shilajit at the concentrations between 0.4 and 1.6 mg/ml increased cell proliferation relative to the controls. These increases were statistically significant for 0.8 (p=0.005), 1.2 (p=0.01), and 1.6 (p=0.017) mg/ml (Table 3.1.1.1). The percentages of the cell proliferation were statistically significantly decreased relative to the low control at concentrations of 2 mg/ml and above (Table 3.1.1.1).

Therefore, the highest concentration of shilajit that did not decrease cell proliferation of the MG63 cells according to the WST-1 assays was 1.6 mg/ml.

LDH assays

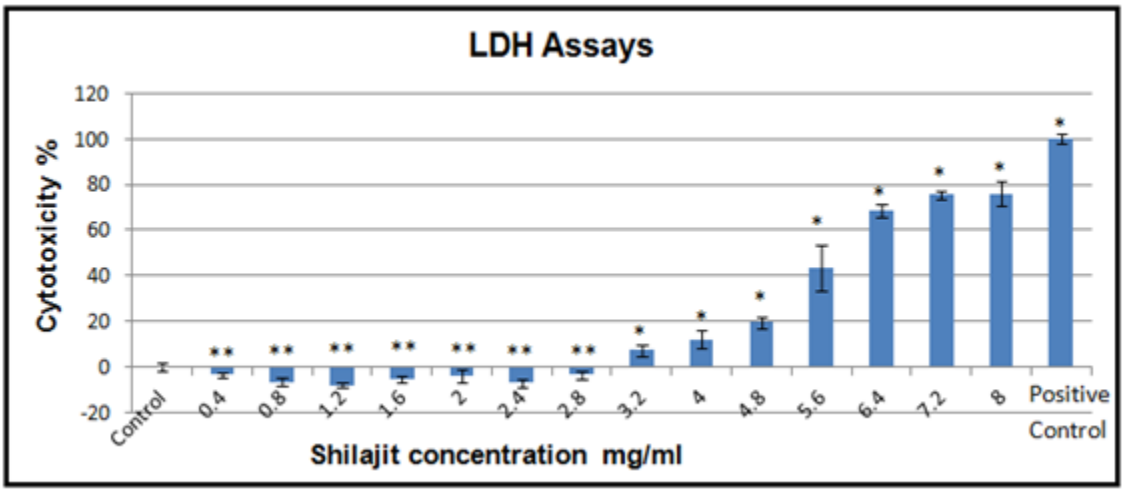
LDH assays (Roche Applied Science, Indianapolis, IN) were utilized to assess membrane damage by measuring the LDH activity released from MG63 cells treated with and without shilajit at different concentrations. The percentages of cytotoxicity for MG63 cells after incubation with the different concentrations of shilajit for 24 hours were calculated and compared relative to the controls (Figure 3.1.1.2 and Table 3.1.1.2). The percentage of cytotoxicity of the negative controls was considered 0%, while the positive control was considered 100%. Shilajit at the concentrations between 0.4 and 2.8 mg/ml statistically significantly decreased the percentages of cytotoxicity relative the control (Table 3.1.1.2). The percentage of cytotoxicity increased relative to the low control at shilajit concentrations of 3.2 mg/ml and above (Table 3.1.1.2). Therefore, the highest non-toxic concentration of shilajit according to LDH assays was 2.8 mg/ml.

Figure 3.1.1.1. Cell proliferation/viability (%) of MG63 evaluated by WST-1 assay after incubation with different concentrations of shilajit for 24 hours. The results are presented as means and the error bars represent standard error (SE). N=3 for each group.



*denote a significant increase relative to the negative control (p<0.05).
**denote a significant decrease relative to the negative control (p<0.05).

Figure 3.1.1.2. Cytoxicity (%) of MG63 evaluated by LDH assay after incubation with different concentrations of shilajit for 24 hours. The results are presented as means and the error bars represent standard error (SE). N=3 for each group.



*denote a significant increase relative to the negative control (p<0.05).
**denote a significant decrease relative to the negative control (p<0.05).

Table 3.1.1.1. Cell proliferation/viability (%) of MG63 evaluated by WST-1 assays. The results are presented as means ± standard errors (SE) and *p* values. N=3 for each group.

Shilajit concentration mg/ml	**% Proliferation/viability relative to the control ± SE**	***p* value**
0.4	102.40±4.39	0.317
0.8	112.44±2.87	0.005*
1.2	110.54±5.2	0.010*
1.6	109.26±5.33	0.017*
2	91.14±5.12	0.018**
2.4	72.18±0.67	0.003**
2.8	59.75±3.07	0.000**
3.2	54.36±0.75	0.001**
4	59.37±1.66	0.000**
4.8	35.31±3.26	0.000**
5.6	12.69±1.29	0.000**
6.4	-4.96±1.98	0.000**
7.2	-7.15±0.45	0.000**
8	-7.23±0.25	0.000**

*denote a significant increase relative to the negative control ($p<0.05$).
**denote a significant decrease relative to the negative control ($p<0.05$).

Table 3.1.1.2. Cytotoxicity (%) of MG63 evaluated by LDH assays. The results are presented as means ± standard errors (SE) and *p* values. N=3 for each group.

Shilajit concentrations mg/ml	**% Cytotoxicity relative to the control ± SE**	***p* value**
0.4	-3.4±0.99	.016**
0.8	-6.54±1.54	.002**
1.2	-8.22±1.06	.001**
1.6	-5.11±1.41	.004**
2	-3.75±2.83	.048**
2.4	-7.18±1.76	.001**
2.8	-3.6±1.9	.019**
3.2	7.23±2.5	.003*
4	12.1±3.92,	.006*
4.8	19.39±2.5	.000*
5.6	43.48±10.11	.006*
6.4	68.61±2.61	.000*
7.2	75.4±2.1	.000*
8	76.06±5.52	.000*

*denote a significant increase relative to the negative control ($p<0.05$).
**denote a significant decrease relative to the negative control ($p<0.05$).

1.2: MG63 Viability/Proliferation and LDH Activity up to 33 Days

WST-1 assays

WST-1 assays were used to measure mitochondrial dehydrogenase activities for MG63 cells with and without shilajit at different time points up to 33 days of incubation without media exchange. The percentages of cell proliferation for the MG63 cells after incubation with and without shilajit for up to 33 days were calculated and compared relative to controls at day 3 (Figure 3.1.2.1 and Table 3.1.2.1). The percentage of cell proliferation for the controls at day 3 was considered as 100%. Shilajit increased MG63 proliferation on days 3 (8.4±4.66%, p=0.788), 6 (32±5.41%, p=0.035), and 9 (10.4±4.49%, p=0.678) when compared to the untreated control at 3 days (Figure 3.1.2.1). The 32% increase in cell proliferation/viability on day 6 was statistically significant relative to 3 day control (Table 3.1.2.1). 47.1±2.35% of shilajit treated cells were alive on day 27 and 14.5±1.87% on day 30 (Figure 3.1.2.1). Total cell death of shilajit treated cells occurred on day 33 (Figure 3.1.2.1). In contrast, the cell viability for the untreated cells started to decrease gradually to 92.5±0.93% and 75.4±3.47% on days 6 and 9, respectively and dramatically to 15.3±2.1% on day 12. Total cell death of untreated cells was observed on day 15.

LDH assays

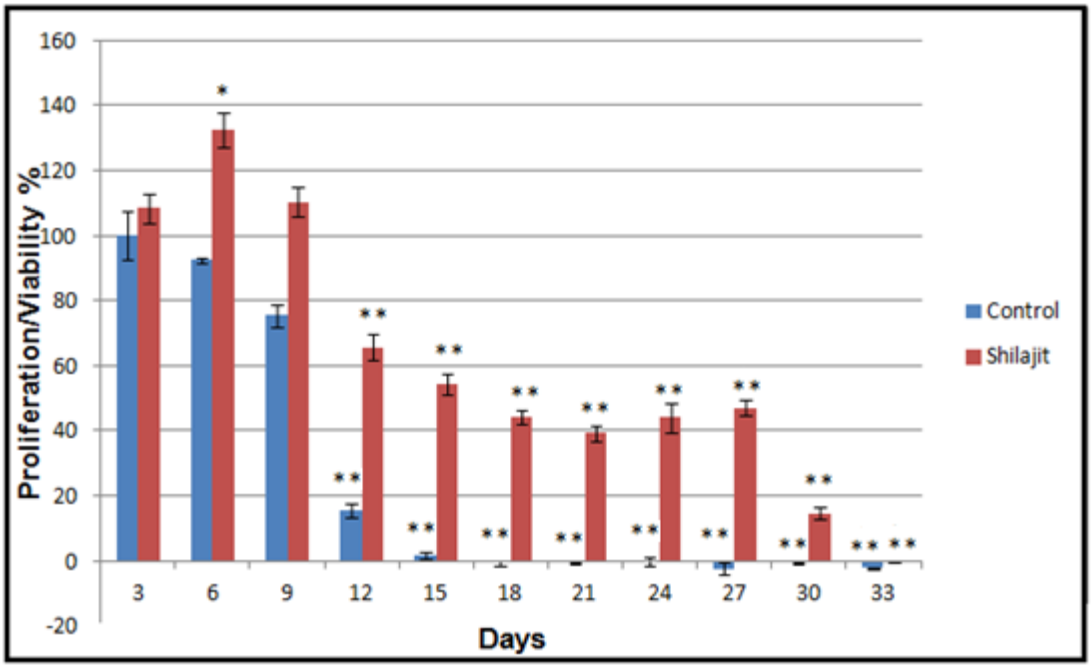
LDH assays were utilized to assess membrane damage by measuring the LDH activity for MG63 cells with and without shilajit at different time points up to

33 days of incubation without media exchange. The percentages of the cytotoxicity for MG63 cells after incubation with and without shilajit for up to 33 days were calculated and compared relative to controls at day 3 (Figure 3.1.2.2 and Table 3.1.2.2). The percentage of the cytotoxicity of the negative controls at day 3 was considered 0%, while the positive controls at day 3 was considered 100%. The statistically significant cytotoxicity for the treated cells appeared on day 9, while it occurred for the untreated cells as early as day 6. Although the cytotoxicity of the untreated cells reached 100% on day 15, the treated cells did not show 100% cytotoxicity until the end of the experiment.

Microscopic examinations further confirmed the results of the WST-1 and revealed the survival of shilajit treated cells up to 30 days. Figure 3.1.2.3. is representative pictures of the treated and untreated cells on days 1, 3, 10, 27, and 30. After 10 days of incubation, many of the cells in the control groups (Figure 3.1.2.3.E) appeared unhealthy, while the cells in the treated groups looked healthy and normal (Figure 3.1.2.3.F). All of the cells in the control groups appeared dead at day 15, whereas most of the cells in the treated groups still appeared healthy and normal. At the end of 27 days, around 40-50% of the treated cells were still attached to the surface of the 6-well cell culture plates, whereas almost all of the control cells were floating (Figure 3.1.2.3.H). When DMEM containing serum was re-introduced to the treated cells after 30 days, the remaining viable cells started to proliferate again (Figure 3.1.2.4). Shilajit treated

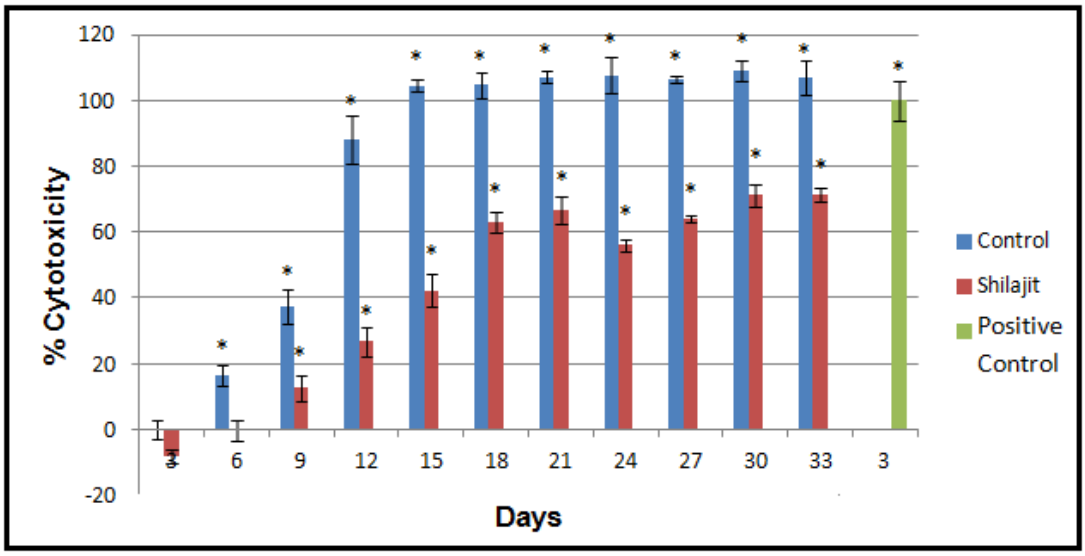
HGFs and a chondrosarcoma cell line (SW1353) appeared to behave similar to MG63 cells (data not shown).

Figure 3.1.2.1. Cell proliferation/viability (%) of MG63 evaluated by WST-1 assay after incubation with 0.8 mg/ml of shilajit for different time points. The results are presented as means and the error bars represent standard error (SE). N=3 for each group.



*denote a significant increase relative to the negative control at day 3 (p<0.05).
**denote a significant decrease relative to the negative control at day 3 (p<0.05).

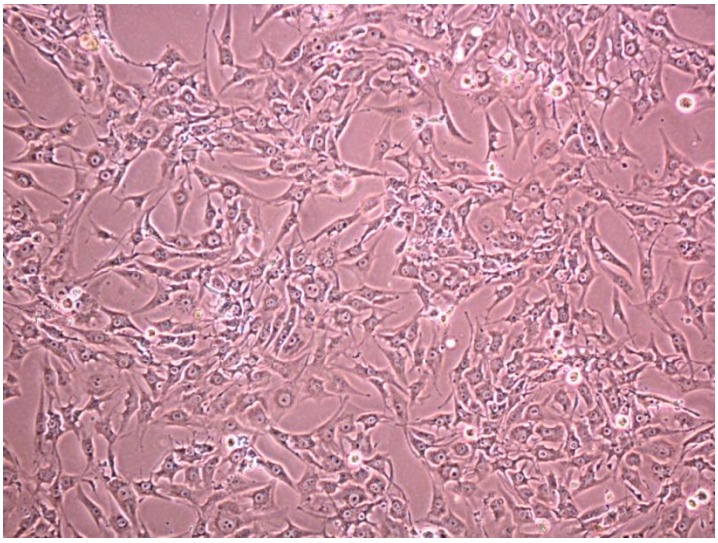
Figure 3.1.2.2. Cytotoxicity (%) of MG63 evaluated by LDH assay after incubation with 0.8 mg/ml of shilajit for different time points. The results are presented as means and the error bars represent standard error (SE). N=3 for each group.



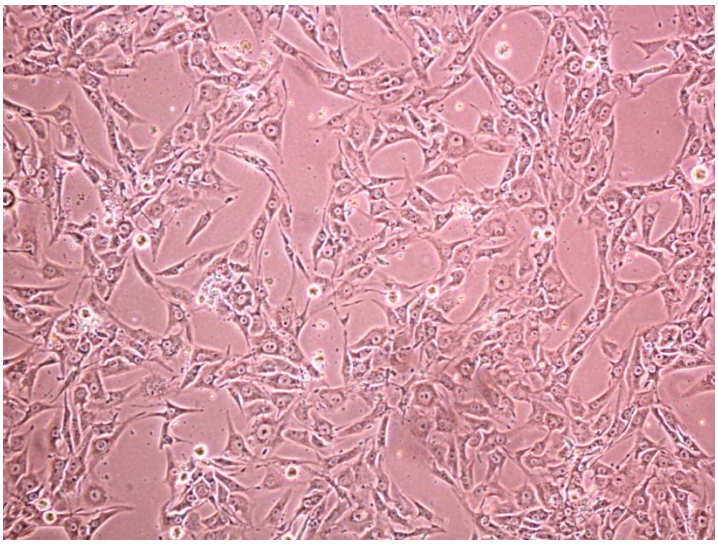
*denote a significant increase relative to the negative control at day 3 (p<0.05).

Figure 3.1.2.3.

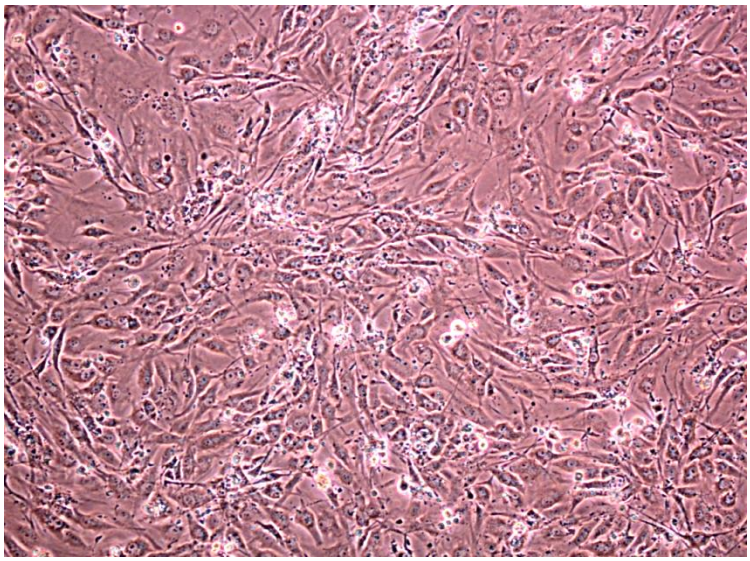
a) Microscopic image (10X magnification) for the MG63 cells after 1 day of incubation without shilajit and in the absence of serum (negative control).



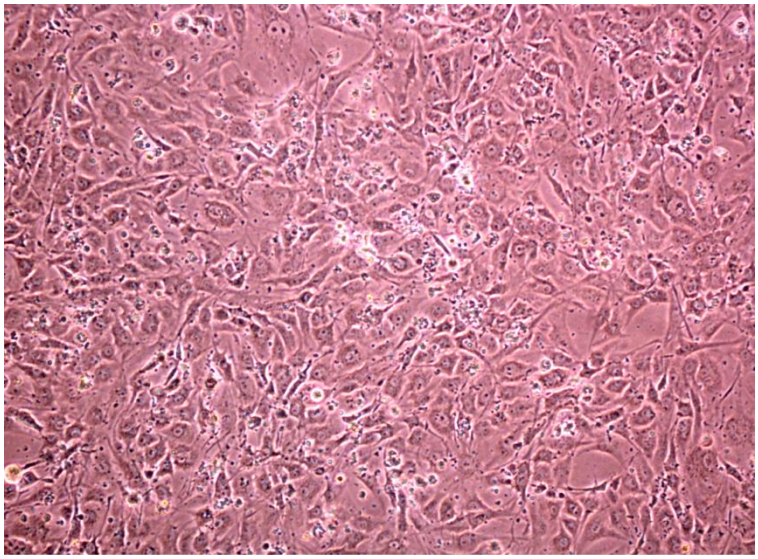
b) Microscopic image (10X magnification) for the MG63 cells after 1 day of incubation with 0.8 mg/ml shilajit and in the absence of serum (shilajit treated group).



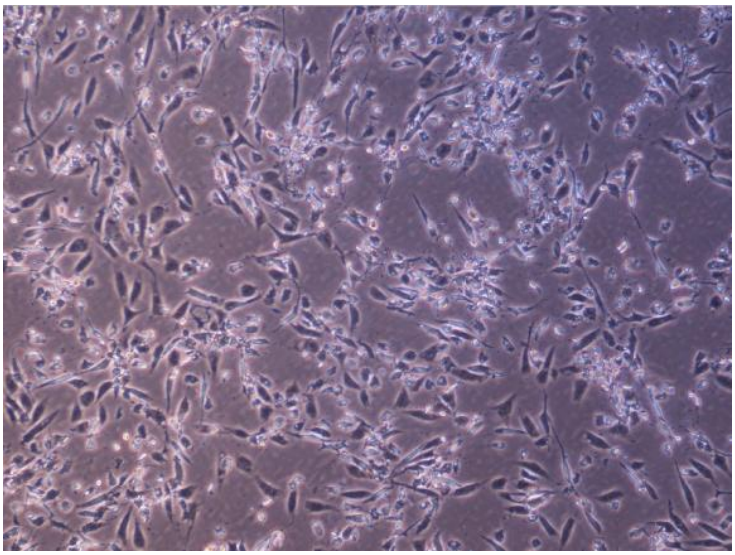
c) Microscopic image (10X magnification) for the MG63 cells after 3 days of incubation without shilajit and in the absence of serum (negative control).



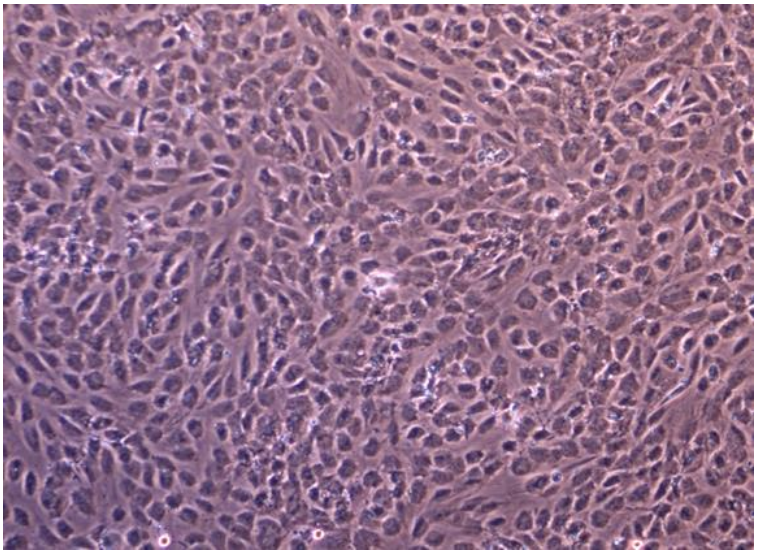
d) Microscopic image (10X magnification) for the MG63 cells after 3 days of incubation with 0.8 mg/ml shilajit and in the absence of serum (shilajit treated group).



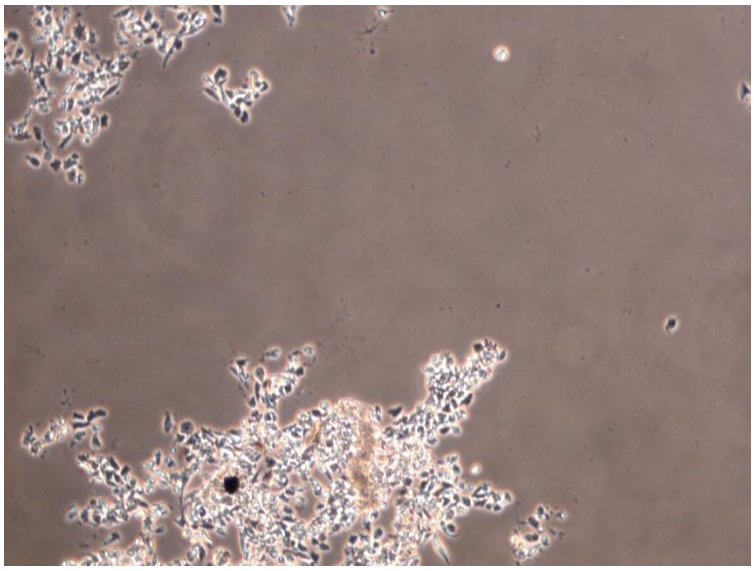
e) Microscopic image (10X magnification) for the MG63 cells after 10 days of incubation without shilajit and in the absence of serum (negative control).



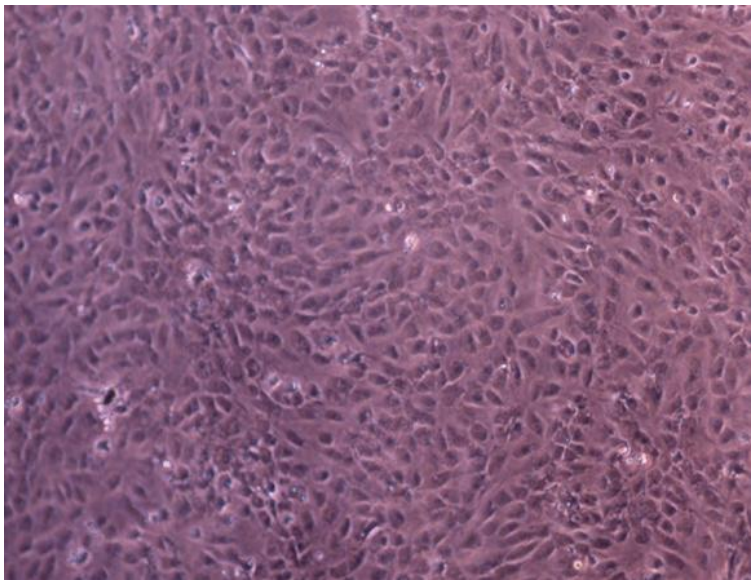
f) Microscopic image (10X magnification) for the MG63 cells after 10 days of incubation with 0.8 mg/ml shilajit and in the absence of serum (shilajit treated group).



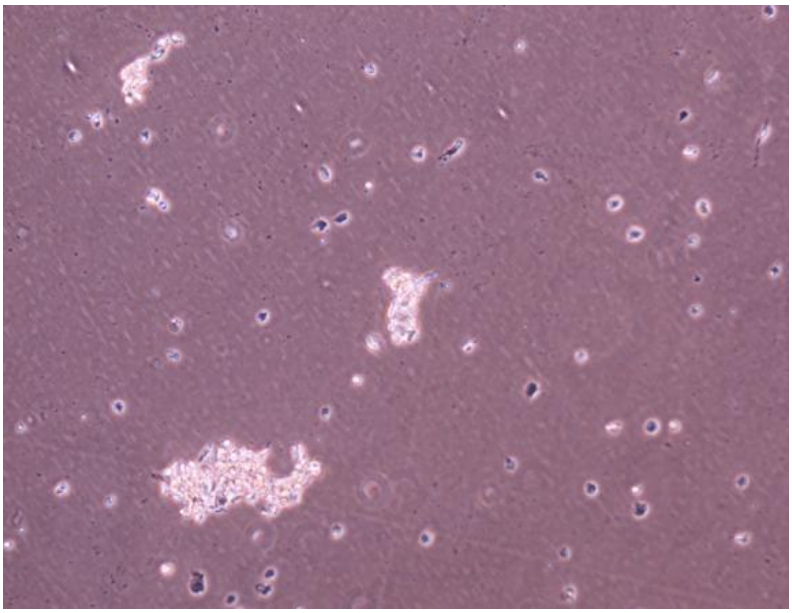
g) Microscopic image (10X magnification) for the MG63 cells after 27 days of incubation without shilajit and in the absence of serum (negative control).



h) Microscopic image (10X magnification) for the MG63 cells after 27 days of incubation with 0.8 mg/ml shilajit and in the absence of serum (shilajit treated group).



i) Microscopic image (10X magnification) for the MG63 cells after 30 days of incubation without shilajit and in the absence of serum (negative control).



j) Microscopic image (10X magnification) for the MG63 cells after 30 days of incubation with 0.8 mg/ml shilajit and in the absence of serum (shilajit treated group).

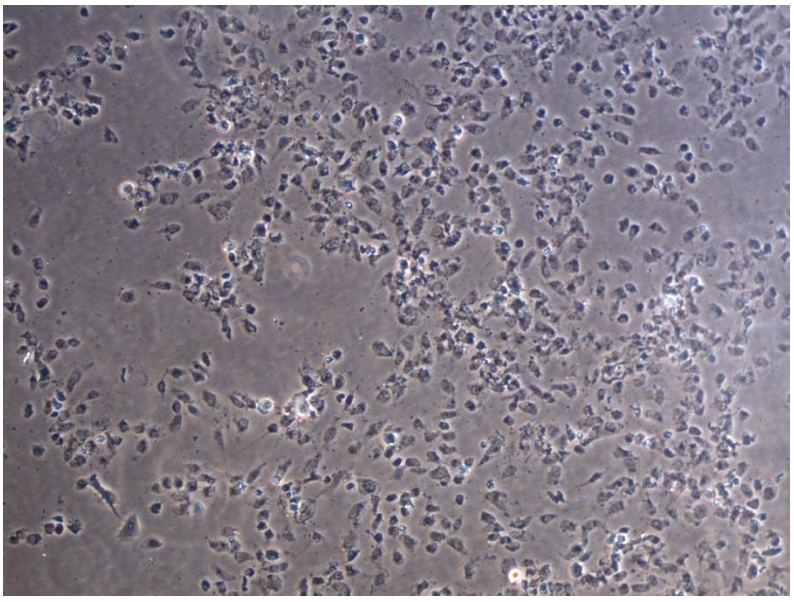


Figure 3.1.2.4. Microscopic image (10X magnification) for the proliferating/growing MG63 cells after being incubated with 0.8 mg/ml for 30 days before serum was re-introduced into the media.

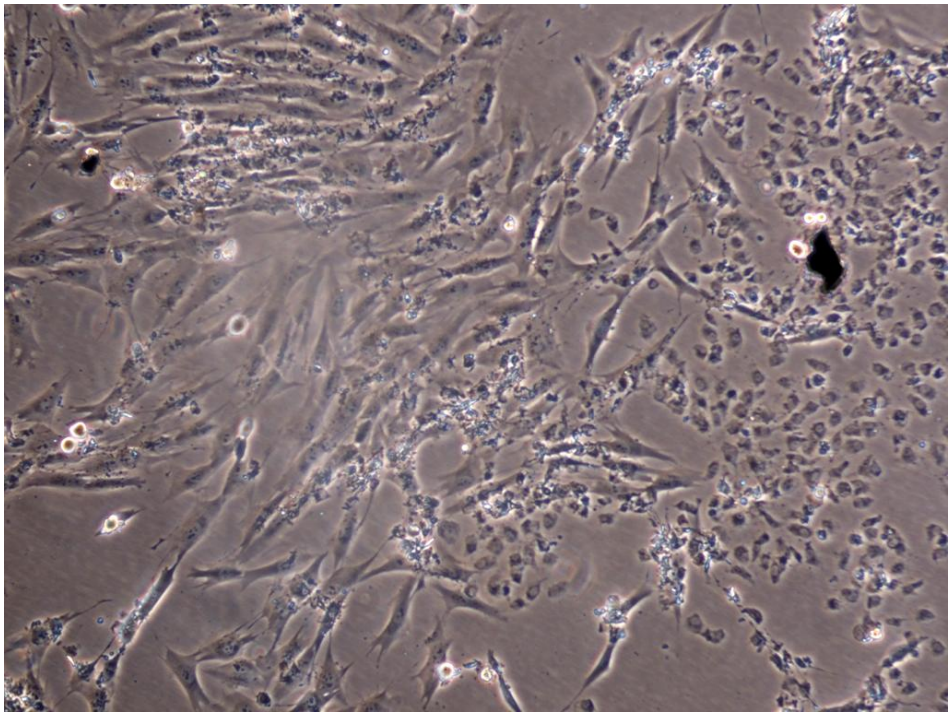


Table 3.1.2.1. Cell proliferation/viability (%) of MG63. The results are presented as means ± standard errors (SE) and *p* values. N=3 for each group.

Group	**% Proliferation/viability relative to the 3 day control ± SE**	***p* value**
Shilajit 3 days	108.4±4.66	.788
Control 6 days	92.5±0.93	.630
Shilajit 6 days	132.6±5.41	.035*
Control 9 days	75.4±3.47	.080
Shilajit 9 days	110.4±4.49	.678
Control 12 days	15.3±2.1	.007**
Shilajit 12 days	65.6±3.98	.033**
Control 15 days	1.6±0.96	.008**
Shilajit 15 days	54.3±3.25	.020**
Control 18 days	-0.7±0.61	.008**
Shilajit 18 days	44.3±2.04	.019**
Control 21 days	-0.6±0.21	.008**
Shilajit 21 days	39.2±2.49	.012**
Control 24 days	-0.01±1.27	.009**
Shilajit 24 days	44.1±4.54	.047**
Control 27 days	-2.5±1.92	.005**
Shilajit 27 days	47.1±2.35	.035**
Control 30 days	-0.6±0.27	.008**
Shilajit 30 days	14.5±1.87	.008**
Control 33 days	-2.1±0.27	.008**
Shilajit 33 days	-0.1±0.34	.008**

*denote a significant increase relative to the negative control at day 3 ($p<0.05$).
**denote a significant decrease relative to the negative control at day 3 ($p<0.05$).

Table 3.1.2.2. Cytotoxicity (%) of MG63 evaluated by LDH assays. The results are presented as means ± standard errors (SE) and *p* values. N=3 for each group.

Group	**% Cytotoxicity relative to the 3 day control ± SE**	***p* value**
Shilajit 3 days	-8.3±1.98	0.221
Control 6 days	16.2±3.18	0.016*
Shilajit 6 days	-0.3±3.09	0.072
Control 9 days	37.6±5.24	0.007*
Shilajit 9 days	12.5±3.92	0.005*
Control 12 days	88.2±7.33	0.003*
Shilajit 12 days	26.7±4.48	0.002*
Control 15 days	104.5±1.71	0.000*
Shilajit 15 days	42.1±5	0.001*
Control 18 days	104.7±3.96	0.000*
Shilajit 18 days	63.1±3.2	0.000*
Control 21 days	107.2±1.82	0.000*
Shilajit 21 days	66.8±4.1	0.000*
Control 24 days	107.6±5.51	0.000*
Shilajit 24 days	55.9±2	0.000*
Control 27 days	106.4±1.28	0.000*
Shilajit 27 days	64.1±1.15	0.000*
Control 30 days	109.6±3.34	0.000*
Shilajit 30 days	71.1±3.43	0.000*
Control 33 days	106.9±5.15	0.000*
Shilajit 33 days	71.3±2.05	0.000*

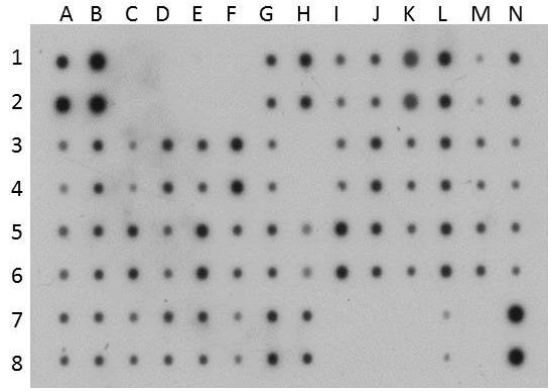
*denote a significant increase relative to the negative control at day 3 ($p<0.05$).

1.3: Evaluation of Apoptotic Protein Expression

RayBio® human apoptosis antibody arrays (Ray Biotech, Norcross, GA) were utilized to assess the relative expression of 43 apoptosis proteins by MG63 cells after 7 days of incubation with and without shilajit. It was determine that the relative expression of multiple pro- and anti-apoptotic proteins were significantly down-regulated in comparison to the control after incubation with shilajit for the 7 days (Figure 3.1.3.1 and Table 3.1.3.1). The down-regulated pro-apoptotic proteins were BIM, Caspase3, Caspase8, CD40, CD40L, HTRA, p53, sTNF-R1, and SMAC. The down-regulated anti-apoptotic proteins were HSP70, bcl-2, IGF-I, IGF-II, and Survivin. These proteins are involved in either the extrinsic or intrinsic pathways of apoptosis. Shilajit did not induce any increases in the expression of any of the 43 apoptotic proteins evaluated in this study.

Figure 3.1.3.1. Apoptotic protein expression from MG63 after 7 days of incubation with and without shilajit. The density and size of each dot indicate relative expression level of specific apoptotic protein and were analyzed by quantity one analysis software.

a) Untreated group (control)



b) Shilajit treated group

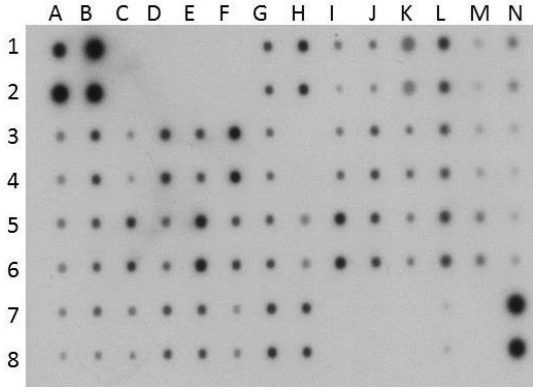


Table 3.1.3.1. The results for the down-regulated apoptotic proteins are presented as the percentages of the down-regulation ± standard deviation and the *p* values. N=4 for each group. Pro-apoptotic Anti-apoptotic

Apoptotic	Percentage of down-regulation ±	*p*
Bad	40±0.82	.14
Bax	32±0.51	.051
Bcl-2	36±0.58	.07
Bcl-w	32±0.37	.03*
BID	61±0.77	.09
BIM	27±0.23	.01*
Caspase 3	32±0.22	.01*
Caspase 8	21±0.43	.04*
CD40	26±0.39	.03*
CD40L	19±0.30	.02*
cIAP-2	29±0.51	.06
CytoC	30±0.54	.06
DR6	60±1.21	.24
Fas	33±0.73	.12
FasL	48±0.91	.15
HSP27	57±0.69	.07
HSP60	35±0.52	.051
HSP70	32±0.42	.03*
HTRA	15±0.26	.02*
IGF-I	21±0.16	.00*
IGF-II	20±0.36	.03*
IGFBP-1	36±0.79	.13
IGFBP-2	35±0.75	.12
IGFBP-3	37±0.76	.12
IGFBP-4	63±0.99	.15
IGFBP-5	35±0.70	.10
IGFBP-6	48±0.84	.13
IGF-1sr	45±0.73	.10
Livin	112±1.92	.35
P21	38±0.56	.06
P27	29±0.49	.053
P53	23±0.37	.03*
SMAC	11±0.28	.02*
Survivin	6±0.26	.02*
sTNF-r1	2±0.38	.05*
sTNF-r2	81±1.80	.42
TNF-alpha	26±0.70	.12
TNF-beta	41±0.64	.08
TRAIL- R1	43±0.68	.09
TRAIL- R2	39±0.65	.08
TRAIL- R3	67±0.99	.14
TRAIL- R4	31±0.52	.06
XIAP	29±0.54	.06

* denotes statistically significant different from control (equal to 1) ($p<0.05$).

Study 2: Effects of shilajit on the expression of osteogenic and connective tissue biomarkers

2.1: Gene expression analysis of MG63 and HGF cells

This experiment was conducted in order to determine which genes in MG63 and HGF cells were being up-regulated or down-regulated relative to the controls after incubation with shilajit. The mRNA gene expression was evaluated using Microarray Illumina Technology (SABiosciences, a QIAGEN company, Frederick, MD). Based on this technology, the mRNA expression of more than 47,000 human genes were compared between the groups. Multiple genes were up-regulated and others were down-regulated. The gene expression was considered significantly up- or down-regulated for those which had a minimum of 2 fold or more change. Table 3.2.1.1 contains some of the most significant up-regulated proteins in both MG63 and HGFs. Of interest, the mRNAs of multiple bone and connective tissue proteins, including several collagens, were up-regulated in the shilajit treated groups. Specifically, the mRNA for type I collagen alpha I chain was increased 23.56 fold in HGFs and 7.96 fold in MG63 cells (Table 3.2.1.1). In addition, gene analyses determined that genes associated with the Wnt pathway were significantly enhanced in both cell types.

Table 3.2.1.1. Fold increase of connective tissue proteins after treatment with shilajit in HGFs and MG63 using Microarray Illumina Technology.

Definition	Fold change in HGFs	Fold change in MG63
Homo sapiens collagen, type I, alpha 1 (COL1A1)	23.56	7.96
Homo sapiens collagen, type III, alpha 1 (COL3A1)	10.87	5.73
Homo sapiens collagen, type I, alpha 2 (COL1A2)	4.90	4.93
Homo sapiens collagen fibrillin 2 (FBN2)	4.63	NS
Homo sapiens collagen, type XI, alpha 1 (COL11A1)	3.73	NS
Homo sapiens collagen, type V, akpha 2 (COL5A2)	3.62	7.49
Homo sapiens fibronectin type III domain containing 1 (FNDC1)	3.53	NS
Homo sapiens collagen, type V, alpha 1 (COL5A1)	3.35	2.09
Homo sapiens collagen, type XII, alpha 1 (COL12A1)	3.14	2.35
Homo sapiens secreted protein, acidic, cysteine-rich (osteonectin) (SPARC)	NA	9.35
Homo sapiens collagen, type XXIII, alpha 1 (COL23A1)	NS	4.79

NS: Not significantly altered, NA: Not applicable.

2.2: ECM proteins

The ECM protein secretion of MG63 cells after incubation with shilajit was compared relative to the controls. MG63 cells (200,000 cells per well) were seeded into 6-well plate and then incubated with serum free DMEM with and without shilajit at a final concentration of 0.8 mg/ml for 33 days. On day 33, treated and untreated cells were washed 3 times with PBS, trypsinized, discarded and the plates stained with Coomassie blue to assess and compare the ECM proteins between the groups. It was determined that the density of the Coomassie blue stained proteins in the wells of the shilajit treated group after the cells were removed was significantly higher than the untreated controls by 1.8-fold after 33 days of incubation in serum free media (Figure 3.2.2.1, Table 3.2.2.1).

In addition, the ECM proteins in each well were dissolved by 6 M urea and their concentrations measured using a Bio-Rad Protein Assay kit (Hercules, CA, USA). It was determined that the ECM proteins concentrations secreted by the cells in the shilajit treated group increased significantly (2.7-fold) relative to untreated control (Table 3.2.2.1).

Figure 3.2.2.1. Six well plate image for the Coomassie blue stained ECM proteins for the untreated and shilajit treated MG63 cells after 33 days of incubation in the absence of serum.

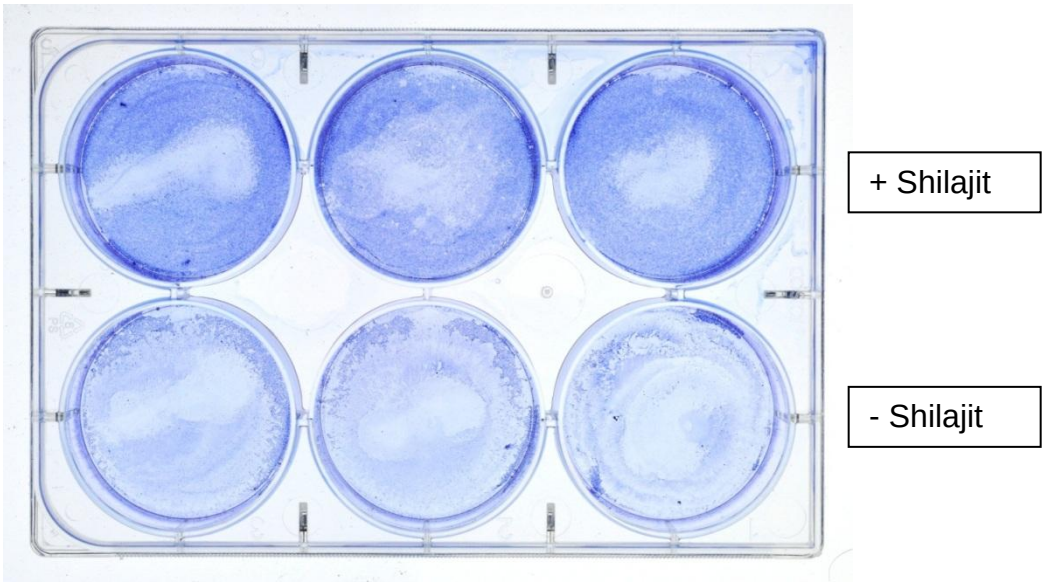


Table 3.2.2.1. The ECM proteins secretion in the untreated and shilajit treated groups after 33 days of incubation presented as means±standard deviation (SD) and the *p* values. N=3 for each group.

Method of measurement	**Untreated Cells Mean±SD**	**Shilajit Treated Cells Mean±SD**	***p* Value**
ECM stain density	34.6±4.3	61.4±3.0	0.0009*
ECM protein concentrations µg/ml	2.6±0.45	7.0±0.32	0.00015*

* denote a significant difference relative to the control ($p<0.05$).

Study 3: Role of shilajit on chondrogenesis

3.1: Non-critical size defect Xenopus model system

The effects of shilajit on enhancing cartilage formation during the second stage (soft callus) of endochondral ossification were evaluated using a non-critical size bone defect Xenopus model system. Each frog had a small segment of the anterior hemisection of the tarsus bone excised to serve as a fracture model. The frogs were divided randomly into 3 groups in which they received a daily subcutaneous injection of PBS, 3 mg/kg of shilajit, or 6 mg/ml of shilajit in the tarsus limb segment area for 4 weeks. The frogs were then euthanized and the hindlimbs were processed, cryosectioned and stained with hematoxylin/eosin.

All the PBS treated animals (control) displayed histology typical of a standard long-bone fracture repair process. On the cut tarsus hemisection (cut tarsus segment labeled with asterisk in the Figure 3.3.1.1.A), cartilage arising from the periosteum began distal to the bone cut site and extended into the region of the cut and into the small gap generated by removal of the small portion of bone and resulted in the small gap being completely bridged by cartilage (Figure 3.3.1.1.A). At one month, no ossification centers were observed in the cartilage at the center of the defect and this cartilage appeared devoid of blood vessels. Some of the uncut tarsus hemisection (uncut tarsus segment labeled with arrowheads in Figure 3.3.1.1.A) displayed modest periosteal cartilage

formation due to the creation of bone fractures in these areas, which may have occurred due to overloading issues.

The cartilage mass formed along the cut tarsus for the both 3 mg/kg and 6 mg/kg shilajit treated samples was statistically significantly larger than the cartilage mass formed in the controls (Figure 3.3.1.1.B and Figure 3.3.1.1.C). In most of the samples, the periosteal cartilage extended almost completely along the length of the fractured tarsus. As in the control samples, the periosteal cartilage bridged the small gap of excised bone. Some blood vessels appeared to be penetrating the cartilage bridging the bone gap (Figure 3.3.1.2). As in the PBS treated samples, there were no ossification centers clearly identified in this cartilage. The uncut tarsus bone segment also displayed robust periosteal cartilage formation in the fracture areas. The cartilage response in the 6 mg/kg shilajit treated samples was so robust that the cartilage masses frequently merged with one another along the interosseous ligament (Figure 3.3.1.1.C).

The mean ratios for the total cartilage area/total section area for the PBS, 3 mg/kg shilajit, and 6 mg/kg shilajit treated groups were 0.084±0.004, 0.216±0.046, and 0.22±0.035, respectively. Shilajit at both 3 and 6 mg/kg of shilajit statistically significantly increased the ratios of total cartilage area/total section area around 2.6 fold (p=0.008 and p=0.003, repectively), thus documenting higher cartilage formation in these groups relative to the controls (Figure 3.3.1.3 and Table 3.3.1.1). There were no significant differences in the

mean ratios of the total cartilage area/total section area between 3 mg/kg and 6 mg/kg shilajit treated groups (p=0.93).

Aside from the periosteal cartilage response and alterations in the uncut tarsus bone tissue, no other notable differences in the limb tissues were observed between the PBS, 3 mg/kg shilajit, or 6 mg/kg shilajit treated samples. Muscle, nerve, vasculature, dermis and epidermis appeared unaltered. There was no observable inflammation or other immune responses in any of the evaluated sections.

Figure 3.3.1.1. Histology of the non-critical size defect demonstrating the periosteal cartilage response in the cut tarsus segments labeled with asterisks and the uncut tarsus segments labeled with arrowheads. A) Low magnification cross sections proximal to cut site, at the cut site, and in the center of the defect for the PBS treated Xenopus. B) Low magnification cross sections proximal to cut site, at the cut site, and in the center of the defect for the 3 mg/kg shilajit treated Xenopus. C) Low magnification cross sections proximal to cut site, at the cut site, and in the center of the defect for the 6 mg/kg shilajit treated Xenopus.

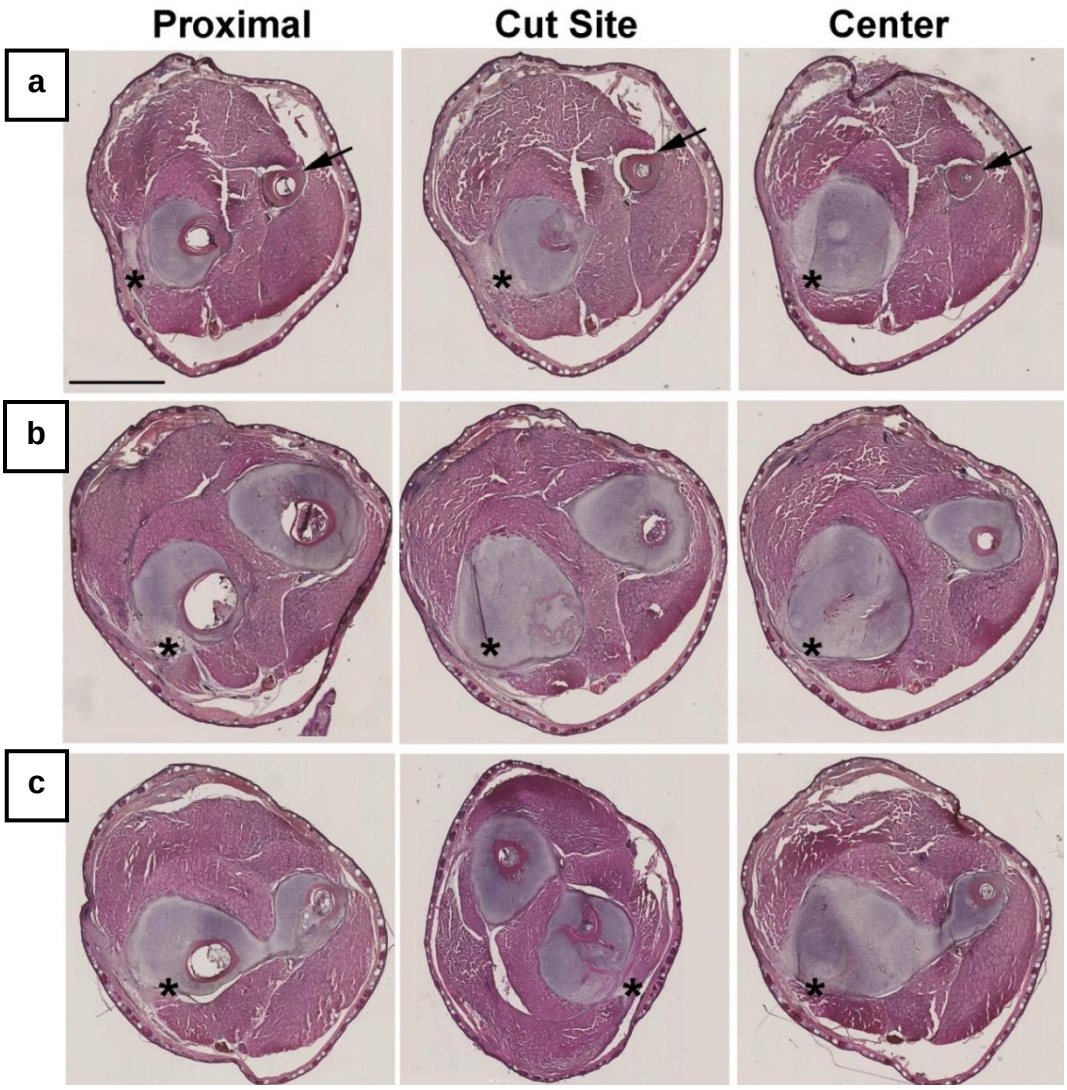


Figure 3.3.1.2. High magnification (5X) of cross sectional image for shilajit treated sample showing some blood vessels penetrating the cartilage bridging the bone gap labeled with arrowheads.

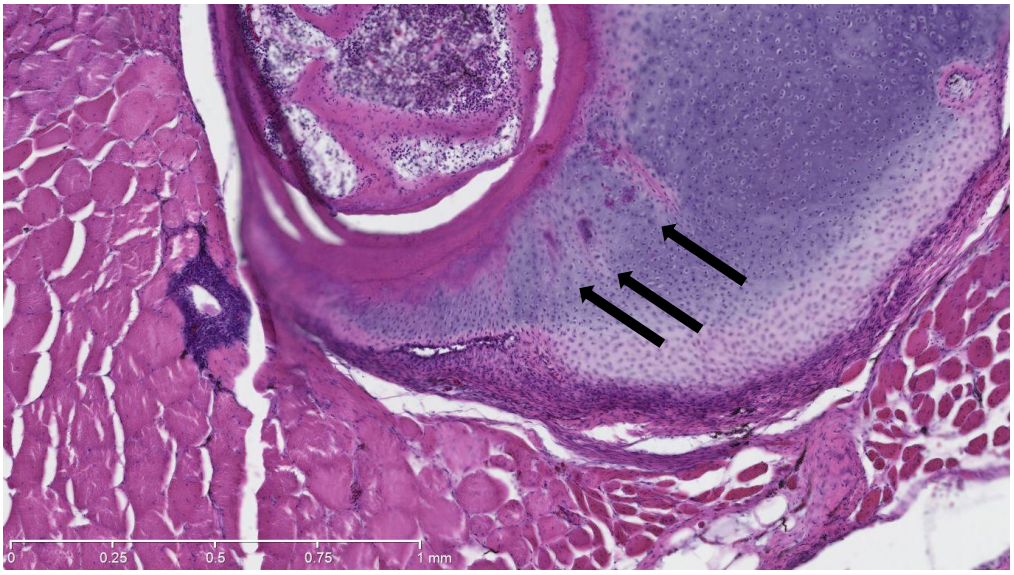
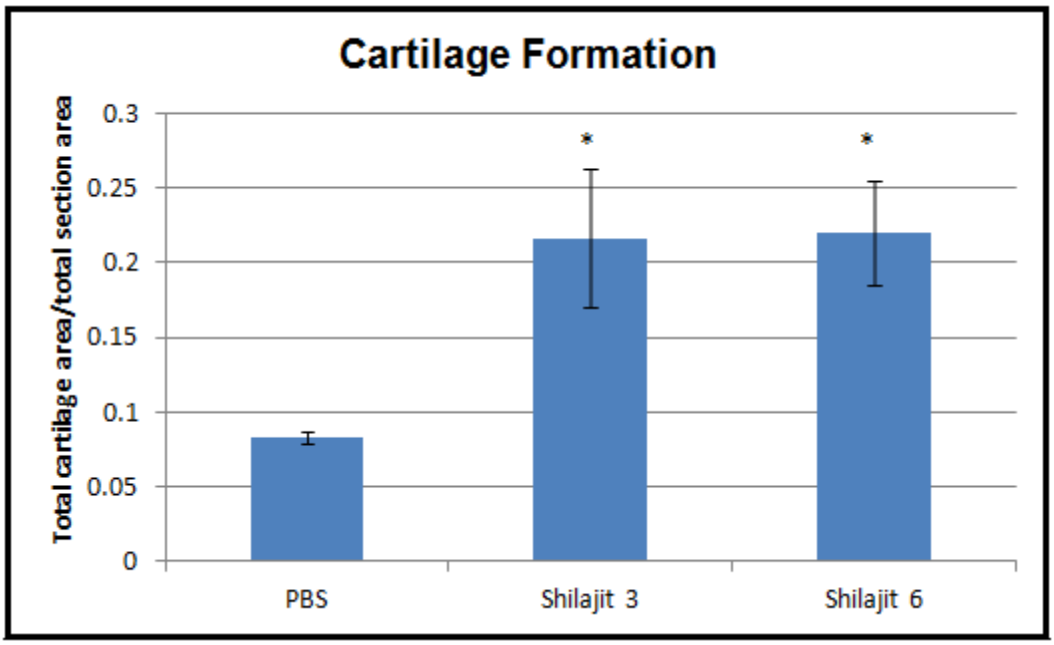


Figure 3.3.1.3. Ratios of the total cartilage area/total section area calculated from the histological cross-sectional images for the PBS, 3 mg/ml or 6 mg/ml shilajit treated groups' samples. The results are presented as means and the error bars represent standard deviation (SD). N=3 to 4 for each group.



*denote a significant difference relative to the PBS treated animals (control) (p<0.05).

Table 3.3.1.1. The changes in the ratios of the total cartilage area/total section area are presented as fold increase relative to the PBS treated samples (control). N=3 to 4 for each group.

	Fold increase in cartilage area/total section area ratio relative to the control	***p* value**
Shilajit 3	2.6	0.008*
Shilajit 6	2.6	0.003*

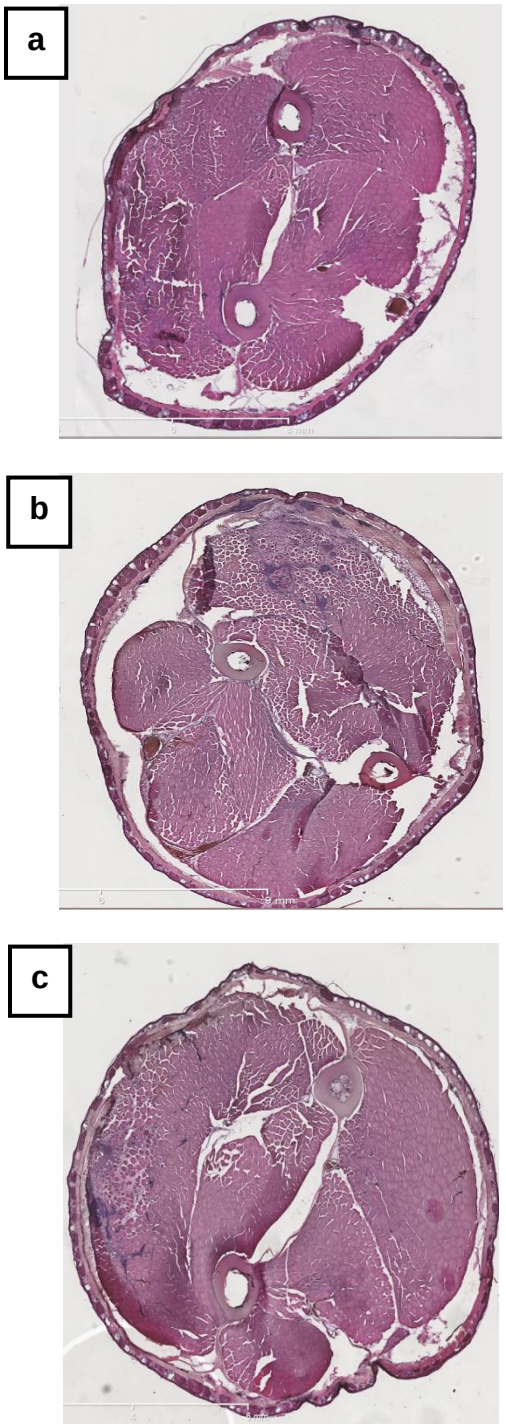
* denote a significant difference relative to the control ($p<0.05$).

3.2: Non-injured Xenopus model system

The effects of shilajit on initiating cartilage formation were evaluated using the same processes as in the non-critical size defect Xenopus model system, except no surgeries/fractures were performed on the animals. The frogs were divided randomly into 3 groups in which they received a daily subcutaneous injection of PBS, 3 mg/kg shilajit, or 6 mg/ml shilajit in the tarsus limb area for 4 weeks. The frogs were then euthanized and the hindlimbs were processed, cryosectioned and stained with hematoxylin/eosin.

The PBS, 3 mg/kg shilajit, and 6 mg/kg shilajit treated groups displayed no signs of periosteal cartilage formation in any of the areas of two tarsus bones in any animal (Figure 3.3.2.1). There appeared to be no notable differences in the limb tissues between the PBS, 3 mg/kg shilajit, and 6 mg/kg shilajit treated samples. Muscle, nerve, vasculature, dermis and epidermis appeared unaltered. There was no observable inflammation or other immune responses in any of the evaluated sections.

Figure 3.3.2.1. Histology of the non-injured tarsus samples. A) Low magnification cross section for the PBS treated Xenopus. B) Low magnification cross section for the 3 mg/ml shilajit treated Xenopus. C) Low magnification cross section for the 6 mg/ml shilajit treated Xenopus.



Study 4: Effects of shilajit on osteoporosis

4.1: Osteoporotic rat model

The anabolic effects of shilajit on bone mass was assessed using ovariectomized rats. The rats had ovariectomy (OVX) or sham operations at the beginning of the study. After 4 weeks from the surgeries, the rats were randomized into 8 groups to receive a daily IP injection of saline, PTH, or different concentrations of shilajit for 4 weeks. PTH 1-34 treatment was used as a positive control. The rats were then euthanized, and the bones collected and used for Micro-CT and bone histomorphometric analyses.

Micro-CT measures were used to assess bone volume (BV/TV) and were calculated for trabecular bone (Figure 3.4.1.1). Bone volumes for the different groups were: sham (25±2.9), OVX (14.9±2.5), PTH (17.6±5.1), 3 mg/kg shilajit (14.2±3.1) and 30 mg/kg shilajit (14.1±4.4). OVX statistically significantly reduced bone volume compared to SHAM (Table 3.4.1.1). PTH therapy improved bone volume by 18±5.1%, while both doses of shilajit showed no improvement in bone volume (Table 3.4.1.1). Overall, there were not any statistically significant differences in bone volume between the OVX group and PTH, shilajit 3, or shilajit 30 groups (Table 3.4.1.1).

Dynamic histomorphometric measures were used to determine the amount of active bone formation in both cortical and trabecular bone. Mineral

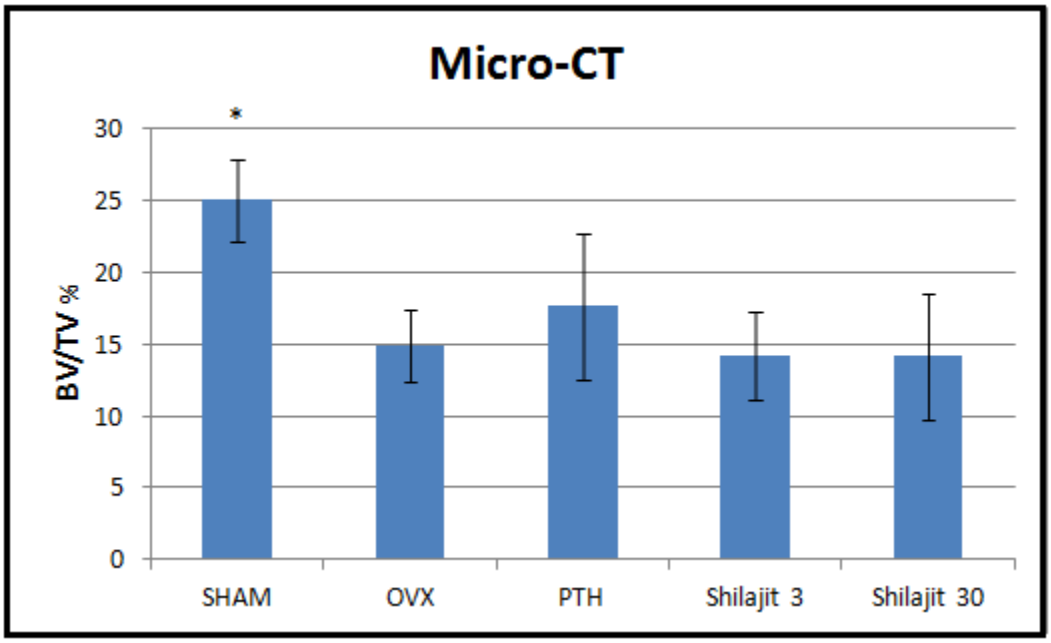
apposition rate (MAR) and mineralizing surface per unit bone surface (MS/BS) were utilized to calculate the bone formation rates (BFR) for each surface. The BFR on the cortical periosteal bone surface of the midshaft tibia were: sham (383.2±193.7), OVX (223.9±74.2), PTH (308.8±112.3), 3 mg/kg shilajit (304.9±117.2) and 30 mg/kg shilajit (370.9±161.3) (Figure 3.4.1.2 and Figure 3.4.1.5). Therefore, PTH, shilajit 3, and shilajit 30 treatments increased cortical periosteal bone formation relative to OVX by 38%, 36%, and 66%, respectively (Table 3.4.1.2). Based on *ANOVA* and a 0.05 significance level, none of the increases in PFR were statistically significant from the OVX group. Completing the second cohort of animals may yield statistically significant effects on cortical periosteal BFR.

The BFR on the cortical endocortical bone surface of the midshaft tibia were: sham (213.2±128), OVX (228.7±106), PTH (288.5±46.34), 3 mg/kg shilajit (232±155.4) and 30 mg/kg shilajit (261.3±206.3) (Figure 3.4.1.3 and Figure 3.4.1.5). Although PTH and shilajit at the higher dose enhanced the BFR of the endocortical surface by 26% and 14%, respectively relative to OVX, these slight changes were not statistically significant in the first cohort of animals (Table 3.4.1.3).

The BFR rates for the trabecular surfaces of proximal metaphysic tibia were: sham (82.2±21.1), OVX (113±46.1), PTH (212.1±86.6), 3 mg/kg shilajit (157±59.4) and 30 mg/kg shilajit (165±62.4) (Figure 3.4.1.4). PTH, shilajit 3, and

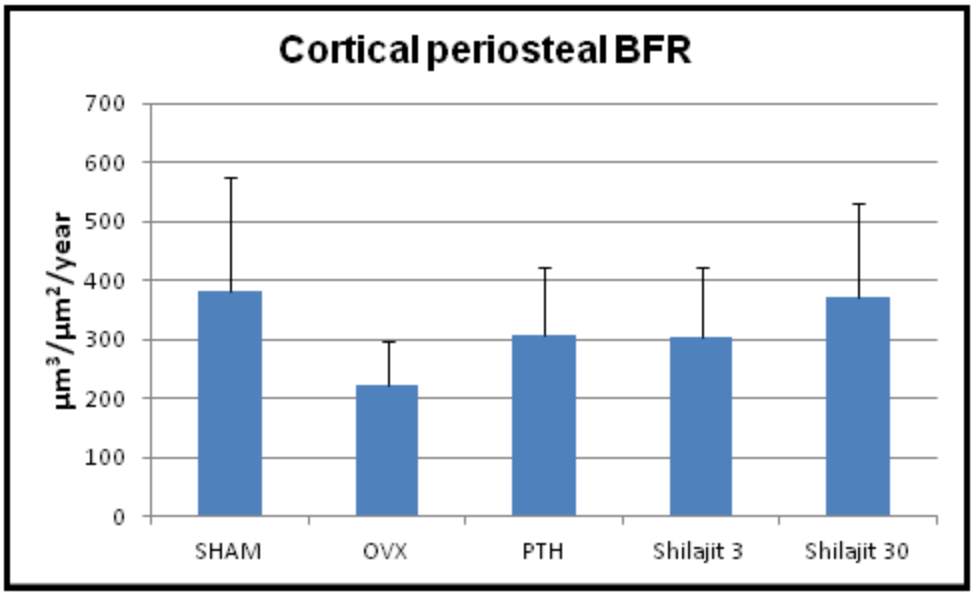
shilajit 30 increased the trabecular PFR relative to OVX group by 88%, 39%, and 46%, respectively (Table 3.4.1.4). None of these increases in PFR were statistically significant relative to the OVX group in the first cohort of animals.

Figure 3.4.1.1. Tibial trabecular bone volume (BV/TV) of the different groups measured by Micro-CT. The results are presented as means and the error bars represent standard deviation (SD). N=6 for each group.



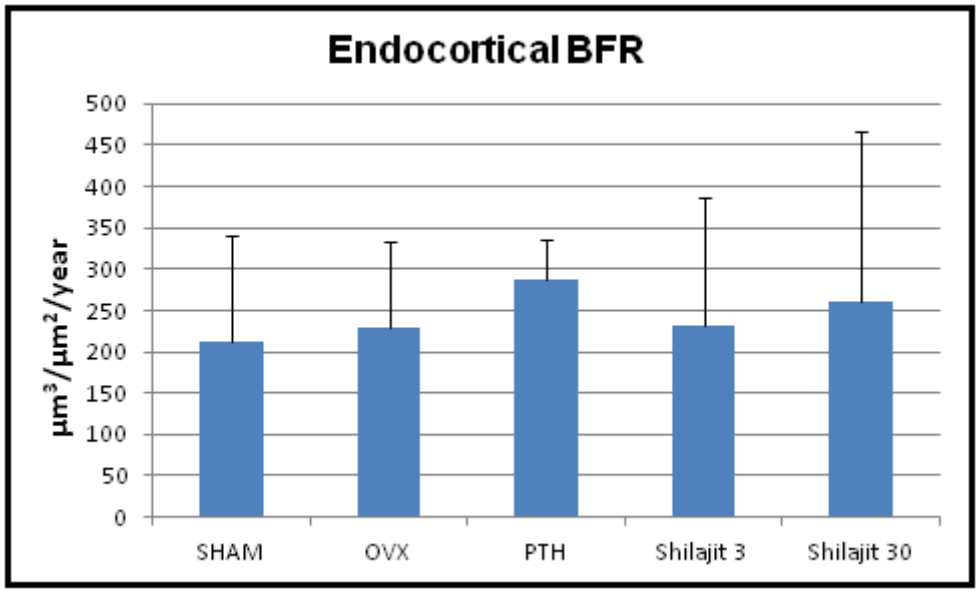
* denote a significant difference relative to the OVX group (p<0.05).

Figure 3.4.1.2. Tibial cortical periosteal BFR of the different groups calculated by dynamic histomorphometric analyses. The results are presented as means and the error bars represent standard deviation (SD). N=6 for each group.



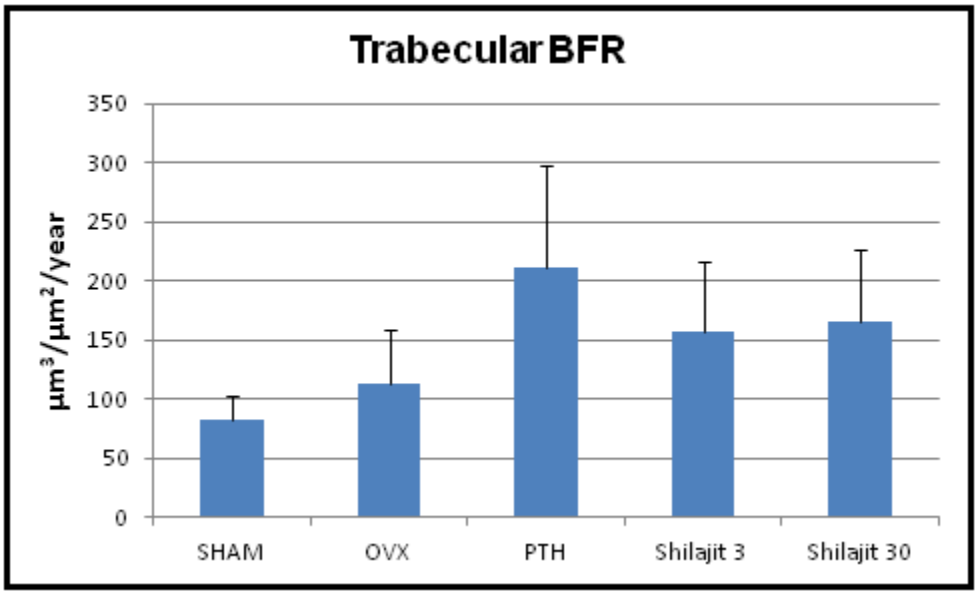
The level of significance was set at $p<0.05$.

Figure 3.4.1.3. Tibial endocortical BFR of the different groups calculated by dynamic histomorphometric analyses. The results are presented as means and the error bars represent standard deviation (SD).



N=6 for each group. The level of significance was set at $p<0.05$.

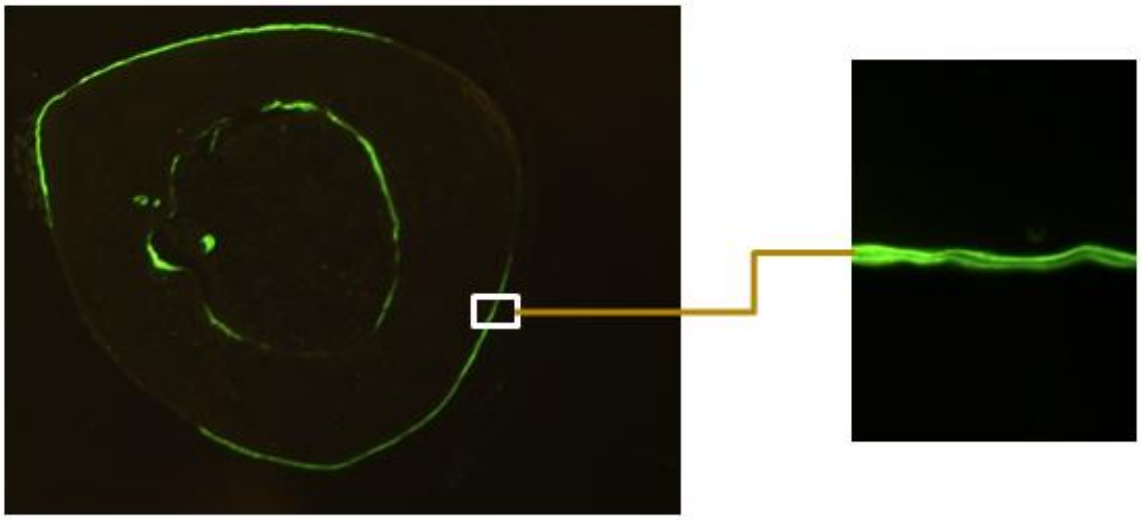
Figure 3.4.1.4. Tibial trabecular BFR of the different groups calculated by dynamic histomorphometric analyses. The results are presented as means and the error bars represent standard deviation (SD). N=6 for each group.



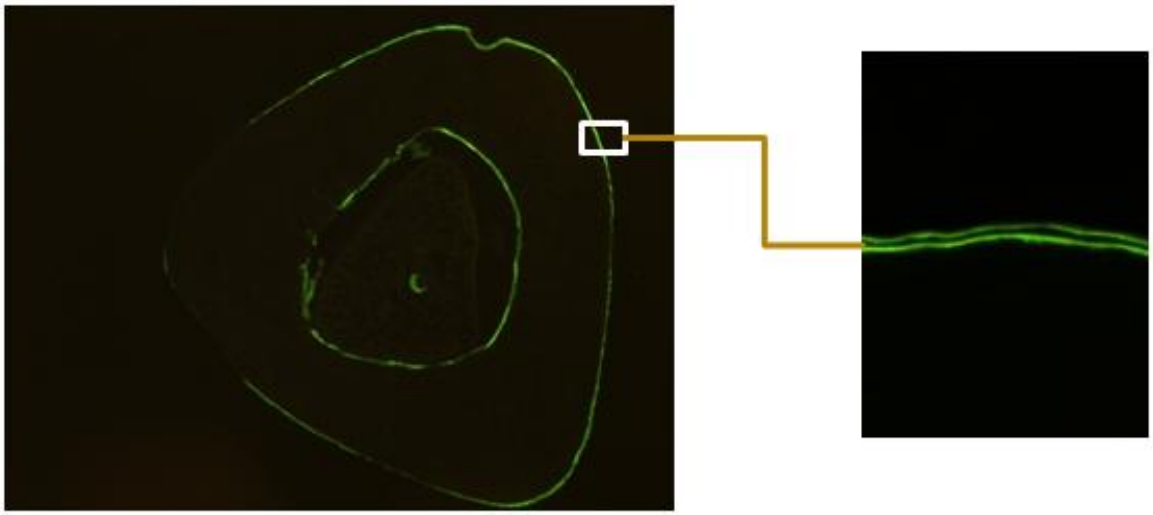
The level of significance was set at $p<0.05$.

Figure 3.4.1.5. Representative cross sectional images for the cortical bone of the midshaft of the right tibia labeled with fluorescent calein for OVX group (panel A), PTH group (panel B), and shilajit 30 (panel C). The green color indicates the active bone formation areas. Panel C shows that shilajit treated samples had more active bone formation areas and the distance between the two green lines resulted from the two calcein injections was larger than the ones for OVX and PTH groups.

A) OVX



B) PTH



C) Shilajit 30

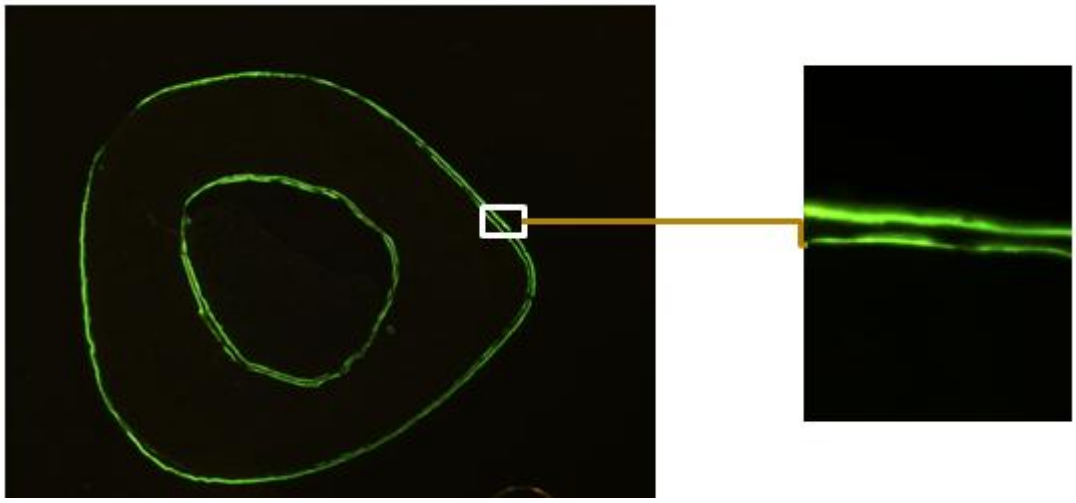


Table 3.4.1.1. The changes (%) in tibial trabecular bone volume (BV/TV) of the different groups measured by Micro-CT. The results are presented as percentages and the *p* values. N=6 for each group.

Group	**% change in BV/TV relative to OVX**	***p* value**
Sham	67.79% (increase)	0.001*
PTH	18.12% (increase)	0.908
Shilajit 3	4.9% (decrease)	1
Shilajit 30	5.7% (decrease)	1

* denote a significant difference relative to the OVX group ($p<0.05$).

Table 3.4.1.2. The changes (%) in cortical periosteal BFR of the different groups calculated by dynamic histomorphometric analyses. The results are presented as percentages and the *p* values. N=6 for each group.

Group	**% Change in cortical periosteal BFR relative to OVX**	***p* value**
PTH	38% (increase)	0.720
Shilajit 3	36% (increase)	0.742
Shilajit 30	66% (increase)	0.373

The level of significance was set at $p<0.05$.

Table 3.4.1.3. The changes (%) in endocortical BFR of the different groups calculated by dynamic histomorphometric analyses. The results are presented as percentages and the *p* values. N=6 for each group.

Group	**% Change in endocortical BFR relative to OVX**	***p* value**
PTH	26% (increase)	0.805
Shilajit 3	1.44% (increase)	1
Shilajit 30	14% (increase)	1

The level of significance was set at $p<0.05$.

Table 3.4.1.4. The changes (%) in trabecular BFR of the different groups calculated by dynamic histomorphometric analyses. The results are presented as percentages and the *p* values. N=6 for each group.

Group	**% Change in trabecular BFR relative to OVX**	***p* value**
PTH	88% (increase)	0.072
Shilajit 3	39% (increase)	0.848
Shilajit 30	46% (increase)	0.836

The level of significance was set at $p<0.05$.

CHAPTER FOUR

Discussion

Although shilajit has been used as a natural remedy for thousands of years to treat many diseases such as bone healing, supporting scientific evidence based on well-designed studies are inadequate. Therefore, the overall objective of the studies in this dissertation was to investigate shilajit's bone/cartilage healing effects using molecular, cell-based and *in vivo* approaches.

Study 1: Effects of shilajit on osteoblast proliferation and survival

1.1: Effects of shilajit on cytotoxicity

Published information about this agent is very limited in providing the concentrations of shilajit to be utilized *in vitro*. Therefore, different concentrations of an aqueous solution of shilajit were tested to identify the highest non-toxic concentration that could be used in the *in vitro* studies on human cells.

The cytotoxic levels of shilajit on MG63 cells were determined by measuring mitochondrial dehydrogenase activities during cell proliferation and LDH activity after membrane damage utilizing a dose response. Toxicity can be manifested in a number of different mechanisms such as: a) a decrease in cell viability in which the cells' growth and proliferation is altered, b) necrosis in which

the cells lose their membrane integrity and release their contents such as the LDH enzyme into the surrounding environment, and c) apoptosis in which programmed cell death occurs with no loss of membrane integrity. Assessing the cytotoxicity of any agent based on the use of at least two techniques results in more accurate evaluations. For example, if the agent inhibits the growth and proliferation of the cells, its cytotoxicity will not be detected based on the evaluation of LDH enzyme in the media. Therefore, two different assays (WST-1 and LDH) were utilized to evaluate the cytotoxicity of shilajit. WST-1 was selected to evaluate the effects of this agent on cell proliferation/viability after 24 hours of incubation. The results showed an increase in cell proliferation/viability after incubation with shilajit relative to the untreated control at concentrations between 0.4 and 1.6 mg/ml with a maximum at 0.8 mg/ml. The increase in cell proliferation was statistically significant at shilajit concentrations of 0.8, 1.2, and 1.6 mg/ml. Shilajit at concentrations starting from 2 mg/ml and up significantly decreased cell proliferation/viability. Shilajit at a concentration of 1.6 mg/ml was the highest non-toxic concentration of shilajit based on cell proliferation.

The LDH assay was utilized to assess the cytotoxicity of shilajit based on the LDH activity following the damage to the cell membranes. The results showed statistically significantly increases in the cytotoxicity with shilajit at concentrations of 3.2 mg/ml and above relative to the untreated control. This is slightly different than the WST-1 results, which demonstrated significant cytotoxicity at concentrations starting from 2 mg/ml. The cell viability of the

treated groups was significantly deceased at shilajit concentrations between 2.0 and 2.8 with no increase in LDH activity. One explanation could be that shilajit had an inhibitory effect on cell growth and proliferation before it produced cell death and loss of membrane integrity leading to the release of LDH enzyme into the media. The percentages of cell proliferation/viability were significantly decreased at shilajit concentrations of 3.2 and above with an increase in LDH activity indicating that shilajit caused cell death. Therefore, the use of LDH assay method will only detect the cytotoxic level when membrane damage has reached the level of leakage of cytosolic components.

Although the chemical composition and physical properties of the shilajit samples from the different countries are very comparable, their components are present at different ratios (Galimov 1986, Schepetkin, Xie et al. 2009). Preliminary results showed that shilajit purchased from the different countries had different cytotoxic levels (data not shown). Therefore, the cytotoxicity needs to be determined for every shilajit sample used.

Although 1.6 mg/ml was the highest non-toxic concentration of shilajit according to the WST-1 assay, a concentration of 0.8 mg/ml was selected to be used in the following experiments. This concentration produced the highest percentage of cell proliferation/viability relative to the untreated control. In addition, the mitochondrial dehydrogenase and LDH activities of MG63 were evaluated after 24 hours incubation with shilajit according to manufacturer's

recommendation for the evaluation of cytotoxicity. All of the other experiments involved in this dissertation were designed to have longer incubation times with the cells than 24 hours in order to evaluate the survival of MG63 cells, which extended up to 33 days. According to the available toxicological studies, shilajit should be provided in low doses when used chronically (Kelginbaev NS 1973, Anisimov VE 1982, Fortan MH 1984, Al-Hamaidi AR 2003, Biswas, Pandit et al. 2010). These studies showed that the LD50 (per oral) for shilajit was >2000 mg/kg and a dose of ≤1000 mg/kg was found to be safe if it is used chronically. The information provided in the literature regarding the cytotoxic levels of shilajit extract in culture was inadequate. For example, Jung et al. (Jung CR 2002) examined the effects of shilajit extract on mesenchymal stem cells in culture. This study did not determined the cytotoxic levels but a much lower dose range of shilajit (200 ng/ml-5 µg/ml) was utilized.

1.2: MG63 Viability/Proliferation and LDH activity up to 33 days

Osteoblasts are one of the three major cell types in bone and responsible for bone formation and then mineralization. Enhancement of some of the osteoblastic activities such as proliferation and survival is one of the therapeutic approaches to treat bone diseases such as osteoporosis (Jung CR 2002). Therefore, the effects of shilajit on MG63 viability/proliferation in serum free conditions and without the media being exchanged for up to 33 days were evaluated using the WST-1 assay.

The untreated and shilajit treated groups were compared at different time points up to 33 days. The percentage of the viable cells of the untreated group on day 3 was used as a baseline (100% viable) and utilized in all comparisons. The results showed that shilajit increased cell proliferation relative to the baseline group up to 9 days with a statistically significant increase at day 6 (32.6±5.41%). On day 12 and 15, the percentages of the viable cells in the shilajit treated group significantly decreased to 65.6±3.98% and 54.3±3.25%, respectively. From day 18 and up to 27 days, the percentages of the viable cells remained stable with an average 43.7±2.86%. Very interestingly, shilajit significantly increased the survival of MG63 up to 30 days relative to the controls at which 14.5±1.87% of baseline cell numbers were still viable. In addition, these cells retained their ability to proliferate when serum and fresh media was re-introduced to them (Figure 3.1.2.4). In contrast, the cell proliferation was not enhanced at any time points after 3 days in the untreated group. In addition, only 15.3±2.1% of the

baseline cell number was viable on day 12 and complete cell death occurred on day 15. According to published literature, this is the first study to evaluate the effects of this agent on cell viability/proliferation and survival in culture. The effects of shilajits on cell survival could be due to multiple mechanisms. For instance, shilajit might have inhibited the expression of one or multiple pro-apoptotic proteins and/or enhanced the expression of the anti-apoptotic ones. Another explanation could have been that this agent increased the expression of some of the proteins involved in the cell survival pathways such as Akt 1. Shilajit might also have maintained the cells in resting state (G0 phase) until serum was re-introduced into the cultures. Increasing the ability of bone cells to proliferate and survive should increase their ability to express extracellular matrix proteins required during bone healing. The results of this study could partially explain the findings of some of the published studies evaluating the effects of shilajit on bone healing. In a study by Kelginbaev et al, shilajit improved bone healing in children after fractures (Kelginbaev NS 1973). Another study has also found that a daily dose of 0.1 g/kg of shilajit (orally) accelerated callus formation and phosphorus uptake using a bone fracture model systems *in vivo* (Ismailova 1965, Shakirov 1965).

In addition to cell proliferation and survival, LDH activities were evaluated for both shilajit treated and untreated groups using the same time points. During group comparisons for LDH activities, the cell numbers should be standardized among the groups so the results are not susceptible to be false positives or false

negatives. For example, if the tested agent enhances cell proliferation during the incubation time before starting the LDH assay, the LDH activity of the agent treated groups might be higher than the untreated control due the greater number of cells and not due to the increase of the cytotoxicity of this agent. For this reason, WST-1 values of the current experiment were used to standardize the LDH values before comparisons. The WST-1 is a colorimetric assay, which is based on the cleavage of a tetrazolium salt into formazan by mitochondrial dehyrogenases in viable cells. If the number of the metabolically active viable cells is higher, the amount of the formazan created after adding WST-1 agent will be increased. By quantifying the amount of the formazan product, it is possible to indirectly quantify the number of the cells in the plates. In the current study, WST-1 assays showed that the increase in cell viability of shilajit treated cells was 8.4% at day 3 and reached its maximum at day 6 by 32.6%. Therefore, the LDH value for shilajit treated group at 3 days was divided by 8.4% while the values at other time points for the same group were divided by 32.6%.

The LDH activity of the untreated group was significantly higher than the baseline group on day 6 and reached a significant level for the shilajit treated group on day 9. In agreement with the WST-1 results, the LDH activity reached 100% for the untreated group on day 15. Of interest, the LDH activity in the shilajit treated group didn’t reach the 100% level until the end of the experiment and even with the occurrence of total cell death. This could be explained by that the death of some of the cells was not due to necrosis but due to apoptosis.

Sufficient energy is always necessary for the cells to proceed with the process of apoptosis, while no energy is required in case of the necrosis process. This might further support the effects of shilajit on enhancing cellular process. The main differences between apoptosis and necrosis are listed in Table 4.1.2.1.

1.3: Evaluation of apoptotic protein expression

Shilajit significantly increased MG63 survival and viability in culture. Therefore, it was hypothesized that either one or multiple of the pro-apoptotic proteins was/were down-regulated or that anti-apoptotic proteins was/were up-regulated or both. This could be one possible mechanism of how this agent enhances cell survival. Therefore, the relative expression of 43 apoptotic proteins were assessed between a shilajit treated group and untreated group using protein arrays.

Unlike necrosis, apoptosis is a programmed and energy dependent cell death with no loss of membrane integrity. The two major pathways of apoptosis are the intrinsic and extrinsic pathways (Figure 1.1.1). There is a balance between anti-apoptotic and pro-apototic proteins within the cells. Any alterations in this balance decides if the cell dies or lives (Yang, Zhu et al., Tsujimoto 1998, Coultas and Strasser 2003).

Shilajit significantly decreased the expression of multiple pro-apoptotic and anti-apoptotic proteins relative to the control after 7 days of incubation (Figure 3.1.3.1 and Table 3.1.3.1). There were statistically significant decreases in the expression of 5 anti-apoptotic proteins and 9 pro-apoptotic proteins. Therefore, shilajit might have an inhibitory effect on cell apoptosis since it down-regulated more pro-apoptotic than anti-apoptotic proteins, which could disturb the balance between these groups of proteins. This might be one explanation for the

WST-1 findings that showed increased cell survival after adding shilajit in culture. Another explanation could be that shilajit might have maintained the cells in a resting period (G0) until serum was re-introduced into the cultures. This requires future investigations.

The down-regulated pro-apoptotic proteins in this study are involved in the extrinsic pathway, intrinsic pathway, or both (Figure 4.1.3.1). Shilajit significantly down-regulated two of the cell surface death receptors (sTNF-r1 and CD40) in the extrinsic pathway. The CD40 ligand for CD40 receptor was also down-regulated. This could also explain why down-regulation occurred consequently for caspase 8 and 3. The down-regulation of caspase 8 can also inactivate the apoptotic process via the intrinsic pathway by down-regulating the pro-apoptotic protein (Bid). Shilajit significantly down-regulated BIM, SMAC, and HTRA within the intrinsic pathway and thus lead to the down-regulation of the expression of caspase 3. Caspase 3 is a key factor for apoptosis via both the extrinsic and intrinsic pathways. Its inhibition is an important sign of the inhibition of the apoptotic process (Kim, Emi et al. 2006). In a study by Kwon et al investigating the effects of aromatic hydrocarbons on apoptosis demonstrated that apoptosis of MG63 cells is preceded by the activation of caspase-3 (Kwon, Ueda et al. 2002). In another study by Woo et al. (Woo, Hakem et al. 1998), it was noted that peripheral T cells with defective caspase 3 were less susceptible to activation induced cell death and CD3 and/or Fas receptor induced apoptosis than normal T cells. Similar findings were also detected in different studies when the caspase

3 gene was deleted in the human MCF-7 breast carcinoma cell line (Janicke, Sprengart et al. 1998, Porter and Janicke 1999).

In the current study, shilajit also down-regulated p53. This protein is a cell cycle regulator and its activation can lead to either cell division arrest or apoptosis. For example in response to stress activation due to DNA damage or severe growth factor deprivation, the expression of p53 protein increases. If the DNA damage can be repaired, p53 induces cell cycle arrest (at G1 and/or G2 phase) until the damage is fixed. But if the DNA damage is not repairable, the apoptotic process will be initiated instead via the extrinsic or intrinsic pathways by p53. According to the WST-1 findings, the amount of the viable cells in the untreated group did not show any increases after day 3. It started to decrease as early as 6 days. This could be due to either enhancing cell death or arresting the growth of the cells or both. The expression of p53 decreased in the shilajit treated group allowing them to grow, proliferate, and survive longer. Chen et al. showed that cell death can be delayed after the down-regulation of p53 with HPV E6 (Chen, Merrett et al. 2002). Another study investigated the effect of 3-methylcholanthrene on apoptosis of MG63 and reported that p53-deficient MG63 cells did not undergo apoptosis in response to 3-methylcholanthrene (Kwon, Ueda et al. 2002). Therefore, it was hypothesized that p53 was responsible for 3-methylcholanthrene induced apoptosis. The current study is the first to investigate the effects of shilajit on apoptosis.

Study 2: Effects of shilajit on the expression of osteogenic and connective tissue biomarkers

2.1: Gene expression analysis of MG63 and HGF cells

Shilajit stimulated the cell survival and proliferation of MG63 cells suggesting that it might enhance bone repair. Therefore, the working hypothesis was that a number of genes were up-regulated including the ones for the collagens and osteogenic biomarkers, and others were down-regulated such as the genes for the pro-apoptotic proteins after incubation with shilajit. By analyzing the mRNA expression of these genes, it should be possible to identify pathways that are affected by this agent. Therefore, the gene expression of MG63 and HGFs were analyzed using Microarray Illumina technology after treatment with shilajit in culture.

In addition to osteoblasts, fibroblasts are also capable of synthesizing extracellular matrix and collagens. Therefore, enhancing the activities of fibroblasts such as proliferation and ECM secretion is also an approach to treat connective tissue diseases and wound healing. Based on Microarray Illumina technology, the mRNA expression of more than 47000 human genes was compared between shilajit treated and untreated cells. Due to its high cost and limited funds, only one sample for each group was examined to compare the effects of shilajit on MG63 and HGF cells.

The effects of shilajit were similar in both MG63 cells and HGFs. It was clear that the mRNAs of multiple bone and connective tissue proteins were up-regulated including several collagens such as collagen type I, III, V, XI, XII, and XXIII. It has been shown by many studies that the collagen matrix is a very important key factor for bone toughness (Bailey, Wotton et al. 1992, Boskey, Wright et al. 1999, Zioupos, Currey et al. 1999, Thompson, Kindt et al. 2001, Wang, Bank et al. 2001, Zioupos 2001, Viguet-Carrin, Garnero et al. 2006). The mRNA for type I collagen alpha I chain was increased 23.56 fold in HGFs and 7.96 fold in MG63 cells (Table 3.2.1.1). While collagen provides elasticity and enhances tissue structure in all connective tissues, collagen type I is mainly associated with the mechanical properties of bone (Viguet-Carrin, Garnero et al. 2006). Type I collagen is the most abundant collagen type in practically all of the connective tissues (Viguet-Carrin, Garnero et al. 2006). It represents around 95% of the total collagen in bone and approximately 80% of all the proteins in bone (Niyibizi and Eyre 1994, Viguet-Carrin, Garnero et al. 2006).

In addition to the collagens, shilajit significantly up-regulated the expression of osteonectin, an osteoblast differentiation marker. Osteonectin has a role in forming a connection between the organic phases and the mineral of the bone by binding to hydroxyapatite, calcium, and collagen (Kelm, Swords et al. 1994, Long 2001). In a study by Jung et al, the effects of shilajit on osteoblastic differentiation were investigated using human bone marrow mesenchymal stem cells (hMSCs). Similar to the findings of the current study, it was determined that

osteoblastic differentiation markers such as osteocalcin, ALP, ERK and Cbfa1 were increased (Jung CR 2002).

In addition to the single gene alteration, the analyses of this study showed that the Wnt pathway was significantly enhanced in both cell types. The role of this pathway on bone formation and healing is well documented. Kim et al (2007) created holes in the proximal tibia of mice and noticed that the healing relied on Wnt mediated B-catenin signaling (Kim, Leucht et al. 2007). During intramembranous bone formation, the genes involved in Wnt signaling were up-regulated and peaked after 10 days before leveling out. In a study in mice lacking the soluble Wnt inhibitor by Bodine et al., the roles of Wnt signaling in the regulation of trabecular bone formation and bone mass were also supported. They found that osteoblast and osteocyte apoptosis were reduced in these mice (Bodine, Zhao et al. 2004). In addition, it has been suggested that Wnt signaling may increase bone via the stimulation of osteoblastic differentiation.

2.2: ECM proteins

Shilajit significantly increased MG63 cells survival and viability in culture and enhanced their gene expression of several collagens and bone tissue biomarkers. Increasing the ability of these cells to proliferate and survive increases their cell numbers and extends the length of time that these cells are actively expressing extracellular matrix proteins such as during bone formation. Therefore, the ECM proteins secreted by MG63 cells after incubation with shilajit for 33 days were measured using two methods, Coomassie Blue staining and a Bio-Rad Protein Assay.

Although the Bio-Rad Assay is a more accurate method, the findings of both methods demonstrated that the ECM secretion was significantly increased with shilajit treatment for 33 days in serum free media and without media exchange. The effects of shilajit on ECM protein concentrations could be due to: 1) direct effects by enhancing the gene expression of ECM proteins, 2) indirect effects by increasing cell proliferation and survival, which increases the cell numbers and extends the length of time that these cells are actively expressing ECM proteins, or 3) both. Osteoblasts are one of the two major cell types in bone and have a major role during bone formation and healing. Therefore, enhancing some of their activities such as proliferation, survival, and ECM secretion are potential therapeutic approaches to treat bone diseases. This study is the first study to explore the effects of shilajit on the amount of ECM secretion from cells in culture. The 2.7 fold increase in ECM did not reflect the fold increase in cell

numbers of the shilajit treated group relative to the untreated control. For instance, the cell number of the treated group on day 12 was 4.3 fold higher than the one for the untreated group. This variation between the amount of ECM and the number of cells could be simply explained by that in the absence of serum most of the cells preserved their energy for survival rather than using it for ECM production.

Study 3: Role of shilajit on chondrogenesis

3.1: Non-critical size defect Xenopus model system

Shilajit enhanced the gene expression of several collagens *in vitro*. Consequently, the working hypothesis for this study was that shilajit may accelerate the endochondral ossification process by enhancing chondrogenesis during callus formation. Therefore, the anabolic properties of shilajit on enhancing cartilage formation (chondrogenesis) were assessed using a non-critical size bone defect Xenopus model system.

The findings showed that the ratio of total cartilage area/total section area significantly increased around 2.6 fold in shilajit treated animals at both doses relative to the controls, thus demonstrating higher cartilage formation in shilajit treated groups. The results of this study supported the hypothesis that shilajit enhanced cartilage formation. The process of endochondral ossification is a type of healing that occurs mainly in long-bone fractures with small size defects (Stocum 2006, Young 2006, Feng, Milner et al. 2011). This process is characterized by callus formation, in which a cartilage is first formed before being followed by woven and lamellar bone formation. Therefore, the early phases of bone healing by endochondral ossification can be accelerated indirectly by enhancing the cartilage formation during callus formation. If the fracture defect of the mammalian species consisted of a large gap between the two bones, it will be filled with a fibrous scar tissue without bone formation and be called a critical

size defect. In this study, young adult Xenopus were utilized. Similar to mammalian animal species, long bone fracture repair in Xenopus occurs by endochondral ossification and the defect cannot be regenerated if it is a critical size defect (Miura, Hanaoka et al. 2008, Feng, Milner et al. 2011). In a study by Feng et al. (Feng, Milner et al. 2011), a Xenopus model system was used to investigate the regeneration of a critical size defect in the tarsus bone. According to that study, the Xenopus model system has several advantages in comparison to mammalian models. Xenopus have very similar bone biology to mammals. The cost, surgery, general animal care, and postoperative care of these animals are simple and inexpensive. In addition, no external stabilization device is needed because the tarsus bone is a dual skeletal element structure. Consequently, the surgical and post-surgical complications are avoided.

Tkachenko et al. showed that the effects of shilajit on bone healing varies based on the dose and the length of time of delivery after the time of surgery (Tkachenko SS 1979, Jung CR 2002). The most favorable bone healing results were noticed when shilajit was giving at oral doses between 260 to 300 mg/kg daily starting as early as possible after inducing the fractures and stopped at less than 7 days (Tkachenko SS 1979, Jung CR 2002). In the current study, Xenopus were injected with shilajit for 4 weeks and then sacrificed at the end of the injection cycle and cartilage formation between the groups was then compared. The dose range of shilajit used in the current study was not as high as the one utilized by Tkachenko et al. (Tkachenko SS 1979, Jung CR 2002). In the current

study, a lower dose range (3 mg/kg and 6 mg/kg) was selected because shilajit was administered to the animals via subcutaneous injections. According to the published toxicological studies, shilajit should be provided in low doses when used chronically and this was taken into consideration for the selection of doses utilized in the current study (Kelginbaev NS 1973, Anisimov VE 1982, Fortan MH 1984, Al-Hamaidi AR 2003, Biswas, Pandit et al. 2010).

The Xenopus model system has an overall slower metabolic rate in comparison to mammals. Therefore, possible drawbacks of using this system is slow fracture healing process, which may require longer study designs. In other preliminary data, cartilage production during endochondral ossification in Xenopus peaked between 4 and 6 weeks after surgery (data not shown). After 6 weeks, this cartilage mass began to shrink and was replaced by bone tissue. Ossified tissues were easily identified by 12 weeks after surgery (data not shown). According to the literature, this study is the first study assessing the effects of shilajit on chondrogenesis using a Xenopus model system. Another Xenopus study will be needed to evaluate the effects of shilajit on bone formation and its mechanical properties at longer time points (e.g., 12 weeks) from the time of surgery.

The uncut tarsus segment of some of the PBS treated animals showed modest periosteal cartilage response in the bone fracture areas opposite to the cut tarsus bone defect only when higher magnification (5X) was used (Figure

4.3.1.1). Both doses of shilajit displayed a robust periosteal cartilage response at 0.96X magnification (Figure 4.3.1.2). Following the creation of the surgical defect in one of the tarsus bones, the uncut tarsus bone appeared to might have been overloaded. Several fracture points could then have developed along this bone, which may have initiated the chondrogenesis process involved in early stages of bone remodeling. Therefore, one explanation could be that shilajit enhanced the cartilage formation made during bone remodeling of the uncut tarsus bone, which may result in an increase in bone thickness. This could consequently increase its ability to support the cut tarsus bone segment until the bone repair process in completed. In a study by Kelginbaev, shilajit improved bone healing in children after fractures (Kelginbaev NS 1973). In addition, it has been found that a daily dose of 0.1 g/kg of shilajit (orally) accelerated callus formation and phosphorus uptake in other bone fracture model systems *in vivo* (Ismailova 1965, Shakirov 1965, Jung CR 2002).

3.2: Non-injured Xenopus model system

Shilajit enhanced the cartilage formation (chondrogenesis) during the endochondral ossification process following the creation of a non-critical size bone defect in Xenopus. According to that experiment, the role of shilajit on the initiation of cartilage formation was undetermined whether it requires bone trauma to occur. Therefore, the aim of this study was to evaluate the effects of shilajit on the initiation of cartilage formation (chondrogenesis) using a non-injured Xenopus model system.

The results revealed no visible histological changes in the tarsus bones or the surrounding tissues in any of the shilajit treated groups or the controls. This showed that shilajit was unable to initiate the chondrogenesis process in the absence of trauma. The current study is the first study evaluating the effects of shilajit on bone using a non-injured model system.

Study 4: Effects of shilajit on osteoporosis

4.1: Osteoporotic rat model

Shilajit significantly increased MG63 survival and viability in culture, as well as enhanced their gene expression for several collagens and bone tissue biomarkers. Enhancing the activities of these cells are one of the therapeutic approaches to treat bone and connective tissue diseases such as osteoporosis. Therefore, the working hypothesis was that shilajit has anabolic effects on bone healing/repair in osteoporosis.

The bone quality of the ovariectomized rats was analyzed and assessed after receiving different treatment modalities. The ovariectomized rat model has been widely used in many studies to investigate postmenopausal osteoporosis (Komrakova, Stuermer et al. 2010). PTH is the only current anabolic agent approved for osteoporosis treatment. Therefore, it was utilized in the study as a positive control in order to evaluate the bone anabolic properties of shilajit. According to the Micro-CT results, the PTH group demonstrated a trend toward an increase in bone volume (+18%) in comparison to the OVX group, which was not statistically significantly different in the first cohort of animals. The effects of PTH therapy on enhancing bone repair are well documented in the literature (Barnes, Kakar et al. 2008, Komrakova, Stuermer et al. 2010). The range for the effective doses of PTH used in rats is from 1.5 to 200 µg/kg/day (Shirota, Tashiro et al. 2003, Barnes, Kakar et al. 2008, Komrakova, Stuermer et al. 2010). In this

study, a daily dose of 10 µg/kg of PTH was selected and injected for 4 weeks to improve bone formation in six month old ovariectomized rats. In the 1st cohort of animals in this study, only 6 rats were involved in each group. A minimum of 12 animals per group as determined by power analyses are needed for the PTH therapy to obtain statistically significant effects on bone volume. In contrast, none of the two doses of shilajit showed any signs of improvement in bone volume after 4 weeks of treatment according to Micro-CT findings.

Dynamic histomorphometric measures showed promising results in regards the effects of shilajit on bone healing. It was noted that shilajit at low dose (3 mg/kg/day) increased cortical periosteal PFR (36%) equivalent to the PTH group (38%), while the high dose (30 mg/kg/day) produced higher cortical periosteal PFR (66%) than both the PTH and the low dose shilajit treated groups. PTH and the high dose of shilajit slightly enhanced the PFR of the endocortical surface by 26% and 14%, respectively, relative to OVX. Unlike the cortical bone, PTH demonstrated the highest trabecular PFR (+88%) relative to OVX group followed by shilajit 30 (+46%) and shilajit 3 (+39%). Overall, both the trabecular and cortical bone formation rates were increased by shilajit but with better results on cortical periosteal bone surface than PTH. *Based* on *ANOVA* and 0.05 significance level, none of the above increases were statistically significant different from the OVX bone formation rate. Shilajit demonstrated a dose-responsive trend. Higher doses of shilajit and completion the second cohort of

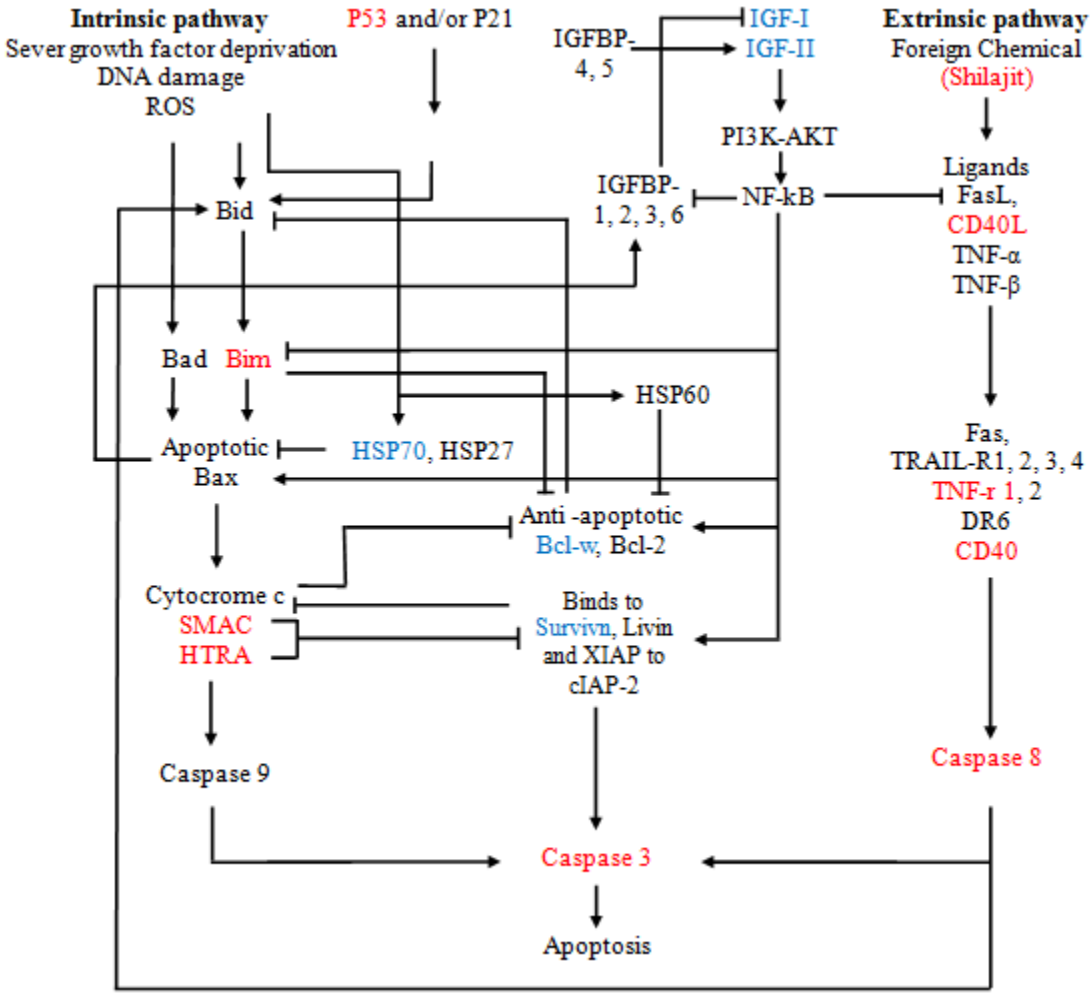
animals of this study may yield greater statistically significant effects on both the cortical and trabecular bone formation rates.

Shilajit was shown to be an inhibitor of osteoclastogenesis and potent stimulator of osteoblastic differentiation of mesenchymal stem cells in culture (Jung CR 2002). In another study, shilajit improved bone healing in children after fractures (Kelginbaev et al., 1973). It has also been found that a daily dose of 0.1 g/kg of shilajit (orally) accelerated callus formation and phosphorus uptake in bone fracture model systems *in vivo* (Ismailova 1965, Shakirov 1965, Jung CR 2002). Tkachenko et al. (Tkachenko SS 1979) showed that the most favorable bone healing effects were noticed in animals when shilajit was giving at oral doses between 260 to 300 mg/kg daily starting as early as possible after inducing fracture and for less than 7 days (Tkachenko SS 1979, Jung CR 2002).

The current study is the first one to evaluate the bone anabolic effects of shilajit using the ovariectomized rat model. This is a different model from the bone fracture one used by others to investigate the bone anabolic effects of shilajit on bone repair. The ovariectomized rat model lacks the callus formation process preceding bone formation. In the current study, shilajit increased the cortical and trabecular bone formation rate but with no changes in trabecular bone volume. It is possible that shilajit's primary effects are on bone healing by improving chondrogenesis that occurrs during callus formation. Another explanation is that there was a delay in the anabolic responses of shilajit

indicating that longer treatment durations might yield greater bone formation and volume. Finally, the dose range used in the current study was different to the one used by Tkachenko et al. In the current study, lower dose ranges (3 mg/kg and 30 mg/kg) was selected because shilajit was administered to the animals via I.P injections and long term injection cycles were used (4 weeks). According to the toxicological studies, shilajit should be provided in low doses when used chronically (Kelginbaev NS 1973, Anisimov VE 1982, Fortan MH 1984, Al-Hamaidi AR 2003, Biswas, Pandit et al. 2010). Higher doses or different time regimens in future studies might result in higher bone formation and volume simultaneously. In the second cohort of animals for this study, the sample size of each group will be increased and additionally a higher dose of shilajit will be utilized. Since the effects of shilajit on BFR appeared to be dose-responsive according to the results of the 1st cohort of animals, it was decided that a higher concentration of shilajit (150 mg/kg) should be assessed. In addition, two additional groups of animals will be added to evaluate the effects of short term application of shilajit on osteoporosis.

Figure 4.1.3.1. The effects of shilajit on extrinsic and intrinsic apoptotic pathways.
Down-regulated pro-apoptotic proteins
Down-regulated anti-apoptotic proteins



Batarseh, Ghada thesis 2011. TEGDMA INDUCTION OF APOPTOTIC PROTEINS IN PULP FIBROBLASTS

Figure 4.3.1.1. High magnification (5X) of cross sectional image for PBS treated sample showing modest periosteal cartilage formation response in the uncut tarsus bone labeled with asterisk.

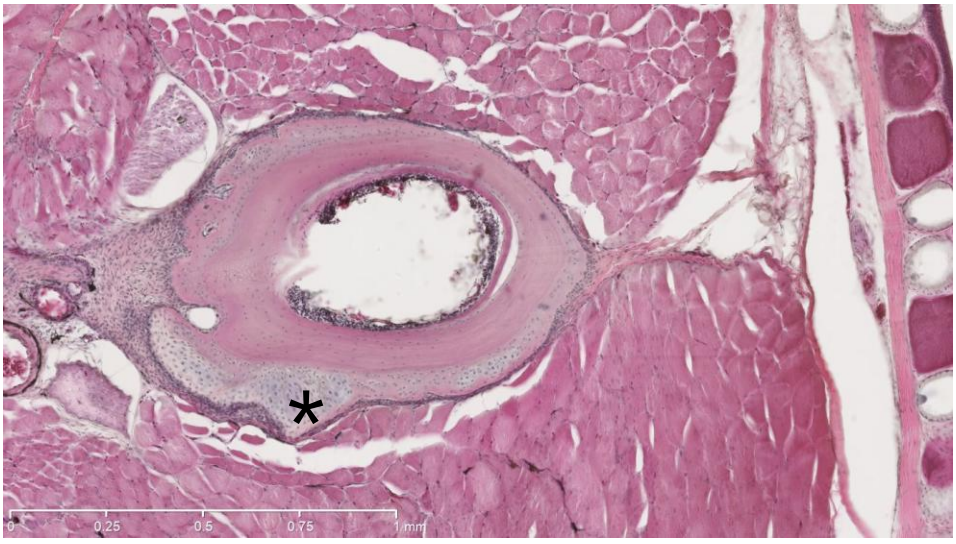


Figure 4.3.1.2. Low magnification (0.96X) of cross sectional image for shilajit treated sample showing robust periosteal cartilage formation response in the uncut tarsus bone labeled with asterisk.

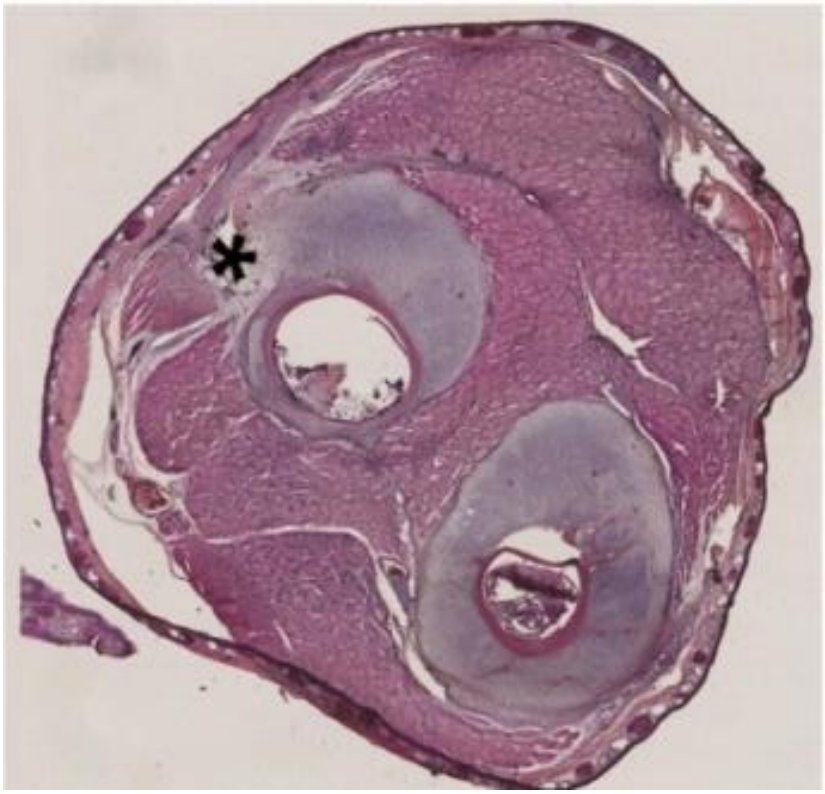


Table 4.1.2.1. The main differences between necrosis and apoptosis.

Necrosis	**Apoptosis**
Pathological uncontrolled process	Physiological controlled process,
Cells swell followed by cell lysis)	Cells shrink leading to apoptotic bodies
No energy required	Energy dependent
DNA dissolution	DNA fragmentation
Inflammatory	Non inflammatory
Loss of membrane integrity	No loss of membrane integrity

CHAPTER FIVE

Summary and Conclusion

A general summary and the conclusions from the different experiments described in this dissertation are discussed in this chapter. In addition, the general significance of these experiments and the ongoing/future studies are mentioned.

The overall objective of these experiments were to better understand if and how shilajit regulates bone and cartilage regeneration/repair during bone fracture and osteoporosis using molecular, cell-based and *in vivo* approaches.

The results from the cytotoxicity assays showed that 1.6 mg/ml was the highest non-toxic concentration of shilajit that could be utilized with MG63 cells. Lower dose ranges may be needed when longer incubation times are needed in cell culture. Other types of cells might demonstrate a different range of cytotoxicity levels when they are evaluated using the same assays. Therefore, cytotoxicity levels need to be determined for each cell type.

According to the findings of the WST-1 assays, shilajit significantly increased the cell proliferation of MG63 cells up to 9 days relative to the untreated control. In addition, it significantly increased their survival up to 30 days and these cells retained their ability to proliferate when serum and fresh media

were re-introduced. Therefore, shilajit might have the ability to increase the number of cells and extend the length of time that they are actively expressing extracellular matrix proteins. Enhancing the activities such as proliferation and survival of bone forming cells (osteoblasts) accelerates the process of bone healing. The LDH activities of these cells during 33 days of shilajit incubation in cell culture were also assessed. Unlike the control group, the LDH activity for shilajit treated group did not reach 100%, although the WST-1 assays for the same group revealed complete cell death. Therefore, it was determined that the cell death in the control group was mainly due to necrosis while a combination between necrosis and apoptosis might have caused the cell death in the shilajit treated group. The design of the WST-1 assays utilized in this experiment is a simple method, which can be utilized to evaluate the active ingredients in shilajit *in vitro* once they are identified.

According to the apoptotic array results, it was determined that shilajit inhibited the apoptosis of MG63 by possible altering the balance between pro-apoptotic and anti-apoptotic proteins. Therefore, shilajit's ability to increase cell survival of the MG63 could be due to its ability to inhibit the apoptotic processes of these cells. Another explanation could be that shilajit might also have maintained the cells in a resting period (G0) until serum was re-introduced. More studies are needed to explain all the mechanisms by which shilajit increases cell survival such as evaluating the effects of shilajit on Protein Kinase B (PKB) that plays a key role in cellular survival pathways.

The findings of the Microarray Illumina Technology mRNA array showed that shilajit enhanced the gene expression for several bone and connective tissue biomarkers in both MG63 and HGF cells. The Wnt pathway was also significantly up-regulated in both cell types. This experiment demonstrated that HGFs were affected by shilajit similarly to MG63 (data not shown).

The ECM protein secretion analysis showed significantly increased around 2.7 folds after 33 days of incubation with shilajit in culture. This supported the findings of the WST-1 assays because shilajit increased the cell number and extend the length of time that these cells are actively expressing extracellular matrix proteins. This further confirms the ability of this agent to enhance the activities of osteoblasts, which should accelerate bone healing.

According to the Xenopus studies, shilajit increased the healing responses as evident by the increased levels of cartilage formation relative to the controls. In addition, these studies showed that shilajit had the ability to enhance but not to induce the chondrogenesis process with bone fracture. The bone healing effects of shilajit documented in the literature could be mainly due to its indirect effects on enhancing the chondrogenesis process that occurs during endochondral ossification.

In the ovariectomized rat model, no post-operative complications or side effects were observed after 4 weeks of daily injections of 3 mg/kg or 30 mg/kg of

shilajit. Additional studies are needed to assess the full safety of different doses and treatment durations. The findings of the Micro-CT showed that both doses of shilajit did not improve trabecular bone volume. In contrast, dynamic histomorphometric analysis showed that both trabecular and cortical bone formation rates were increased by shilajit. The effects of shilajit on periosteal bone formation rate were greater than PTH. In addition, the effects of shilajit on BFR appeared to be dose-responsive. Higher doses of shilajit, as well as completion the second cohort of animals, may yield statistically significant effects on both cortical and trabecular bone formation. Therefore, the trends in the results from shilajit-treated animals suggest that shilajit may possess anabolic properties.

Significance

According to Gullberg et al. (Gullberg, Johnell et al. 1997), the worldwide incidence of hip fracture in men and women in 2050 are projected to increase to 310% and 240%, respectively. The combined life time risk for hip, forearm and vertebral fractures is around 40%, which is equivalent to the risk for cardiovascular disease (Kanis 2002). Another interesting statistics is that the life time risk for hip fracture is 1 to 6 in white women, while it is 1 to 9 risk for breast cancer (Cummings and Melton 2002). In Europe, the disability due to osteoporosis is greater than that caused by cancer (Johnell and Kanis 2006). The disadvantages of osteoporosis-related treatment strategies are the high cost and the associated side effects. In addition to osteoporosis, osteoarthritis affects around twenty seven million Americans and 12% of the adults in the United States (Lawrence, Felson et al. 2008, Smith, Martins et al. 2012). Also, it occurs mainly in individuals over 65 years of age (Walker-Bone, Javaid et al. 2000). By the year 2030, it is estimated that osteoarthritis will affect seventy two million and twenty percent of the adult population in the United States (Elders 2000, Smith, Martins et al. 2012). Osteoarthritis is ranked as the fourth in health impact among women and eighth among men (Walker-Bone, Javaid et al. 2000). It is also a major cause of disability and pain among the Western population. By 2020, it is estimated that the combined annual cost of medical care and lost wages due to osteoarthritis will reach US $100 billion (Elders 2000, Leigh, Seavey et al. 2001, Bitton 2009, Smith, Martins et al. 2012). Posttraumatic osteoarthritis usually occurs after an intra-articular cartilage fracture and treatment is estimated to cost

around $12 billion each year (Brown, Johnston et al. 2006). It has been reported that articular cartilage fractures occurs in patients younger than 45 years old about 76% of the time. Individuals who develop posttraumatic osteoarthritis are about 9 to 14 years younger than the individuals who have osteoarthritis (Brown, Johnston et al. 2006, McKinley, Borrelli et al. 2010). Approximately between 23% and 44% of patients develop posttraumatic osteoarthritis after an intra-articular cartilage fracture of the knee and more than 50% of the patients after an intra-articular cartilage fracture of the distal tibial articular surface also develop posttraumatic osteoarthritis (Ovadia and Beals 1986, Volpin, Dowd et al. 1990, Teeny and Wiss 1993, Honkonen 1995, McKinley, Borrelli et al. 2010). Unfortunately, the outcomes of the intra-articular cartilage fractures and posttraumatic osteoarthritis have not changed much over the years.

The goal here is to evaluate shilajit for its ability to enhance bone and cartilage formation. It would be a significant discovery and could eventually impact patient care if these studies provide scientific data needed to justify further exploring shilajit for its bone promoting properties. The results of the series of experiments described in this dissertation are very important because they help provide scientific evidence needed to develop a possible new approach to treat and prevent osteoporosis, as well as osteoarthritis and posttraumatic osteoarthritis, that may have little to no side effects. Given shilajit's human use for thousands of year and its apparent ability to promote regeneration/repair, it should be easily translated into practice and could become the standard of care

for bone/cartilage tissue pathologies, as well as it may have other applications. Some of these other applications are bone and soft tissue grafting, periodontal disease and bone-implant interface healing. Great discoveries have been made by scientifically examining remedies/treatments that people have used for years based only on the fact that they work especially if the active agent can be identified.

Ongoing/Future studies

The results obtained from the studies of this dissertation are preliminary. More studies are required to investigate the properties of this agent to provide enough scientific evidence needed to advance the use of shilajit as a treatment option for bone and cartilage healing. Some of the ongoing and possible future studies are outlined below.

1) Assessing the anabolic properties of shilajit on bone mass using an ovariectomized rat model (cohort 2).

A second cohort of animals will be employed to repeat the same experiment in order to increase the sample size and assessing higher concentrations of shilajit (150 mg/kg). In addition, two additional groups of animals will be added to evaluate the effects of short term application of shilajit on osteoporosis and comparing it with the long term groups.

2) Evaluating the effects of shilajit on cell survival, extracellular matrix (ECM) expression, and cell differentiation using other cell types such mesenchymal stem cells, chondrocytes, and osteoclast cells in culture.

MG63 (osteoblast-like cells) was the main type of cells utilized in the current *in vitro* studies. HGFs were examined in some of these studies. It is very important

that other types of cells are evaluated to further confirm the bone/cartilage healing or side effects of shilajit.

3) Investigating the bone-healing effects of shilajit using a rat cortical defect model.

Some of the limitations of the simple fracture or segmental bone defect models are slow bone healing rate, technique sensitivity, and difficulty to standardize the defect among the animals. These always require longer studies, higher cost, and larger sample sizes. Therefore, a cortical defect model will be selected instead because it requires shorter healing duration and it is easier to standardize its defect among the animals.

4) Evaluating the efficacy of shilajit on the expression of osteogenic markers in three dimensional cultures.

Cells might react differently when they are cultured into three dimensional environment. Mono-plane cultures were utilized in all of the current *in vitro* studies. Therefore, the goal here is to confirm the positive effects of shilajit obtained using three dimensional cultures.

5) Investigating the effects of shilajit on cartilage repair by:

a- Evaluating the effects of shilajit on chondrogensis using mesenchymal stem cells in culture.

b- Evaluating how shilajit affects cartilage healing using an osteoarthritis mice model.

According to the preliminary results, it was demonstrated that shilajit enhanced cartilage formation during the bone fracture repair process using in the *in vivo* Xenopus model system. Therefore, it is hypothesized that shilajit induces or enhances the chondrogenesis processes needed to treat cartilage diseases such as osteoarthritis.

In summary, the findings of the studies of this dissertation suggest that shilajit promotes osteoblast survival due to its effects on altering the balance between pro-apoptotic and anti-apoptotic proteins. This could extend the length of time that these cells are actively expressing ECM proteins, which should accelerate bone healing. In addition, *in vivo* studies revealed that shilajit enhanced cartilage formation in Xenopus and BFRs in rats. Therefore, shilajit may possess anabolic bone/cartilage properties.

REFERENCES

(2010). "Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society." Menopause 17(1): 25-54; quiz 55-26.

Abrahamsen, B. (2010). "Adverse effects of bisphosphonates." Calcif Tissue Int 86(6): 421-435.

Acharya, S. B., M. H. Frotan, R. K. Goel, S. K. Tripathi and P. K. Das (1988). "Pharmacological actions of Shilajit." Indian Journal of Experimental Biology 26(10): 775-777.

Agarwal, S. P., R. Khanna, R. Karmarkar, M. K. Anwer and R. K. Khar (2007). "Shilajit: a review." Phytotherapy Research 21(5): 401-405.

Agzamov RA, A. S., Vakhidova GA. (1988). "Mumie as a pathogenetic agent in the treatment of tuberculosis." Probl Tuberk 7: 49-52.

Ahlborg, H. G., O. Johnell, C. H. Turner, G. Rannevik and M. K. Karlsson (2003). "Bone loss and bone size after menopause." N Engl J Med 349(4): 327-334.

Al-Hamaidi AR, U. M. (2003). "Safe use of Shilajit during pregnancy of female mice." Online J Biol Sci 3: 681-684.

Allen, M., Burr, DB (2006). "Parathyroid Hormone and Bone Biomechanics." Clin Rev Bone Min Metab 4: 259-268.

Allen, M. R., J. M. Hock and D. B. Burr (2004). "Periosteum: biology, regulation, and response to osteoporosis therapies." Bone 35(5): 1003-1012.

Alves de Oliveira, E. C., V. L. Szejnfeld, N. Pereira da Silva, L. E. Coelho Andrade and C. Heldan de Moura Castro (2010). "Intermittent PTH1-34 causes DNA and chromosome breaks in osteoblastic and nonosteoblastic cells." Calcif Tissue Int 87(5): 424-436.

Anisimov VE, S. R. (1982). "Application of Mumie in therapeutic practice." Kazan Med Zh 63: 65-68.

Arboleya, L., M. Alperi and S. Alonso (2011). "[Adverse effects of bisphosphonates]." Reumatol Clin 7(3): 189-197.

Ashkenazi, A. (2002). "Targeting death and decoy receptors of the tumour-necrosis factor superfamily." Nat Rev Cancer 2(6): 420-430.

Atik, O. S. (2011). "What do we know about the ocular adverse effects associated with intravenous bisphosphonates?" Eklem Hastalik Cerrahisi 22(1): 1.

Bailey, A. J., S. F. Wotton, T. J. Sims and P. W. Thompson (1992). "Post-translational modifications in the collagen of human osteoporotic femoral head." Biochemical and Biophysical Research Communications 185(3): 801-805.

Bandyopadhyay, A., K. Tsuji, K. Cox, B. D. Harfe, V. Rosen and C. J. Tabin (2006). "Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis." PLoS Genet 2(12): e216.

Barbara Obermayer-Pietsch, V. S. (2011). Principles of Osteoimmunology: Molecular Mechanisms and Clinical Applications, Springer.

Barhanpurkar, A. P., N. Gupta, R. K. Srivastava, G. B. Tomar, S. P. Naik, S. R. Joshi, S. T. Pote, G. C. Mishra and M. R. Wani (2012). "IL-3 promotes osteoblast differentiation and bone formation in human mesenchymal stem cells." Biochem Biophys Res Commun 418(4): 669-675.

Barnes, G. L., S. Kakar, S. Vora, E. F. Morgan, L. C. Gerstenfeld and T. A. Einhorn (2008). "Stimulation of fracture-healing with systemic intermittent parathyroid hormone treatment." Journal of Bone and Joint Surgery 90 Suppl 1: 120-127.

Benglis, D., M. Y. Wang and A. D. Levi (2008). "A comprehensive review of the safety profile of bone morphogenetic protein in spine surgery." Neurosurgery 62(5 Suppl 2): ONS423-431; discussion ONS431.

Bhattacharya, S. K. (1995). "Shilajit attenuates streptozocin induced diabetes mellitus and decrease in pancreatic islet superoxide dismutase activity in rats." Phytother Res 9: 41-44.

Bhattacharya SK, S. A. (1995). "Effects of shilajit on biogenic free radicals." Phytother Res. 9: 56-59.

Bhosale, A. M. and J. B. Richardson (2008). "Articular cartilage: structure, injuries and review of management." Br Med Bull 87: 77-95.

Biswas, T. K., S. Pandit, S. Mondal, S. K. Biswas, U. Jana, T. Ghosh, P. C. Tripathi, P. K. Debnath, R. G. Auddy and B. Auddy (2010). "Clinical evaluation of spermatogenic activity of processed Shilajit in oligospermia." Andrologia 42(1): 48-56.

Bitton, R. (2009). "The economic burden of osteoarthritis." Am J Manag Care 15(8 Suppl): S230-235.

Black, D. M., S. R. Cummings, D. B. Karpf, J. A. Cauley, D. E. Thompson, M. C. Nevitt, D. C. Bauer, H. K. Genant, W. L. Haskell, R. Marcus, S. M. Ott, J. C. Torner, S. A. Quandt, T. F. Reiss and K. E. Ensrud (1996). "Randomised trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture Intervention Trial Research Group." Lancet 348(9041): 1535-1541.

Blaney Davidson, E. N., P. M. van der Kraan and W. B. van den Berg (2007). "TGF-beta and osteoarthritis." Osteoarthritis Cartilage 15(6): 597-604.

Bodine, P. V., W. Zhao, Y. P. Kharode, F. J. Bex, A. J. Lambert, M. B. Goad, T. Gaur, G. S. Stein, J. B. Lian and B. S. Komm (2004). "The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice." Molecular Endocrinology 18(5): 1222-1237.

Boskey, A. L., T. M. Wright and R. D. Blank (1999). "Collagen and bone strength." Journal of Bone and Mineral Research 14(3): 330-335.

Brown, T. D., R. C. Johnston, C. L. Saltzman, J. L. Marsh and J. A. Buckwalter (2006). "Posttraumatic osteoarthritis: a first estimate of incidence, prevalence, and burden of disease." J Orthop Trauma 20(10): 739-744.

Burr, D. B., T. Hirano, C. H. Turner, C. Hotchkiss, R. Brommage and J. M. Hock (2001). "Intermittently administered human parathyroid hormone(1-34) treatment increases intracortical bone turnover and porosity without reducing bone strength in the humerus of ovariectomized cynomolgus monkeys." J Bone Miner Res 16(1): 157-165.

Cahill, K. S., J. H. Chi, A. Day and E. B. Claus (2009). "Prevalence, complications, and hospital charges associated with use of bone-morphogenetic proteins in spinal fusion procedures." JAMA 302(1): 58-66.

Carlisle, E. and J. S. Fischgrund (2005). "Bone morphogenetic proteins for spinal fusion." Spine J 5(6 Suppl): 240S-249S.

Carrasco-Gallardo, C., L. Guzman and R. B. Maccioni (2012). "Shilajit: a natural phytocomplex with potential procognitive activity." Int J Alzheimers Dis 2012: 674142.

Chaudhary, L. R. and L. V. Avioli (1998). "Identification and activation of mitogen-activated protein (MAP) kinase in normal human osteoblastic and bone marrow stromal cells: attenuation of MAP kinase activation by cAMP, parathyroid hormone and forskolin." Mol Cell Biochem 178(1-2): 59-68.

Chen, D., M. Zhao and G. R. Mundy (2004). "Bone morphogenetic proteins." Growth Factors 22(4): 233-241.

Chen, G., C. Deng and Y. P. Li (2012). "TGF-beta and BMP signaling in osteoblast differentiation and bone formation." Int J Biol Sci 8(2): 272-288.

Chen, Q. M., J. B. Merrett, T. Dilley and S. Purdom (2002). "Down regulation of p53 with HPV E6 delays and modifies cell death in oxidant response of human diploid fibroblasts: an apoptosis-like cell death associated with mitosis." Oncogene 21(34): 5313-5324.

Cole, A. G. (2011). "A review of diversity in the evolution and development of cartilage: the search for the origin of the chondrocyte." Eur Cell Mater 21: 122-129.

Cornejo, A., J. M. Jimenez, L. Caballero, F. Melo and R. B. Maccioni (2011). "Fulvic acid inhibits aggregation and promotes disassembly of tau fibrils associated with Alzheimer's disease." J Alzheimers Dis 27(1): 143-153.

Coultas, L. and A. Strasser (2003). "The role of the Bcl-2 protein family in cancer." Semin Cancer Biol 13(2): 115-123.

Cowin, S. C. (2002). "Mechanosensation and fluid transport in living bone." J Musculoskelet Neuronal Interact 2(3): 256-260.

Cummings, S. R., D. M. Black, D. E. Thompson, W. B. Applegate, E. Barrett-Connor, T. A. Musliner, L. Palermo, R. Prineas, S. M. Rubin, J. C. Scott, T. Vogt, R. Wallace, A. J. Yates and A. Z. LaCroix (1998). "Effect of alendronate on risk of fracture in women with low bone density but without vertebral fractures: results from the Fracture Intervention Trial." JAMA 280(24): 2077-2082.

Cummings, S. R. and L. J. Melton (2002). "Epidemiology and outcomes of osteoporotic fractures." Lancet 359(9319): 1761-1767.

Dahl, S. G., P. Allain, P. J. Marie, Y. Mauras, G. Boivin, P. Ammann, Y. Tsouderos, P. D. Delmas and C. Christiansen (2001). "Incorporation and distribution of strontium in bone." Bone 28(4): 446-453.

Datta, N. S. (2011). "Osteoporotic fracture and parathyroid hormone." World J Orthop 2(8): 67-74.

Ducy, P., C. Desbois, B. Boyce, G. Pinero, B. Story, C. Dunstan, E. Smith, J. Bonadio, S. Goldstein, C. Gundberg, A. Bradley and G. Karsenty (1996). "Increased bone formation in osteocalcin-deficient mice." Nature 382(6590): 448-452.

Einhorn, T. A. (2011). "Systemic administration of PTH 1-84 for fracture repair: an exciting prospect: Commentary on an article by Peter Peichl, MD, et al.: "Parathyroid hormone 1-84 accelerates fracture-healing in pubic bones of elderly osteoporotic women"." J Bone Joint Surg Am 93(17): e102(101-102).

Elders, M. J. (2000). "The increasing impact of arthritis on public health." J Rheumatol Suppl 60: 6-8.

Farley, J., H. P. Dimai, B. Stilt-Coffing, P. Farley, T. Pham and S. Mohan (2000). "Calcitonin increases the concentration of insulin-like growth factors in serum-free cultures of human osteoblast-line cells." Calcif Tissue Int 67(3): 247-254.

Feng, L., D. J. Milner, C. Xia, H. L. Nye, P. Redwood, J. A. Cameron, D. L. Stocum, N. Fang and I. Jasiuk (2011). "Xenopus laevis as a novel model to study long bone critical-size defect repair by growth factor-mediated regeneration." Tissue Eng Part A 17(5-6): 691-701.

Fortan MH, A. S. (1984). "Pharmacological studies of Shilajit. ." Indian J Pharmacol 16: 45-48.

Fortier, L. A., J. U. Barker, E. J. Strauss, T. M. McCarrel and B. J. Cole (2011). "The role of growth factors in cartilage repair." Clin Orthop Relat Res 469(10): 2706-2715.

Franceschi, R. T. (1999). "The developmental control of osteoblast-specific gene expression: role of specific transcription factors and the extracellular matrix environment." Crit Rev Oral Biol Med 10(1): 40-57.

Fulda, S. and K. M. Debatin (2006). "Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy." Oncogene 25(34): 4798-4811.

Gaikwad, N. S., A. V. Panat, M. S. Deshpande, K. Ramya, P. U. Khalid and P. Augustine (2012). "Effect of shilajit on the heart of Daphnia: A preliminary study." J Ayurveda Integr Med 3(1): 3-5.

Galimov, E. M., Kodina, L.A., Vlasova LN, Velyukhanova TK, Bazilevskaja OL. (1986). "Geochemistry of Mummiyo." Geochemistry: 1494-1505

Ghosal S., L. J., Singh S.K. (1989). "Mast cell protecting effects of shilajit and its constituents." Phytotherapy Research 3: 249-252.

Ghosal S., L. S., Kumar Y., Gaur B., and Misra N. (1995). "Interaction of shilajit with biogenic free radicals." Indian Journal of Chemistry and Biology 34: 596-602.

Ghosal S., S. S. K., Kumar S. (1988.). "Shilajit. 3. Antiulcerogenic of fulvic acids and 4-methoxy-6-carbomethoxybiphenyl isolated from shilaji." Phytotherapy Research 2: 187-191.

Goel, R. K., R. S. Banerjee and S. B. Acharya (1990). "Antiulcerogenic and antiinflammatory studies with shilajit." Journal of Ethnopharmacology 29(1): 95-103.

Gu, K., L. Zhang, T. Jin and R. B. Rutherford (2004). "Identification of potential modifiers of Runx2/Cbfa1 activity in C2C12 cells in response to bone morphogenetic protein-7." Cells Tissues Organs 176(1-3): 28-40.

Gullberg, B., O. Johnell and J. A. Kanis (1997). "World-wide projections for hip fracture." Osteoporos Int 7(5): 407-413.

Guo, X. and X. F. Wang (2009). "Signaling cross-talk between TGF-beta/BMP and other pathways." Cell Research 19(1): 71-88.

Gupta, P. K., A. K. Das, A. Chullikana and A. S. Majumdar (2012). "Mesenchymal stem cells for cartilage repair in osteoarthritis." Stem Cell Res Ther 3(4): 25.

Hanamura, H., Y. Higuchi, M. Nakagawa, H. Iwata and M. R. Urist (1980). "Solubilization and purification of bone morphogenetic protein (BMP) from Dunn osteosarcoma." Clin Orthop Relat Res(153): 232-240.

Handagama, P., D. F. Bainton, Y. Jacques, M. T. Conn, R. A. Lazarus and M. A. Shuman (1993). "Kistrin, an integrin antagonist, blocks endocytosis of fibrinogen into guinea pig megakaryocyte and platelet alpha-granules." J Clin Invest 91(1): 193-200.

Harada, H., S. Tagashira, M. Fujiwara, S. Ogawa, T. Katsumata, A. Yamaguchi, T. Komori and M. Nakatsuka (1999). "Cbfa1 isoforms exert functional differences in osteoblast differentiation." J Biol Chem 274(11): 6972-6978.

Harris, S. T., N. B. Watts, H. K. Genant, C. D. McKeever, T. Hangartner, M. Keller, C. H. Chesnut, 3rd, J. Brown, E. F. Eriksen, M. S. Hoseyni, D. W. Axelrod and P. D. Miller (1999). "Effects of risedronate treatment on vertebral and nonvertebral fractures in women with postmenopausal osteoporosis: a randomized controlled trial. Vertebral Efficacy With Risedronate Therapy (VERT) Study Group." JAMA 282(14): 1344-1352.

Hashimoto, S., K. Takahashi, D. Amiel, R. D. Coutts and M. Lotz (1998). "Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis." Arthritis Rheum 41(7): 1266-1274.

Hirano, T., D. B. Burr, R. L. Cain and J. M. Hock (2000). "Changes in geometry and cortical porosity in adult, ovary-intact rabbits after 5 months treatment with LY333334 (hPTH 1-34)." Calcif Tissue Int 66(6): 456-460.

Hirano, T., D. B. Burr, C. H. Turner, M. Sato, R. L. Cain and J. M. Hock (1999). "Anabolic effects of human biosynthetic parathyroid hormone fragment (1-34), LY333334, on remodeling and mechanical properties of cortical bone in rabbits." J Bone Miner Res 14(4): 536-545.

Hock, J. M. and I. Gera (1992). "Effects of continuous and intermittent administration and inhibition of resorption on the anabolic response of bone to parathyroid hormone." J Bone Miner Res 7(1): 65-72.

Holroyd, C., C. Cooper and E. Dennison (2008). "Epidemiology of osteoporosis." Best Pract Res Clin Endocrinol Metab 22(5): 671-685.

Honkonen, S. E. (1995). "Degenerative arthritis after tibial plateau fractures." J Orthop Trauma 9(4): 273-277.

Huang, W., S. Yang, J. Shao and Y. P. Li (2007). "Signaling and transcriptional regulation in osteoblast commitment and differentiation." Frontiers in Bioscience 12: 3068-3092.

Huang, Z., P. G. Ren, T. Ma, R. L. Smith and S. B. Goodman (2010). "Modulating osteogenesis of mesenchymal stem cells by modifying growth factor availability." Cytokine 51(3): 305-310.

Ignotz, R. A. and J. Massague (1986). "Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix." Journal of Biological Chemistry 261(9): 4337-4345.

Islam K., S. A., and Gropp J.M. (2005). "Humic acid substances in animal agriculture." Pakistan Journal of Nutrition Abstracts and Reviews 4: 126-134.

Ismailova, V. N. (1965). "To a question about the dosage of mumie and special features of the healing of the fractures of the bones during its application." Med J Uzbekistan 9: 69-70.

Jaiswal, A. K., Bhattacharya, S. K. (1992). "Effects of shilajit on memory, anxiety and brain monoamines in rats." Indian J Pharmacol 24: 12-17.

Janicke, R. U., M. L. Sprengart, M. R. Wati and A. G. Porter (1998). "Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis." Journal of Biological Chemistry 273(16): 9357-9360.

Jeffrey, J. E., D. W. Gregory and R. M. Aspden (1995). "Matrix damage and chondrocyte viability following a single impact load on articular cartilage." Arch Biochem Biophys 322(1): 87-96.

Jerome, C. P., C. S. Johnson, H. T. Vafai, K. C. Kaplan, J. Bailey, B. Capwell, F. Fraser, L. Hansen, H. Ramsay, M. Shadoan, C. J. Lees, J. S. Thomsen and L. Mosekilde (1999). "Effect of treatment for 6 months with human parathyroid hormone (1-34) peptide in ovariectomized cynomolgus monkeys (Macaca fascicularis)." Bone 25(3): 301-309.

Johnell, O. and J. A. Kanis (2006). "An estimate of the worldwide prevalence and disability associated with osteoporotic fractures." Osteoporos Int 17(12): 1726-1733.

Johnson, E. E., M. R. Urist and G. A. Finerman (1990). "Distal metaphyseal tibial nonunion. Deformity and bone loss treated by open reduction, internal fixation, and human bone morphogenetic protein (hBMP)." Clin Orthop Relat Res(250): 234-240.

Jung CR, K. K., Kwon BS (2001). "Mesenchymal stem cells and osteogenesis" Immune Network 3: 179-186.

Jung CR, S. I., Woo SB, Kwon BS (2002). "Osteoblastic Differentiation of Mesenchymal Stem Cells by mumie extract." Drug Development Research 57(3): 122-133.

Kadiyala, S., R. G. Young, M. A. Thiede and S. P. Bruder (1997). "Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro." Cell Transplant 6(2): 125-134.

Kanakaris, N. K. and P. V. Giannoudis (2008). "Clinical applications of bone morphogenetic proteins: current evidence." J Surg Orthop Adv 17(3): 133-146.

Kanis, J. A. (2002). "Diagnosis of osteoporosis and assessment of fracture risk." Lancet 359(9321): 1929-1936.

Katagiri, T. and N. Takahashi (2002). "Regulatory mechanisms of osteoblast and osteoclast differentiation." Oral Diseases 8(3): 147-159.

Kawaguchi, H., T. Kurokawa, K. Hanada, Y. Hiyama, M. Tamura, E. Ogata and T. Matsumoto (1994). "Stimulation of fracture repair by recombinant human basic fibroblast growth factor in normal and streptozotocin-diabetic rats." Endocrinology 135(2): 774-781.

Kelginbaev NS, S. V., Stefandu AC, Ismailova VN (1973). "Treatment of long tubular bone fractures with Mumie and preparation in experimental and clinical conditions." Exp Surg Anesth 18: 31-35.

Kelm, R. J., Jr., N. A. Swords, T. Orfeo and K. G. Mann (1994). "Osteonectin in matrix remodeling. A plasminogen-osteonectin-collagen complex." Journal of Biological Chemistry 269(48): 30147-30153.

Kelm, R. J., Jr., N. A. Swords, T. Orfeo and K. G. Mann (1994). "Osteonectin in matrix remodeling. A plasminogen-osteonectin-collagen complex." J Biol Chem 269(48): 30147-30153.

Kennel, K. A. and M. T. Drake (2009). "Adverse effects of bisphosphonates: implications for osteoporosis management." Mayo Clin Proc 84(7): 632-637; quiz 638.

Kharazmi, M., U. Persson and G. Warfvinge (2012). "Pharmacovigilance of Oral Bisphosphonates: Adverse Effects Manifesting in the Soft Tissue of the Oral Cavity." J Oral Maxillofac Surg.

Kim, H. W. and J. S. Jahng (1999). "Effect of intermittent administration of parathyroid hormone on fracture healing in ovariectomized rats." Iowa Orthop J 19: 71-77.

Kim, J. B., P. Leucht, K. Lam, C. Luppen, D. Ten Berge, R. Nusse and J. A. Helms (2007). "Bone regeneration is regulated by wnt signaling." Journal of Bone and Mineral Research 22(12): 1913-1923.

Kim, R., M. Emi and K. Tanabe (2006). "Role of mitochondria as the gardens of cell death." Cancer Chemother Pharmacol 57(5): 545-553.

Komori, T., H. Yagi, S. Nomura, A. Yamaguchi, K. Sasaki, K. Deguchi, Y. Shimizu, R. T. Bronson, Y. H. Gao, M. Inada, M. Sato, R. Okamoto, Y. Kitamura, S. Yoshiki and T. Kishimoto (1997). "Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts." Cell 89(5): 755-764.

Komrakova, M., E. K. Stuermer, C. Werner, M. Wicke, L. Kolios, S. Sehmisch, M. Tezval, F. Daub, T. Martens, P. Witzenhausen, C. Dullin and K. M. Stuermer (2010). "Effect of human parathyroid hormone hPTH (1-34) applied at different regimes on fracture healing and muscle in ovariectomized and healthy rats." Bone 47(3): 480-492.

Kulkarni, N. H., T. Wei, A. Kumar, E. R. Dow, T. R. Stewart, J. Shou, M. N'Cho, D. L. Sterchi, B. D. Gitter, R. E. Higgs, D. L. Halladay, T. A. Engler, T. J. Martin, H. U. Bryant, Y. L. Ma and J. E. Onyia (2007). "Changes in osteoblast, chondrocyte, and adipocyte lineages mediate the bone anabolic actions of PTH and small molecule GSK-3 inhibitor." J Cell Biochem 102(6): 1504-1518.

Kwon, Y. W., S. Ueda, M. Ueno, J. Yodoi and H. Masutani (2002). "Mechanism of p53-dependent apoptosis induced by 3-methylcholanthrene: involvement of p53 phosphorylation and p38 MAPK." J Biol Chem 277(3): 1837-1844.

Lawrence, R. C., D. T. Felson, C. G. Helmick, L. M. Arnold, H. Choi, R. A. Deyo, S. Gabriel, R. Hirsch, M. C. Hochberg, G. G. Hunder, J. M. Jordan, J. N. Katz, H. M. Kremers and F. Wolfe (2008). "Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II." Arthritis Rheum 58(1): 26-35.

Leigh, J. P., W. Seavey and B. Leistikow (2001). "Estimating the costs of job related arthritis." J Rheumatol 28(7): 1647-1654.

Lian, J. B., G. S. Stein, J. L. Stein and A. J. van Wijnen (1998). "Osteocalcin gene promoter: unlocking the secrets for regulation of osteoblast growth and differentiation." J Cell Biochem Suppl 30-31: 62-72.

Linkhart, T. A., J. C. Jennings, S. Mohan, G. K. Wakley and D. J. Baylink (1986). "Characterization of mitogenic activities extracted from bovine bone matrix." Bone 7(6): 479-487.

Lo, M. Y. and H. T. Kim (2004). "Chondrocyte apoptosis induced by collagen degradation: inhibition by caspase inhibitors and IGF-1." J Orthop Res 22(1): 140-144.

Long, M. W. (2001). "Osteogenesis and bone-marrow-derived cells." Blood Cells, Molecules, and Diseases 27(3): 677-690.

Long, M. W., J. A. Robinson, E. A. Ashcraft and K. G. Mann (1995). "Regulation of human bone marrow-derived osteoprogenitor cells by osteogenic growth factors." J Clin Invest 95(2): 881-887.

Lubis, A. M. and V. K. Lubis (2012). "Adult bone marrow stem cells in cartilage therapy." Acta Med Indones 44(1): 62-68.

Marie, P. J. (2006). "Strontium ranelate: a dual mode of action rebalancing bone turnover in favour of bone formation." Curr Opin Rheumatol 18 Suppl 1: S11-15.

Marie, P. J. (2010). "Strontium ranelate in osteoporosis and beyond: identifying molecular targets in bone cell biology." Mol Interv 10(5): 305-312.

Mashiba, T., D. B. Burr, C. H. Turner, M. Sato, R. L. Cain and J. M. Hock (2001). "Effects of human parathyroid hormone (1-34), LY333334, on bone mass, remodeling, and mechanical properties of cortical bone during the first remodeling cycle in rabbits." Bone 28(5): 538-547.

Massague, J. (1987). "The TGF-beta family of growth and differentiation factors." Cell 49(4): 437-438.

Mayahara, H., T. Ito, H. Nagai, H. Miyajima, R. Tsukuda, S. Taketomi, J. Mizoguchi and K. Kato (1993). "In vivo stimulation of endosteal bone formation by basic fibroblast growth factor in rats." Growth Factors 9(1): 73-80.

McKinley, T. O., J. Borrelli, Jr., D. D. D'Lima, B. D. Furman and P. V. Giannoudis (2010). "Basic science of intra-articular fractures and posttraumatic osteoarthritis." J Orthop Trauma 24(9): 567-570.

Meena, H., H. K. Pandey, M. C. Arya and Z. Ahmed (2010). "Shilajit: A panacea for high-altitude problems." Int J Ayurveda Res 1(1): 37-40.

Meunier, P. J., C. Roux, E. Seeman, S. Ortolani, J. E. Badurski, T. D. Spector, J. Cannata, A. Balogh, E. M. Lemmel, S. Pors-Nielsen, R. Rizzoli, H. K. Genant and J. Y. Reginster (2004). "The effects of strontium ranelate on the risk of vertebral fracture in women with postmenopausal osteoporosis." N Engl J Med 350(5): 459-468.

Miki, T. and H. Masaki (2009). "[New development in bisphosphonate treatment. Bisphosphonates and gastrointestinal adverse effects]." Clin Calcium 19(1): 91-97.

Miura, S., K. Hanaoka and S. Togashi (2008). "Skeletogenesis in Xenopus tropicalis: characteristic bone development in an anuran amphibian." Bone 43(5): 901-909.

Miyamoto, S., K. Takaoka, K. Yonenobu and K. Ono (1992). "Ossification of the ligamentum flavum induced by bone morphogenetic protein. An experimental study in mice." J Bone Joint Surg Br 74(2): 279-283.

Miyazono, K., S. Maeda and T. Imamura (2005). "BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk." Cytokine and Growth Factor Reviews 16(3): 251-263.

Mohan, S. (1993). "Insulin-like growth factor binding proteins in bone cell regulation." Growth Regul 3(1): 67-70.

Morel, V. and T. M. Quinn (2004). "Short-term changes in cell and matrix damage following mechanical injury of articular cartilage explants and modelling of microphysical mediators." Biorheology 41(3-4): 509-519.

Mulconrey, D. S., K. H. Bridwell, J. Flynn, G. A. Cronen and P. S. Rose (2008). "Bone morphogenetic protein (RhBMP-2) as a substitute for iliac crest bone graft in multilevel adult spinal deformity surgery: minimum two-year evaluation of fusion." Spine (Phila Pa 1976) 33(20): 2153-2159.

Niyibizi, C. and D. R. Eyre (1994). "Structural characteristics of cross-linking sites in type V collagen of bone. Chain specificities and heterotypic links to type I collagen." European Journal of Biochemistry 224(3): 943-950.

Noda, M. and R. Vogel (1989). "Fibroblast growth factor enhances type beta 1 transforming growth factor gene expression in osteoblast-like cells." J Cell Biol 109(5): 2529-2535.

Orsini, L. S., M. D. Rousculp, S. R. Long and S. Wang (2005). "Health care utilization and expenditures in the United States: a study of osteoporosis-related fractures." Osteoporos Int 16(4): 359-371.

Orwoll, E. S. (2003). "Toward an expanded understanding of the role of the periosteum in skeletal health." J Bone Miner Res 18(6): 949-954.

Ovadia, D. N. and R. K. Beals (1986). "Fractures of the tibial plafond." J Bone Joint Surg Am 68(4): 543-551.

Papachroni, K. K., D. N. Karatzas, K. A. Papavassiliou, E. K. Basdra and A. G. Papavassiliou (2009). "Mechanotransduction in osteoblast regulation and bone disease." Trends Mol Med 15(5): 208-216.

Parfitt, A. M., M. K. Drezner, F. H. Glorieux, J. A. Kanis, H. Malluche, P. J. Meunier, S. M. Ott and R. R. Recker (1987). "Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee." J Bone Miner Res 2(6): 595-610.

Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig and D. R. Marshak (1999). "Multilineage potential of adult human mesenchymal stem cells." Science 284(5411): 143-147.

Porter, A. G. and R. U. Janicke (1999). "Emerging roles of caspase-3 in apoptosis." Cell Death and Differentiation 6(2): 99-104.

Power, J., N. Loveridge, N. Rushton, M. Parker and J. Reeve (2002). "Osteocyte density in aging subjects is enhanced in bone adjacent to remodeling haversian systems." Bone 30(6): 859-865.

Price, P. A., A. A. Otsuka, J. W. Poser, J. Kristaponis and N. Raman (1976). "Characterization of a gamma-carboxyglutamic acid-containing protein from bone." Proc Natl Acad Sci U S A 73(5): 1447-1451.

Quinn, T. M., R. G. Allen, B. J. Schalet, P. Perumbuli and E. B. Hunziker (2001). "Matrix and cell injury due to sub-impact loading of adult bovine articular cartilage explants: effects of strain rate and peak stress." J Orthop Res 19(2): 242-249.

Reginster, J. Y., E. Seeman, M. C. De Vernejoul, S. Adami, J. Compston, C. Phenekos, J. P. Devogelaer, M. D. Curiel, A. Sawicki, S. Goemaere, O. H. Sorensen, D. Felsenberg and P. J. Meunier (2005). "Strontium ranelate reduces the risk of nonvertebral fractures in postmenopausal women with osteoporosis: Treatment of Peripheral Osteoporosis (TROPOS) study." J Clin Endocrinol Metab 90(5): 2816-2822.

Roodman, G. D. (1999). "Cell biology of the osteoclast." Experimental Hematology 27(8): 1229-1241.

Saper, R. B., R. S. Phillips, A. Sehgal, N. Khouri, R. B. Davis, J. Paquin, V. Thuppil and S. N. Kales (2008). "Lead, mercury, and arsenic in US- and Indian-manufactured Ayurvedic medicines sold via the Internet." JAMA 300(8): 915-923.

Schepetkin I, K. A., Kwon BS. (2002). "Medical drugs from humus matter: focus on mumie." Drug Dev Res 57: 140-159.

Schepetkin, I. A., A. I. Khlebnikov, S. Y. Ah, S. B. Woo, C. S. Jeong, O. N. Klubachuk and B. S. Kwon (2003). "Characterization and biological activities of humic substances from mumie." Journal of Agricultural and Food Chemistry 51(18): 5245-5254.

Schepetkin, I. A., G. Xie, M. A. Jutila and M. T. Quinn (2009). "Complement-fixing activity of fulvic acid from Shilajit and other natural sources." Phytotherapy Research 23(3): 373-384.

Shakirov, A. S. (1965). "The uptake of radioactive phosphorus [32]P on the focus of bone fracture with mumie treatment." Med J Uzbekistan 10: 30-33.

Shalini (2009). "Antifungal activity screening and hplc analysis of crude extract from Tectona grandis, shilajit, Valeriana wallachi." Electronic Journal of Environmental, Agricultural and Food Chemistry 8: 218-229.

Shapiro, F. (2008). "Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts." Eur Cell Mater 15: 53-76.

Shen, B., A. Wei, S. Whittaker, L. A. Williams, H. Tao, D. D. Ma and A. D. Diwan (2010). "The role of BMP-7 in chondrogenic and osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells in vitro." Journal of Cellular Biochemistry 109(2): 406-416.

Shirota, T., M. Tashiro, K. Ohno and A. Yamaguchi (2003). "Effect of intermittent parathyroid hormone (1-34) treatment on the bone response after placement of titanium implants into the tibia of ovariectomized rats." Journal of Oral and Maxillofacial Surgery 61(4): 471-480.

Shu, B., M. Zhang, R. Xie, M. Wang, H. Jin, W. Hou, D. Tang, S. E. Harris, Y. Mishina, R. J. O'Keefe, M. J. Hilton, Y. Wang and D. Chen (2011). "BMP2, but not BMP4, is crucial for chondrocyte proliferation and maturation during endochondral bone development." Journal of Cell Science 124(Pt 20): 3428-3440.

Shvetskii AG, V. L. (1978). "Effect of the nonspecific biogenic stimulators pentoxyl and mumie on metabolic processes." Vopr Med Khim 24: 102-108.

Siegel, P. M. and J. Massague (2003). "Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer." Nat Rev Cancer 3(11): 807-821.

Silva, B. C. and J. P. Bilezikian (2010). "New Approaches to the Treatment of Osteoporosis." Annu Rev Med 62: 307-322.

Singh, A., A. A. Mehdi, R. N. Srivastava and N. S. Verma (2012). "Immunoregulation of bone remodelling." Int J Crit Illn Inj Sci 2(2): 75-81.

Smith, J. W., T. B. Martins, E. Gopez, T. Johnson, H. R. Hill and T. D. Rosenberg (2012). "Significance of C-reactive protein in osteoarthritis and total knee arthroplasty outcomes." Ther Adv Musculoskelet Dis 4(5): 315-325.

Soltanoff, C. S., S. Yang, W. Chen and Y. P. Li (2009). "Signaling networks that control the lineage commitment and differentiation of bone cells." Critical Reviews in Eukaryotic Gene Expression 19(1): 1-46.

Sporn, M. B. and A. B. Roberts (1985). "Autocrine growth factors and cancer." Nature 313(6005): 745-747.

Stocum, D. L. (2006). Regeneration Biology and Medicine. San Diego, Elsevier/Academic Press; 2006. pp. 363-404.

Tachi, K., M. Takami, H. Sato, A. Mochizuki, B. Zhao, Y. Miyamoto, H. Tsukasaki, T. Inoue, S. Shintani, T. Koike, Y. Honda, O. Suzuki, K. Baba and R. Kamijo (2011). "Enhancement of bone morphogenetic protein-2-induced ectopic bone formation by transforming growth factor-beta1." Tissue Eng Part A 17(5-6): 597-606.

Takeuchi, K. and K. Amagase (2012). "Evaluation of gastric ulcerogenic and healing impairment effects of bisphosphonates: adverse gastric reactions of bisphosphonate." Curr Protoc Toxicol Chapter 21: Unit 21 10 21-29.

Teeny, S. M. and D. A. Wiss (1993). "Open reduction and internal fixation of tibial plafond fractures. Variables contributing to poor results and complications." Clin Orthop Relat Res(292): 108-117.

Termine, J. D., H. K. Kleinman, S. W. Whitson, K. M. Conn, M. L. McGarvey and G. R. Martin (1981). "Osteonectin, a bone-specific protein linking mineral to collagen." Cell 26(1 Pt 1): 99-105.

Thomas, T., F. Gori, T. C. Spelsberg, S. Khosla, B. L. Riggs and C. A. Conover (1999). "Response of bipotential human marrow stromal cells to insulin-like growth factors: effect on binding protein production, proliferation, and commitment to osteoblasts and adipocytes." Endocrinology 140(11): 5036-5044.

Thompson, J. B., J. H. Kindt, B. Drake, H. G. Hansma, D. E. Morse and P. K. Hansma (2001). "Bone indentation recovery time correlates with bond reforming time." Nature 414(6865): 773-776.

Tkachenko SS, R. V., Grachev IR. (1979). "Reparative regeneration of the bone tissue under the effect of mumie-asyl." Ortop Traumatol Protez 11: 49-52.

Toussaint, N. D., G. J. Elder and P. G. Kerr (2009). "Bisphosphonates in chronic kidney disease; balancing potential benefits and adverse effects on bone and soft tissue." Clin J Am Soc Nephrol 4(1): 221-233.

Tsuji, K., K. Cox, L. Gamer, D. Graf, A. Economides and V. Rosen (2010). "Conditional deletion of BMP7 from the limb skeleton does not affect bone formation or fracture repair." Journal of Orthopaedic Research 28(3): 384-389.

Tsujimoto, Y. (1998). "Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria?" Genes Cells 3(11): 697-707.

Urist, M. R. (1965). "Bone: formation by autoinduction." Science 150(698): 893-899.

Urist, M. R., R. J. DeLange and G. A. Finerman (1983). "Bone cell differentiation and growth factors." Science 220(4598): 680-686.

Viguet-Carrin, S., P. Garnero and P. D. Delmas (2006). "The role of collagen in bone strength." Osteoporosis International 17(3): 319-336.

VN., I. (1965). "To a question about the dosage of mumie and special features of the healing of the fractures of the bones during its application." Med J Uzbekistan 9: 69-70.

Volpin, G., G. S. Dowd, H. Stein and G. Bentley (1990). "Degenerative arthritis after intra-articular fractures of the knee. Long-term results." J Bone Joint Surg Br 72(4): 634-638.

Walker-Bone, K., K. Javaid, N. Arden and C. Cooper (2000). "Regular review: medical management of osteoarthritis." BMJ 321(7266): 936-940.

Wang, C., Z. Wang, A. Peng, J. Hou and W. Xin (1996). "Interaction between fulvic acids of different origins and active oxygen radicals." Science in China. Series C, Life Sciences 39(3): 267-275.

Wang, X., R. A. Bank, J. M. TeKoppele and C. M. Agrawal (2001). "The role of collagen in determining bone mechanical properties." Journal of Orthopaedic Research 19(6): 1021-1026.

Weinreb, M., D. Shinar and G. A. Rodan (1990). "Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization." J Bone Miner Res 5(8): 831-842.

Wilson, E., G. V. Rajamanickam, G. P. Dubey, P. Klose, F. Musial, F. J. Saha, T. Rampp, A. Michalsen and G. J. Dobos (2011). "Review on shilajit used in traditional Indian medicine." Journal of Ethnopharmacology 136(1): 1-9.

Woo, M., R. Hakem, M. S. Soengas, G. S. Duncan, A. Shahinian, D. Kagi, A. Hakem, M. McCurrach, W. Khoo, S. A. Kaufman, G. Senaldi, T. Howard, S. W. Lowe and T. W. Mak (1998). "Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes." Genes and Development 12(6): 806-819.

Yang, H., Y. T. Zhu, R. Cheng, M. Y. Shao, Z. S. Fu, L. Cheng, F. M. Wang and T. Hu "Lipopolysaccharide-induced dental pulp cell apoptosis and the expression of Bax and Bcl-2 in vitro." Braz J Med Biol Res 43(11): 1027-1033.

Yin, H., E. J. Yang, S. J. Park and S. K. Han (2011). "Glycine- and GABA-mimetic Actions of Shilajit on the Substantia Gelatinosa Neurons of the Trigeminal Subnucleus Caudalis in Mice." Korean J Physiol Pharmacol 15(5): 285-289.

Young, B. (2006). Skeletal Tissues, in Wheater's Functional Histology: A Text and Color Atlas, San Diego.

Zambonin, G., M. Grano, G. Greco, R. O. Oreffo and J. T. Triffit (1999). "Hydroxyapatite coated with insulin-like growth factor 1 (IGF1) stimulates human osteoblast activity in vitro." Acta Orthop Scand 70(2): 217-220.

Zhou, J. and L. J. Windsor (2006). "Porphyromonas gingivalis affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases." J Periodontal Res 41(1): 47-54.

Zioupos, P. (2001). "Ageing human bone: factors affecting its biomechanical properties and the role of collagen." Journal of Biomaterials Applications 15(3): 187-229.

Zioupos, P., J. D. Currey and A. J. Hamer (1999). "The role of collagen in the declining mechanical properties of aging human cortical bone." Journal of Biomedical Materials Research 45(2): 108-116..

CURRICULUM VITAE

Nawaf Yousef Labban

EDUCATION

Ph.D. in Dental Science (2013)

Indiana University, Indianapolis, IN, U.S.A.

M.S.D./Residency in Prosthodontics (2007)

Indiana University, Indianapolis, IN, U.S.A.

B.D.S. in Dental Sciences (2001)

College of Dentistry, King Saud University, Riyadh, Saudi Arabia.

RESEARCH EXPERIENCE

Ph.D. research

August 2007 - January 2013

Indiana University School of Dentistry, Indianapolis, IN, U.S.A.

Project: Shitajit, a Novel Regulator of Bone/Cartilage Healing.

Advisor: Dr. L. Jack Windsor (Ph.D.)

Masters research

August 2004 - May 2007

Indiana University School of Dentistry, Indianapolis, IN, U.S.A.

Project: Effects of provisional acrylic resins on gingival fibroblast cytokine/growth factor expression.

Advisor: Dr. L. Jack Windsor (Ph.D.)

Bachelor reseach

July 1996 - June 2001

College of Dentistry, King Saud University, Riyadh, Saudi Arabia.

Project: A survey of dental anomalies in a group of Saudi population.

Advisor: Dr. Maysara Al-Shawwaf.

HONORS/AWARDS:

- 2012: George K. Stookey Delta Dental Award for Innovation in Oral Care Research Award: Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.
- 2009: Indiana Dental Association Best Clinical Case Report Award for the Master Students: Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 24th, 2009.
- 2008: A member in the Golden Key International Honour Society
- 2007: 1st place: John F. Johnston Society Scholarship Award
- 2005:1st place: I. Lester Furnas Graduate Prosthodontics Award

ACADEMIC MEMBERSHIPS

- Indiana Section of the American Association for Dental Research (INAADR)
- Advance Graduate Organization
- American College of Prosthodontists (ACP)
- John F. Johnston Society (JFJ)
- Saudi Dental Society

SELECTED PEER REVIEWED PUBLICATIONS

1. A simple technique to reduce the risk of irreversible gingival recession after the final impression. Labban N, J Prosthodont 2011;20:649-51.

2. Effects of cigarette smoke condensate on oral squamous cell carcinoma cells. Allam E, Zhang W, Labban N, Al-Shibani N et al. Arch Oral Biol 2011;56:1154-61.

3. Responses of human neutrophils to nicotine and/or porphyromonas gingivalis. Al-Shibani N, Labban N, Kowolik M et al. J Periodontol 2011;82:1504-1508.

4. Effects of provisional acrylic resins on gingival fibroblast cytokine/growth factor expression. Labban N, Song F, Al-Shibani N et al. J Prosthet Dent 2008;100:390-7.

CHAPTERS

1. Al-Shibani N, Labban N, Allam E, and Windsor LJ. Tobacco: A risk factor for periodontal disease. Periodontal Disease: Symptoms, Treatment and Prevention. Sho L Yamamoto. Nova Science Publishers, Inc 2011. Pp 121-135.

CONFERENCES AND ABSTRACTS

1. *BENS*, bone and connective tissue regeneration agent for osteoporosis. Labban N, Aref M, Brown D et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

2. Resolvin-Dl blocks the effects of *Porphyromona*s *gingivalis* on human gingival fibroblasts. Khaled M, Al-Shibani N, Labban N et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

3. Metronidazole-containing devices: effect on the oxidative burst of human neutrophils. Austin Starr, Al-Shibani N, Labban N et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

4. Oxidative burst of human neutrophils induced by doxycycline-containing devices. Hani Ahdab, Al-Shibani N, Labban N et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

5. Mechano-biological characterization of electrospun composite scaffold for regenerative endodontics. Yassen G, Labban N, Platt J et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

6. Assessing the bone anabolic properties of a traditional medicinal compound. Aref M, Labban N, Windsor J et al. IUPUI Life-Health Sciences Internship Program Spring 2012 Poster Session and Fifth Year Celebration.

7. *BENS*, a novel regulator of bone cell survival and wound healing. Labban N, Song F, Cameron J et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 11th, 2011.

8. Rehabilitation of a fully edentulous patient using implants and CAD/CAM technology. Labban N and Andres C. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 24th, 2009.

9. Effects of provisional acrylic resins on gingival fibroblast cytokine/growth factor expression. Labban N, Song F, Al-Shibani N et al. Hinman Research Symposium. The University of Tennessee, Memphis TN. 2008.

10. Effects of provisional acrylic resins on gingival fibroblast cytokine/growth factor expression. Labban N, Song F, Al-Shibani N et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 9th, 2008.

11. Expression of matrix metalloproteinases (MMPs) during axolotl limb regeneration. Santosh N, Al-Shibani N, Labban N et al. Indiana Section American

Association of Dental Research / Indiana University School of Dentistry Research Day April 9th, 2008.