Effect of Preservation Solution and Period on the Mechanical and Histological Properties of Liver

by

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This is to certify that I have examined this copy of a doctoral dissertation by

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ABSTRACT

In liver transplantation, most of the time, the donor and recipient are in different locations and longer preservation periods are inevitable. Hence, the choice of the preservation solution and the duration of the safe preservation period are critical for the success of the transplant surgery. In this thesis, we examine the mechanical and histological properties of bovine liver stored in Lactated Ringers, HTK and UW solutions as a function of preservation period to investigate the efficiencies and safe preservation periods of these solutions. The mechanical experiments are conducted with a shear rheometer on cylindrical tissue samples extracted from three bovine livers and the change in viscoelastic material properties of the bovine liver is characterized using the fractional derivative KelvinVoigt Model. Also, the histological examinations are performed on the same liver samples under a light microscope. The results show that the preservation solution and period have a significant effect on the mechanical and histological properties of the liver tissue. The storage and loss moduli, the number of the apoptotic cells, the collagen accumulation, and the sinusoidal dilatation increase, and the glycogen deposition decreases as the preservation period is increased. Based on the statistical analyses, we observe that the liver tissue is preserved well in all solutions for up to 11 h. After then, UW solution provides a better preservation up to 29 h. However, for preservation periods longer than 29 h, HTK is a more effective preservation solution if the least amount of change in mechanical properties is considered as the criteria. On the other hand, the highest correlation between the mechanical and histological properties is observed for the liver samples preserved in UW solution.

ÖZETÇE

Karaciğer nakli sırasında, bağışta bulunan donör ile alıcı çoğu zaman farklı yerlerdedir ve nakledilen karaciğerin kimyasal bir solüsyon içinde operasyona kadar korunması gerekmektedir. Bu sebepten, nakledilen karaciğerin hangi koruma solüsyonunda, ne kadar süre ile güvenli bir şekilde saklanabileceği operasyonunun başarısı açısından çok kritiktir. Bu çalışmada, Laktatlı Ringer (kontrol), HTK ve UW koruma solüsyonlarında (karaciğer nakillerinde en çok kullanılan) saklanmış dana karaciğeri dokusunun mekanik ve histolojik materyal özelliklerindeki değişimi saklama zamanına bağlı olarak araştırdık. Böylece kullanılan koruma solüsyonlarının verimini ve karaciğerlerin bu solüsyonlarda ne kadar süre güvenli bir şekilde saklanabileceklerini inceledik. Mekanik deneyleri, taze kesilmiş üç farklı dana karaciğerinin sol lobundan elde edilen ince silindirik doku örnekleri üzerinde reometre kullanarak yaptık ve karaciğer dokusunun viskoelastik materyal özelliklerindeki değişimi Kelvin-Voigt kesirli türev modelini kullanarak karakterize ettik. Aynı karaciğerlerin histolojik özelliklerini, bir ışık mikroskobu kullanılarak, saklama zamanına ve solüsyona bağlı olarak inceledik. Sonuçlar, koruma solüsyonunun ve saklama zamanının dana karaciğerinin mekanik ve histolojik materyal özellikleri üzerinde anlamlı bir etkisinin olduğunu gösterdi. Dokunun koruma ve kayıp modüllerinin, apoptotik hücre miktarının, kollejen doku miktarının ve sinüslerdeki genişleme miktarının saklama süresi ile arttığını, bunun yanında glikojen depolarının saklama süresi ile azaldığını gözlemledik. Yaptığımız istatistiksel analizler sonucunda, karaciğer dokusunun üç koruma solüsyonunda da 11 saate kadar güvenli olarak korunabildiğini, 29'uncu saklama saatine kadar ise UW solüsyonunun en etkin koruma solüsyonu olduğunu tespit ettik. Fakat 29 saatten daha

uzun saklama süreleri için mekanik özelliklerindeki değişiminin az olmasından dolayı HTK solüsyonunun karaciğeri daha etkin bir şekilde koruduğunu gördük. Bununla birlikte, mekanik ve histolojik materyal özellikler arasındaki en yüksek korelasyonun UW solüsyonunda saklanan karaciğer dokularında olduğunu gördük.

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NOMENCLATURE

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α	Order of the Fractional Derivative.
E	Elastic Modulus.
E_L	Loss Modulus.
E_S	Storage Modulus.
G	Shear Modulus.
G_L	Loss Shear Modulus.
G_S	Storage Shear Modulus.
G^*	Complex Shear Modulus.
G_T	Tangent Shear Modulus.
γ^*	Sinusoidal Shear Strain.
γ_A	Amplitude of the Shear Strain.
γ_{LVR}	Linear Viscoelastic Range of the Shear Strain.
H	Height of the Sample.
ν	Poisson's Ratio.
ω	Angular Frequency.
p	Probability.
R	Radius of the Parallel Plate.
R^2	Coefficient of Determination.
r_S	Strentgh of Correlation.
τ^*	Complex Shear Stress.

τ_A	Amplitude of the Complex Shear Stress.
θ	Deflection Angle.
T	Torque.
V	Unique Quasi-property.

Acronyms

ANOVA	Analysis of Variance.
DSL	Dynamic Shear Loading.
EC	Euro Collins.
ECM	Extracellular Matrix.
H&E	Hematoxylene & Eosin.
HFUIB	High Frequency Ultrasound Integrated Backscatter.
HTK	Histidine-Tryptophane-Ketoglutarate.
KVFDM	Kelvin-Voigt Fractional Derivative Model.
LDLT	Living Donor Liver Transplantation.
M	Moderate.
PAS	Periodic-Acid Schiff.
QSSL	Quasi-Static Shear Loading.
S	Strong.
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling.
UW	University of Wisconsin.
VS	Very Strong.
VW	Very Weak.
W	Weak.

Chapter 1

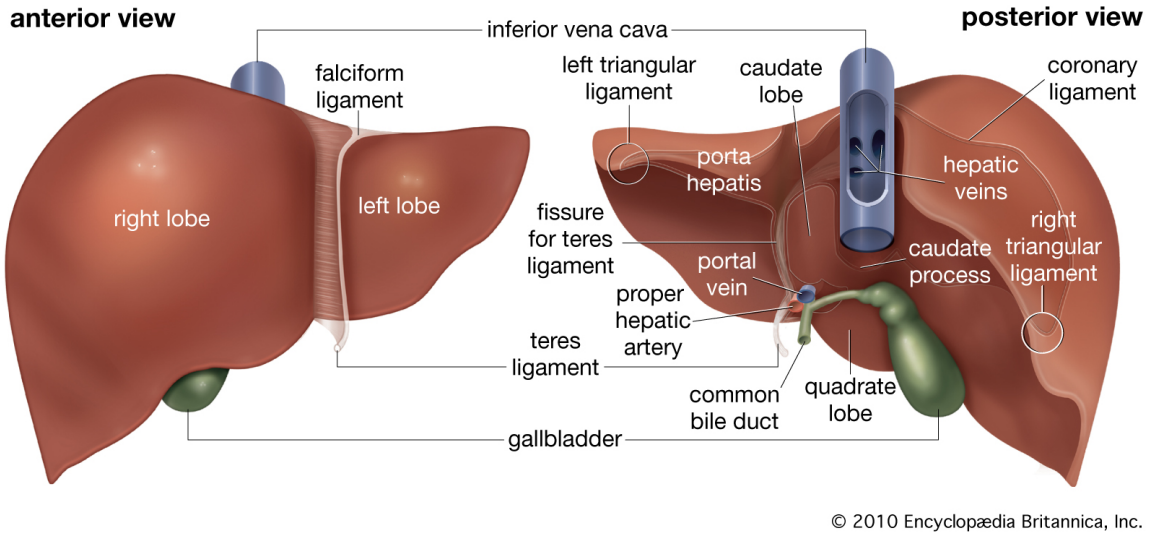
INTRODUCTION

1.1 Liver Anatomy

The liver is a soft and reddish brown organ located in the right upper quadrant of the abdominal cavity, under the diaphragm, right to the stomach, and over the gallbladder. It is protected by the costal cartilage of the ribs. The shape of the liver tends to be triangular which is determined by the surrounding structures. The liver is the largest internal organ and gland in human body. The size and weight of the liver show variations depending on age, sex, body size and shape: 10.5 cm and 1.4-1.5 kg for adult men; and 7 cm and 1.2-1.4 kg for adult women, respectively [71]. The external surface of the liver is enclosed by a tough fibrous membrane, Glisson's capsule, which protects the liver and also reduces friction against other organs.

The liver anatomy is described using two different aspects: morphological anatomy (based on external appearance) and functional anatomy (based on the vascular inflow, outflow, and biliary drainage). In morphological anatomy, the liver is divided into two lobes: left and right when it is observed from the anterior view, and divided into four lobes: left, right, caudate and quadrate if it is observed from the posterior view as shown in Figure 1.1 [14]. In functional anatomy, the liver is divided into eight functionally independent segments [10]. Each segment is self-contained and can be

surgically resected without damaging the rest of the liver.



(a) (b)

Figure 1.1: Liver: (a) anterior and (b) posterior views [14].

The microscopic structure of the liver is shown in Figure 1.2 [8]. The liver is connected to two blood vessels: the hepatic artery and portal vein. The hepatic artery delivers oxygen-rich blood along with cholesterol and other substances required for processing food. In contrast, the portal vein carries venous blood from the entire intestinal region and supplies this nutrient-rich blood to the liver for processing and metabolizing. These blood vessels divide into capillaries (network of tiny channels) and lead to lobules (small divisions of the liver defined at the histological scale). Subsequently, blood flow goes towards sinusoids and, hence hepatocytes absorb proteins and other plasma components through perisinusoidal space.

The liver is responsible for many vital functions related to digestion, metabolism,

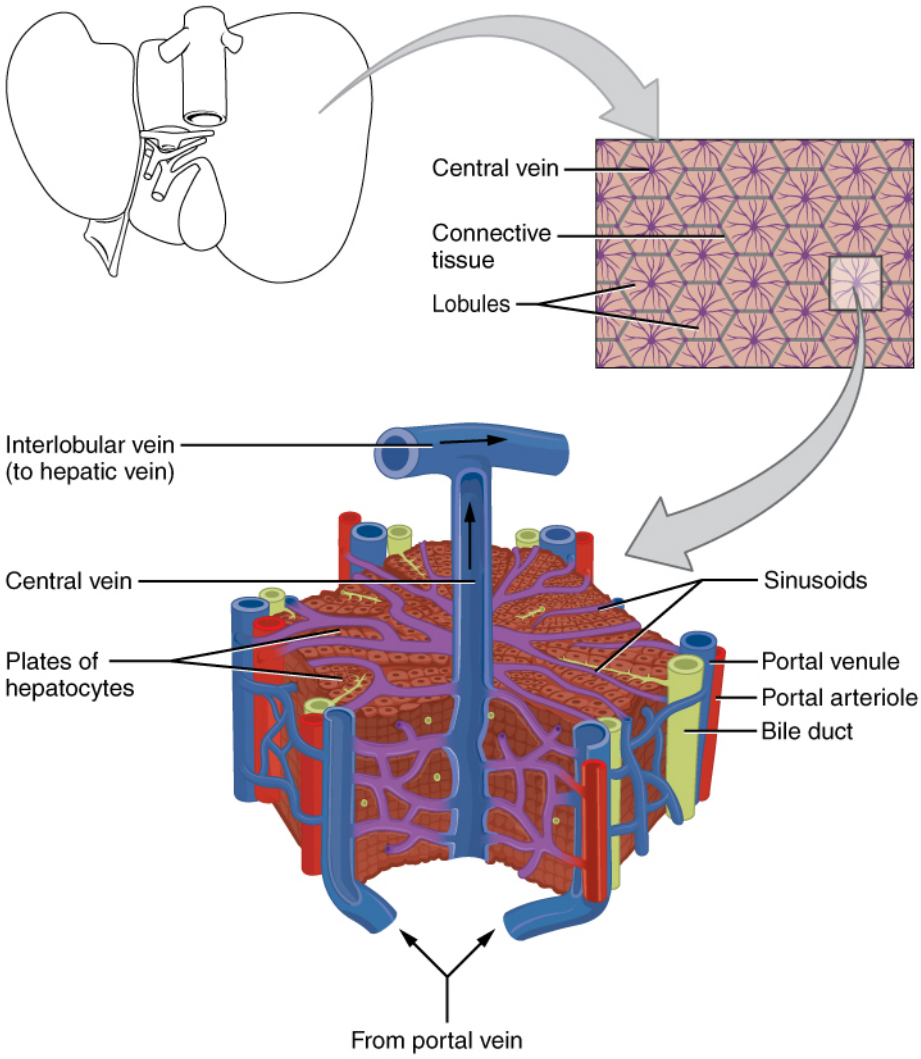


Figure 1.2: Liver: microscopic structure [8].

immunity, and the storage of nutrients within the body. Currently, it is not possible to emulate all the functions of the liver by an artificial organ or device. Hence, a healthy liver is necessary for survival.

1.2 Liver Transplantation

The liver is prone to many diseases such as hepatitis infection, alcohol damage, fatty liver, cirrhosis and cancer due to its numerous functions and critical location. Currently, it is not possible to treat a severe liver failure, and yet the function of liver cannot be compensated by any other ways. Fortunately, the liver is the only organ capable of natural regeneration in the body. As little as 25% of liver can regenerate into a whole liver. Hence, a severely diseased liver must be removed from the patient and replaced with a whole or partial healthy liver. This surgical procedure is called liver transplantation.

First liver transplantation surgery was performed by Thomas Starzl in 1963 [59]. The liver transplantation remained experimental with a one-year patient survival rate of 25% until 1970s. The introduction of cyclosporin, an immunosuppressant drug widely used in organ transplantation to prevent rejection, made the liver transplantation to a patient from a non-living donor, a standard clinical treatment in 1980s. However, the number of the potential recipients have quickly far more outnumbered the livers supplied from non-living donors (usually come from donors who have died from fatal brain injury). This fact promoted the development of the Living Donor Liver Transplantation (LDLT). First successful LDLT was performed by Dr. Christoph Broelsch in 1989, when two-year-old baby received 20% of her mother's liver (Couinaud segments 2 and 3). In mid-1990s, countries with limited number of donor transplants performed adult-to-adult LDLT using the donor's right hepatic lobe. Shah et al. [55] reported the overall complication rate for the donor as 37%, and the survival rate of the recipient as 86-88%.

In transplantation, the liver supplied from a living or a deceased donor, must be preserved and transported to a suitable recipient immediately (Figure 1.3 [34]). However, most of the time, the donor and the recipient are in different locations. Throughout the removal, preservation and transplantation, the stability of the inte-

rior environment of the liver is altered primarily due to the drop in its temperature (hypothermia) and insufficient supply of blood to its vessels (ischemia). As a result, the liver tissue inevitably experiences intracellular and extracellular changes such as cellular edema, cell death (apoptosis), depletion in cellular energy storage, hepatic sinusoidal dilatation, increase in connective tissue, acidosis, oxidation, and changes in ionic composition [2, 17, 20, 58]. The longer the post-mortem time, the more damage develops in the liver. Various preservation methods and solutions are used to maintain the cell integrity, the integrity of the Extracellular Matrix (ECM), and pH balance of the liver tissue from the time of harvesting to transplantation.

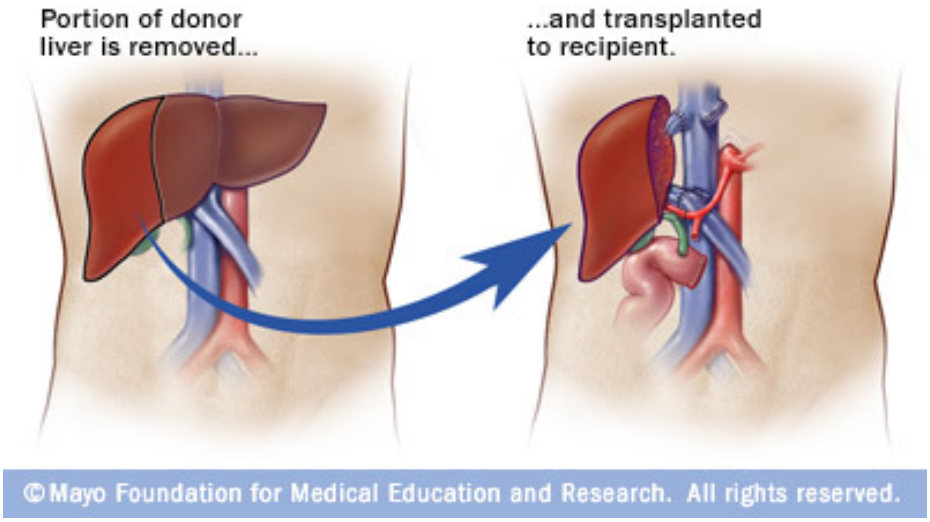


Figure 1.3: Liver transplantation: the liver of the donor is removed partially/wholly, and transplanted to recipient [34].

1.2.1 Preservation Methods

Currently, there are two methods for the liver preservation: cold static preservation and warm machine perfusion. Cold static preservation is the most widely used method for liver preservation [20]. This method involves flushing the liver with a preservation

solution and storing it in a sterile bag immersed in the same solution. This sterile bag is packed in several layers of sterile containers and kept in ice at 0-4 °C until the transplantation. Another method for the liver preservation is the warm machine perfusion. In this approach, the donor's liver is stored in an artificial environment mimicking the human body. The liver is continuously perfused with blood containing oxygenation and nutrition at physiological pressures and at 37 °C. The liver is functional during the preservation and, hence the metabolic performance of the liver can be continuously evaluated. However, preserving the donor's liver with warm machine perfusion is complex, it requires expensive equipment, and cause difficulties during the transportation.

The clinical and cost effectiveness of the preservation methods are controversial in the literature. Bessems et al. [3] reported that machine perfusion is better in the preservation of the steatotic rat liver, as compared to cold preservation. On the other hand, Watson et al. [69] concluded that machine perfusion has no advantage over cold preservation which is cheaper and straightforward. Despite the discussions in the literature, cold preservation is the most popular and universally available method for liver preservation. Hence, the choice of the preservation solution is the one of the main factors affecting the success in cold static preservation.

1.2.2 Preservation Solutions

The first commonly used preservation solution for organ transplantation was Euro Collins (EC) solution, which was introduced in the 1970s [57]. The safe preservation period for storing a liver in this solution was four to eight hours. In the same decade, Marshall Solution was developed for liver and kidney preservation, which offered up to eight hours of safe liver preservation. In late 1980s, University of Wisconsin (UW) solution was introduced for cold storage of the pancreas, liver, and kidney, and quickly become the gold standard for organ preservation in all over the world [56]. Despite the large success of the UW solution, other preservation solutions such as

Histidine-Tryptophane-Ketoglutarate (HTK) and Celsior were also introduced to the market. Over the last two decades, HTK solution has become increasingly widespread especially in developing countries due to its lower cost and comparable outcomes with the UW solution. Today, UW and HTK stand as the most commonly used preservation solutions in the world for the liver transplantation. Although, these solutions vary in chemical composition (Table 1.1 [33]), they all aim to delay the inevitable tissue damage. UW solution mimics an intracellular environment with high potassium and low sodium content. It is effective in protecting the integrity of the cell structure. In contrast, HTK is an extracellular type preservation solution with high sodium and low potassium concentrations and it is effective in preserving the integrity of the ECM structure. In addition, HTK solution has a lower viscosity (1.8 vs. 86.2 mPa·s), osmolarity (310 vs. 320 mOsm/L), and pH level (7.2 vs 7.4 pH) compared to UW solution [17, 16, 66, 21].

1.3 Motivations

The success of the liver transplantation primarily depends on the initial conditions of the transplant organ, type of the preservation solution and length of the preservation period between the procurement and the transplantation [37, 15, 64, 61, 11, 65]. However, there is still a dispute among the physicians on the choice of the preservation solution as well as on the extent of the preservation period.

During the cold ischemic preservation, the histological properties of the liver tissue in micro scale change with increasing post-mortem times and these changes lead alterations in gross mechanical properties at macro scale. As a result, it is possible to exploit these relations to quantitatively evaluate and monitor the state of the liver before the transplantation. However, the links between the mechanical and pathological properties of the liver tissue are still not fully understood.

Table 1.1: The chemical composition of UW and HTK solutions [33].

Component [mmol/L]	UW	HTK
Sodium	40	15
Potassium	120	10
Magnesium	5	4
Calcium		0.015
Ketoglutarate		1
Histidine		198
Mannitol		30
Tryptophan		2
Lactobionate	100	
Phosphate	25	
Sulfate	5	
Raffinose	30	
Adenosine	5	
Glutathione	3	
Allopurinol	1	

1.4 Objectives

The main goals of this dissertation are to (1) examine the effect of preservation solution and post-mortem time on the mechanical and histological properties of liver, and (2) investigate the correlations between these properties to better understand and assess the preservation injury. To achieve these goals, the following aspects have been addressed:

- examination of the mechanical properties of bovine liver samples preserved in Lactate Ringer, UW and HTK preservation solutions as a function of post-mortem time.

- examination of the the histological properties of bovine liver samples (taken from the same livers) preserved in Lactate Ringer, UW and HTK solutions as a function of post-mortem time.

- examination of the correlations between the mechanical and histological properties of bovine liver tissue by Spearmans Rank-Order correlation method.

- assessment of the effectiveness and upper limit of safe preservation period of solutions.

- estimation of the optimum viscoelastic material coefficients of bovine liver tissue as a function of preservation solution and post-mortem time.

1.5 Contributions

In liver transplantation surgery, most of the time, the donor and recipient are in different locations and longer preservation periods are inevitable. Therefore, the determination of the efficiency and safe preservation periods of the preservation solutions commonly used in liver transplantation are important. In this dissertation, we investigate the changes in mechanical (dynamic and quasi-static) and histological (apoptotic cell death, collagen accumulation, sinusoidal dilatation, and glycogen level) properties of the liver tissue simultaneously as a function of preservation period to provide better insight into the effectiveness of HTK and UW solutions during cold ischemic preservation. Also, the relation between the changes in histological and gross mechanical properties during cold preservation is investigated and upper limit of safe preservation period is suggested for each preservation solution. Understanding this relation

is the first step towards to the development of new protocols for the evaluation of the tissue injury during preservation and hence the suitability of the donor liver for transplantation.

1.6 Outline of the Dissertation

The outline of the dissertation has divided in eight chapters:

Chapter 1 has introduced the basic concepts of liver anatomy, transplantation, and preservation. Also, the motivations, main objectives, and contributions of the research work have been presented in this chapter.

Chapter 2 presents a literature review for the preservation solutions, and mechanical and histological properties of liver.

Chapter 3 presents our soft tissue cutting and sampling apparatus for preparing thin cylindrical soft tissue samples. Also, the methodology for sample preparation is explained in this chapter.

In Chapter 4, the mechanical properties of bovine liver samples preserved in Lactate Ringer (control), UW and HTK (most popularly used) solutions for 5 h, 11 h, 17 h, 29 h, 41 h, and 53 h are examined with a shear rheometer. Two types of experiments are conducted: dynamic shear loading (DSL) and quasi-static shear loading (QSSL) experiments. First, the methodology and theory behind these experiments are explained, and then the results of the experiments are given. The effects of preservation solution and period on the mechanical properties are analyzed by two-way ANOVA test. The statistical differences between the individual groups are further analyzed by Bonferroni corrected paired t-tests. Finally, the results are discussed and compared with the findings reported in the literature.

In Chapter 5, histological properties of tissue samples taken from the same bovine livers are examined under a light microscope. The methods for tissue preparation, fixation, sectioning, staining and imaging are explained. The exemplar images of the stained tissue sections are demonstrated. The changes in the apoptotic cell count,

collagen accumulation, sinusoidal dilatation, and glycogen level in the liver cells are quantitatively presented. The effects of preservation solution and period on the histological properties are investigated by two-way ANOVA first, and then the statistical differences between the individual groups are further analyzed by Bonferroni corrected paired t-tests. Finally, the results are discussed and compared with the findings reported in the literature.

Chapter 6 investigates the correlations between the mechanical and histological measurements by Spearmans Rank-Order correlation method. First, the methods of the analyses are explained, and then the results are reported. Then, the findings are discussed and compared with the results reported in the literature.

In Chapter 7, the viscoelastic material properties of liver tissue are characterized for each preservation period and solution. A fractional derivative viscoelastic model is fit to the experimental data and the corresponding model parameters are reported. Eventually, the findings are discussed and compared with the results reported in the literature.

General conclusions, future prospects and academic publications derived from this dissertation are presentation in Chapter 8.

Chapter 2

LITERATURE REVIEW

2.1 Preservation Solutions

In spite of the extended information about the composition and rheological properties of UW and HTK solutions, it is still controversial which solution is better and how long an organ can be preserved safely in these solutions. Some investigators have reported that there are no major differences between the efficiencies of UW and HTK solutions in preservation. A study performed by Feng et al. [17] suggested that when the preservation period is short, there are no significant differences among UW, HTK, and Celsior solutions based on the incidence of biliary complications. Moench et al. [37] showed that there are no major differences between the UW and HTK solutions during in-situ and ex-situ perfusion of liver grafts at different pressure steps on pig models. Erhard et al. [15] examined the data of 60 patients who went through a liver transplantation. They found no significant differences between the HTK and UW groups in terms of survival rate and the number of complications even when the cold ischemia period was more than 15 h. Testa et al. [64] evaluated the efficiencies of the UW and HTK solutions on adult-to-adult living donor liver transplantations. They perfused 30 grafts with either UW or HTK solutions. The authors reported that liver biochemistries, complications, and graft-patient survival rates of the two groups did not show any significant differences 13$\pm$7 months after the transplantation. However,

they stated that using HTK solution was advantageous due to its lower cost. Also, it did not need to be flushed away before reperfusion of the graft.

Compared to the above results, some other studies in the literature reported that UW is a better solution with a longer preservation period. Straatsburg et al. [61] conducted a study to compare the effects of UW, HTK and Celsior solutions on cell death in 69 rat livers. The authors reported that UW and Celsior solutions were equally effective in preserving the rat liver cells for 0-16 h, but they were best preserved in UW solution after 24 h. Den Toom et al. [11] investigated the effects of UW and Euro-Collins (EC) solutions on the morphology and metabolic capacity of rat livers for 18 h to 42 h of preservation. They reported that UW solution provided better morphology and metabolic capacity compared to EC solution after 18 h of preservation. They also reported that the differences between UW and EC solutions were more strengthened after 42 h of preservation. A study conducted by Todo et al. [65] analyzed 185 cadaveric liver homografts preserved for 4 h to 24 h in UW solution and 180 grafts preserved 3 h to 9.5 h in EC solution. The livers stored in UW solution had a higher survival rate and lower primary non-function rate, although the preservation duration of the liver in UW solution was about two times longer than that of the livers in EC solution. In a recent study, Stewart et al. [60] analyzed the data of 21626 patients, who underwent deceased donor kidney transplantation between the years 2004 and 2008, to determine the effects of UW and HTK solutions on the delayed graft function and death-censored graft survival (i.e., graft loss without death). The authors found that HTK preservation showed 20% increase in the death-censored graft loss compared to UW preservation. Other studies [5, 13, 50] also reported that UW is better than HTK in kidney preservation when the preservation time is more than 24 h.

2.2 Mechanical Properties of Liver

Previously, the dynamic [5, 12, 19, 28, 29, 31, 43, 45, 49, 62, 67, 70] and quasi-static [7, 19, 30, 40, 46, 51, 54, 73] mechanical properties of the animal and human livers have examined extensively. Recently, the histological and mechanical analyses of liver are performed together. Mazza et al. [35], measured the mechanical properties of human liver with an aspiration device and analyzed the biopsies of the liver tissue in terms of pathology and connective tissue. They found a correlation between the connective tissue content and stiffness index. Kerdok et al. [27] investigated the effects of perfusion on the viscoelastic and histological characteristics of liver tissue. They reported some minimal changes in cellular integrity beyond 2 h of perfused and unperfused cases. Also they stated that unperfused liver tissue is stiffer and more viscous than the in vivo liver tissue. Also, few studies investigating the effect of the post-mortem time on mechanical properties of liver tissue have been published in the literature. Ocal et al. [45] measured the dynamic viscoelastic material properties of the bovine liver tissue in the frequency range of 0-100 Hz at the room temperature using an impact hammer and time-dependent relaxation modulus of the bovine liver tissue (compressed to 20% normal strain in 0.1 s for 500 s) via a custom-made mechanical testing device between the post-mortem times of 1-48 h. Authors concluded that the stiffness and viscosity of the liver tissue increases with increasing post-mortem times. Garo et al. [19] examined the post-mortem time on the mechanical response of porcine brain tissue using a rheometer. For each sample, authors performed dynamic frequency sweep (at the frequency range of 1-10 Hz and shear strain of 1%) and shear loading-unloading (up to 5% shear strain) experiments at the post-mortem time range of 2.5-10 h. They reported a significant relation between the modulus and post-mortem time after 6 h. In a recent study, Wex et al. [70] analyzed the linear viscoelastic properties of the porcine liver at the post-mortem times in the range of 1-26 h using a rheometer. They reported significant changes in linear mechanical properties of liver with increasing

post-mortem times.

2.3 Histological Properties of Liver

Although, the histological properties of animal and human livers stored in different the preservation solutions during the cold ischemic preservation have been investigated in the past, these finding are not quantitatively related with the material properties of the liver. Natori et al. [39] examined rat livers stored in the UW solution for 24 h at 4 °C to quantify the apoptosis using the using the TUNEL assay. Abrahamse et al. [1] analyzed the cell injury on porcine livers preserved in UW, HTK and Celsior solutions by the induction of histone-associated DNA fragments and cellular caspase-3 activity. Budzinski et al. [6] evaluated the level of apoptosis in porcine livers preserved for 12 h in UW and HTK solutions. Puhl et al. [48] investigated the efficiencies of the single use of UW and HTK solutions and the use of the combination of HTK and UW solutions in the rat liver transplantation. Jain et al. [25] conducted a microscopy study on human livers preserved in UW solution to understand the flow dynamics of sinusoidal perfusion during 24 h hypothermic machine perfusion. Corps et al. [9] perfused rat livers with HTK and UW solutions, and analyzed the adenine triphosphate and metabolites using the high-performance liquid chromatography and histologic analysis. Nowak et al. [44] assessed the glucose concentrations on pig livers cold preservation with UW solution.

2.4 Correlation between the Mechanical and Histological Properties of Liver

Previously, Mazza et al. [35] used aspiration, Sandrin et al. and Yeh et al. [52, 73] used ultrasound elastography (UE), Manduca et al., Huwart et al., and Mori et al. [24, 32, 38] used magnetic resonance elastography (MRE) and Ozcan et al. [45] used an impact hammer to detect diseases and tumors in the liver tissue using the rela-

tions between the increase in the static and dynamic moduli and the fibrosis level of the liver tissue. However, other possible correlations between the material and histological properties are needed to be investigated to understand and evaluate the preservation injury.

Currently, the only way to examine the organ suitability before the transplantation surgery is based on organ appearance and donors medical history. For longer preservation periods, the risk of non-functioning or dysfunction of the transplant organ is fairly high. Furukava et al. [18] reported that after 20 h of cold ischemic preservation, the early graft failure rate increased nonlinearly. Therefore, the non-invasive evaluation of the liver suitability is highly desirable to increase the patient and graft survival rates. But, the studies investigating the organ suitability for transplantation are very limited. Vlad et al. [68] used High Frequency Ultrasound Integrated Backscatter (HFUIB) to assess the cold ischemic liver preservation injury and provided some preliminary data to develop a non-invasive assessment technique. Authors used the correlation between the changes in HFUIB measurements, and the changes in size and acoustical properties of cells induced by osmotic stress following ATP depletion to evaluate state of the liver before the transplantation surgery.

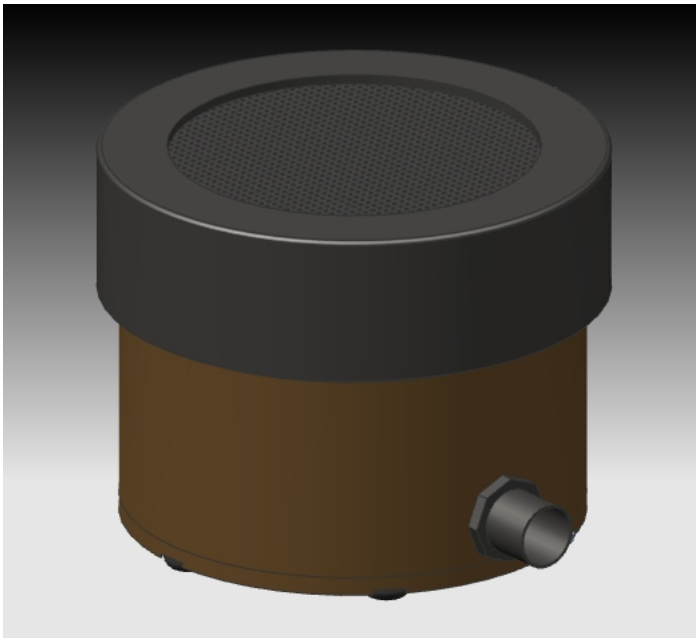
Chapter 3

SOFT TISSUE CUTTING AND SAMPLING APPARATUS

3.1 Design

Thin cylindrical tissue samples having a flat surface are required for rheological shear experiments. However, preparing such samples is challenging due to the viscoelastic nature of the liver tissue. In order to obtain tissue samples from a bovine liver in desired thickness and diameter, a tissue slicing and sampling apparatus has been developed[1] (Figure 3.1a). The commercial devices (e.g., microtome) used in histology and pathology laboratories of hospitals are designed to obtain very small and thin samples. Moreover, before obtaining these samples, soft tissue is fixated to reduce morphological distortions and damage, which makes it easier to obtain very small and thin samples. However, the tissue samples used in our experiments have to be fresh and cylindrical in shape, with a diameter of 20-50 mm and thickness of 2-3 mm as suggested in [36] for rheological experiments. Furthermore, since we investigate the effect of three different preservation solutions and six different preservation periods on the material properties of the liver, we need to obtain several samples from each liver. Obtaining several cylindrical samples from liver tissue in desired diameter and thickness is a challenging task since soft tissue does not keep its form under any force,

[1]The tissue slicing and sampling apparatus was inspired by a similar apparatus developed by Juergen Braun at Charité-Universitätsmedizin Berlin, Germany.



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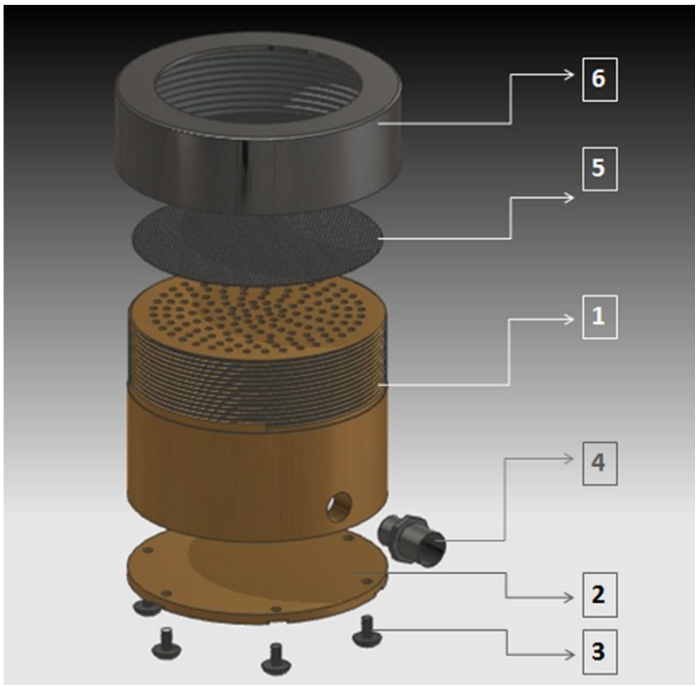


(b)

Figure 3.1: The tissue slicing and cutting apparatus: (a) solid model and, (b) assembly order.

including the one due to gravity. However, it is highly important to apply force to immobilize the tissue during cutting to obtain a slice with a uniform thickness. For this purpose, we use a vacuum pump and immobilize the soft tissue by suction. The assembly order of our design is shown in Figure 3.1b and its part list is given in Table 3.1.

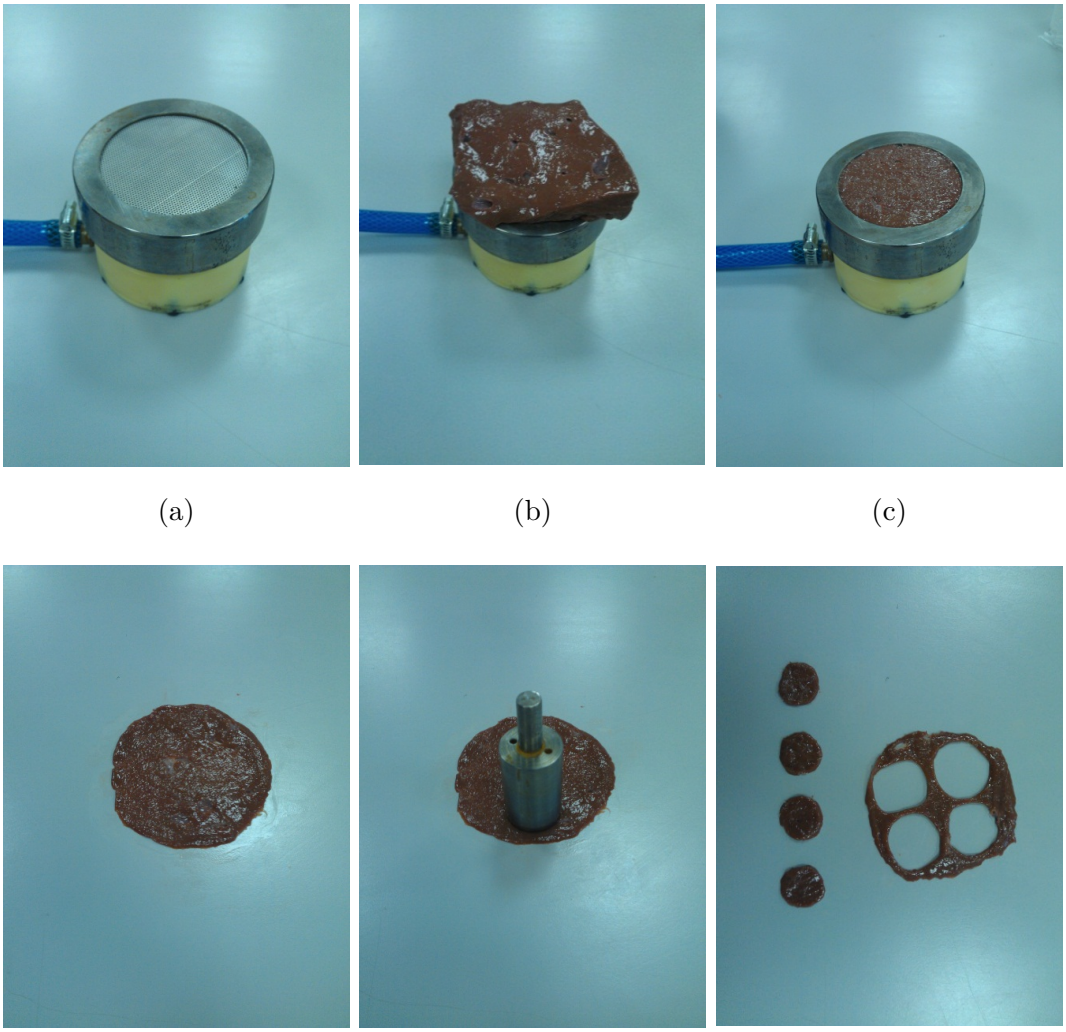
Table 3.1: The part list of our tissue slicing and cutting apparatus

Part No	Part Name	Number of Units
1	Vacuum Chamber	1
2	Lower Plate	1
3	M4 × 8 Screw	6
4	Connecting Adapter	1
5	Perforated Thin Plate	1
6	Cylindrical Nut	1

3.2 Sample Preparation

In this dissertation, three fresh bovine livers are obtained from a slaughterhouse in three consecutive weeks and cold preserved in Lactated Ringers solutions at 4 °C immediately after the harvesting. The livers are transferred from the slaughterhouse to our laboratory in 4 h. In order to prepare soft tissue samples, the following steps are performed; first, the gap between the top surface of the cylindrical nut and the perforated plate of the tissue slicing and cutting apparatus is adjusted to 2 mm (Figure 3.2a). Then, a chunk of liver excluding the Glissons capsule and visible blood vessels are cut and placed on the top surface of the apparatus (Figure 3.2b). The vacuum pump is turned on, the liver tissue is sucked through the holes of the perforated plate into the cylindrical nut, and immobilized. Using a sharp slicing blade, small

oscillatory movements are applied to the immobilized tissue to remove the excessive layer at the top. Hence, a tissue slice with a thickness of 2 mm and a diameter of 80 mm is obtained from the liver tissue as shown in Figure 3.2c. The vacuum pump is turned off and the cylindrical nut is unthreaded. Then, the tissue slice is taken out of the apparatus and put on a flat surface (Figure 3.2d). Finally, a cylindrical blade (Figure 3.2e) is used to obtain 4 cylindrical samples having a diameter of 25 mm and a thickness of 2 ± 0.5 mm from each slice (Figure 3.2f).



(a) (b) (c)

(d) (e) (f)

Figure 3.2: The steps of the tissue slicing and sampling: (a) the gap between the cylindrical nut and the perforated plate is adjusted to 2 mm, (b) the liver is immobilized on the top surface of the apparatus by suction, (c) the excess liver is removed using the sharp slicing blade and the tissue slice with a diameter of 80 mm is left in the apparatus after the liver was cut, (d) the vacuum pump is turned off, the cylindrical nut is unthreaded, the slice is taken out of the apparatus and placed on a flat surface, (e) a sharp cylindrical blade is used to obtain samples from the slice, and then (f) four samples, each with a diameter of 25 mm, were obtained from each slice.

Chapter 4

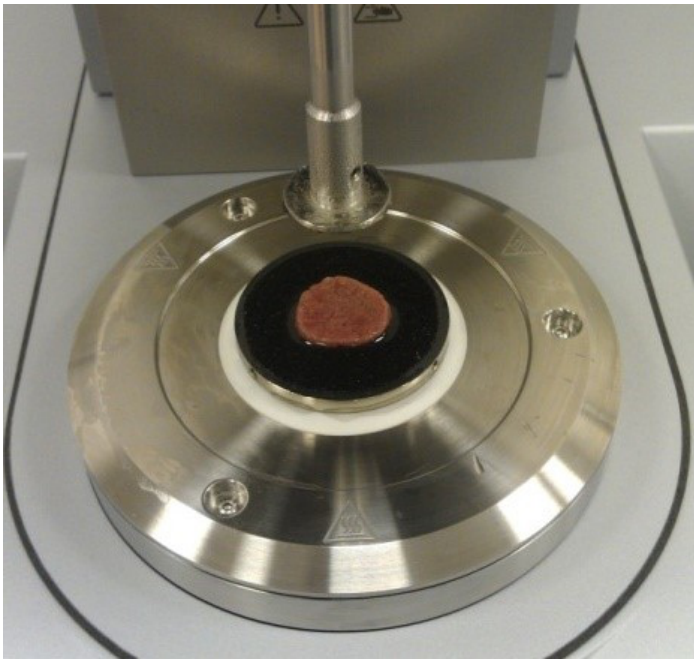
EFFECT OF PRESERVATION SOLUTION AND PERIOD ON MECHANICAL PROPERTIES OF LIVER

4.1 Materials and Methods

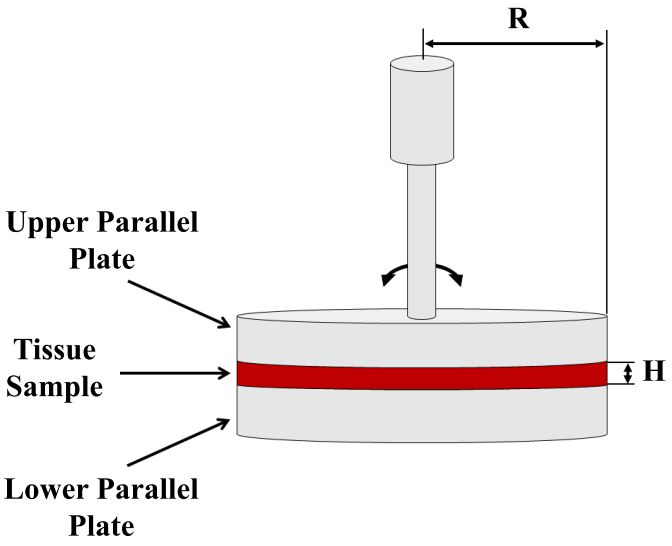
A total of 54 cylindrical tissue samples (3 preservation solutions $\times$ 6 preservation periods $\times$ 3 repetitions) from the left lobe (lobus hepatis sinister) is obtained from each liver. The samples are divided into 3 groups and stored in 3 different solutions at 4 °C: UW and HTK for the preservation purposes and Lactated Ringers solution for the control purposes. The preparation of the cylindrical tissue samples took another hour following the four-hour transportation period. Consequently, the tissue samples placed in Lactated Ringers, UW and HTK solutions are subjected to mechanical measurements at the 5^{th} h, 11^{th} h, 17^{th} h, 29^{th} h, 41^{st} h and 53^{rd} h of the cold ischemic preservation. Note that the tissue slicing process takes no more than one minute (Figure 3.2b-3.2c) and does not cause any significant dehydration. Nicolle and Palierne [41] reported that the dehydration process is fully reversible when the samples are re-hydrated. In our experiments, the samples were put into a preservation solution (Ringer, UW, HTK) right after the preparation of the tissue samples. The holes of the perforated plate are sufficiently small not to cause any damage on the tissue slices during suction. All the slices are cut in the same orientation (parallel to the perforated thin plate shown in Figure 3.1) in order to reduce undesired mea-

surement errors due to anisotropy. Each sample is tested within 20 minutes to ensure that samples are not suffered from dehydration during the rheological experiments as suggested in [41].

We perform DSL and QSSL experiments on the cylindrical tissue samples preserved in Lactated Ringers, UW and HTK solutions for 5 h, 11 h, 17 h, 29 h, 41 h, and 53 h using a rheometer (Anton Paar, MCR 102). The temperature during the experiments is controlled at 4 °C by the Peltier module (P-PTD200/56/AIR) of the rheometer. Each cylindrical liver sample is put between the concentric parallel plates of the rheometer as shown in Figure 4.1a). In order to determine the height of the sample, the upper plate of the rheometer is commanded to move towards the sample with a very slow speed (50 μm/s). When the normal force is reached to 0.5 N, the movement of the upper plate is stopped and the gap between the upper and lower plates is measured and taken as the height (H) of the sample (Figure 4.1b). We observed swelling in the samples due to the influx of preservation solution. Hence, to discharge some of the fluid and bring each sample close to its original height, some initial compression is necessary. Afterwards, a pre-strain of 5% is applied to the samples in the normal direction to maintain full contact between the tissue sample and the parallel plates of the rheometer as suggested in the literature [28, 31, 62]. In addition, sandpapers having a grain size of P80 are attached to the parallel plates of the rheometer using a double-sided tape (3M-9473PC) to ensure that no slippage occurs between the plates and the sample during the experiments as recommended in the literature [23, 31, 42, 70]. Then, pre-conditioning is applied to the sample to reduce the variations among the measurements as recommended by the other groups in the literature [31, 43, 62]. For this purpose, the sample is oscillated at 1 Hz frequency at 0.5% torsional shear strain for 50 cycles before the DSL experiments.



(a)



(b)

Figure 4.1: (a) A cylindrical tissue sample held between two parallel plates of the rheometer, (b) a schematic showing the plates and the sample (R: radius of the parallel plates; H: height of the sample)

4.1.1 Dynamic Shear Loading Experiment

The most common method for characterizing the frequency-dependent viscoelastic material properties of soft tissues is the frequency sweep test. In this experiment, the upper plate of the rheometer oscillates at varying frequencies (ω) with an amplitude of γ_A to introduce a sinusoidal shear strain, γ^* (4.1). The amplitude of the shear strain in a rheometer is calculated based on the deflection angle of the upper plate (θ) as show in Equation (4.2).

$$\gamma^* = \gamma_A e^{i\omega t} = \gamma_A \sin(\omega t) \tag{4.1}$$

$$\gamma_A = \frac{1}{10}\frac{R}{H}\theta \tag{4.2}$$

During the oscillation, the reaction torque (T) is measured (4.3) to calculate the complex shear stress, τ^* (4.4),

$$\tau^* = \tau_A e^{i(\omega t+\delta)} = \tau_A \sin(\omega t + \delta) \tag{4.3}$$

$$\tau_A = \frac{2}{1000}\frac{T}{\pi R^3} \tag{4.4}$$

where, is the amplitude of the shear stress and is the phase angle. Finally, the complex shear ($|G^*|$), storage (G_S) and loss (G_L) moduli are calculated using the Equation (4.5)-(4.6).

$$G^* = \frac{\tau^*}{\gamma^*} \tag{4.5}$$

$$G^* = G_S + iG_L \tag{4.6}$$

In our DSL experiments, the frequency range of the oscillation and the amplitude of the shear strain are set to ω=0.1-20 Hz and γ=0.5%, respectively. The frequency range of the oscillations is determined based on the measurement bandwidth of our rheometer and the recommendations given in [29].

4.1.2 Quasi-Static Shear Loading Experiment

Tissue samples are subjected to SSL at a very slow velocity (0.0005 s^{-1}) up to 15% torsional shear strain (γ_A). The reaction toque measured on the sample are recorded and converted to the torsional shear stress (τ_A) via Equation (4.4). Tangent shear modulus, the slope of the torsional stress-strain curve (G_T), is calculated for 0.1%, 5% and 15% shear strains. This procedure is completed for each sample stored in each preservation solution at 5 h, 11 h, 17 h, 29 h, 41 h, and 53 h preservation periods.

4.2 Results

4.2.1 Dynamic Shear Loading Experiment

The storage and loss moduli of the liver samples preserved in Ringer, UW and HTK solutions for 5 - 53 h are plotted as a function of torsional shear strain (ω=20 Hz) in Figure 4.2. The amplitude of the shear strain (used in the frequency sweep experiments is selected within the linear regime based on the results of the amplitude sweep experiments. These experiments are performed at ω=20 Hz for the shear strain varying between γ_A=0.1-2%. The linear viscoelastic regime is determined as γ_{LVR}=1% based on 10% deviation of either the storage or the loss modulus from the initial value (the value at γ_A=0.1%).

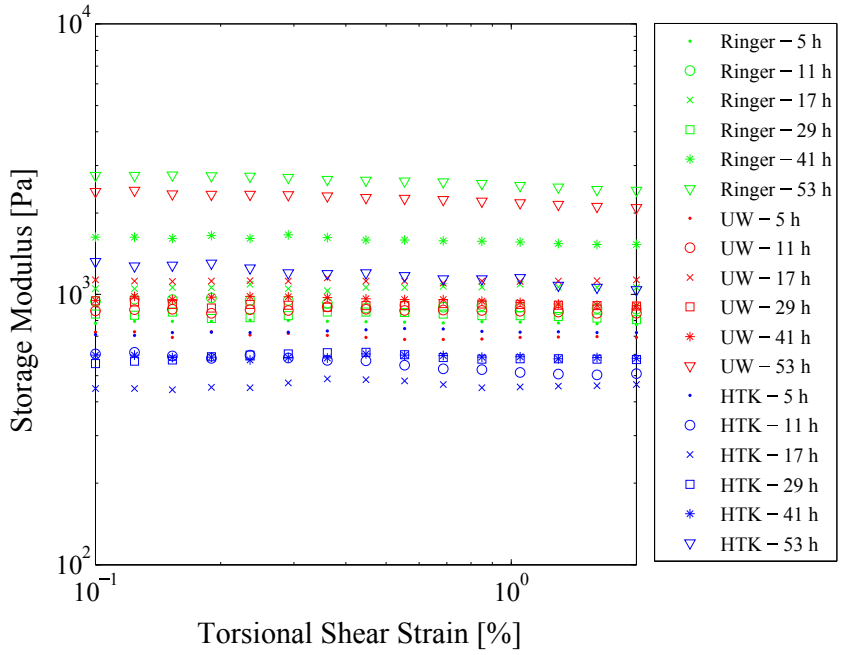
The average storage and loss moduli of the liver samples preserved in Ringer, UW and HTK solutions for 5 - 53 h are plotted as a function of all frequencies tested (ω=0.1-20 Hz) in Figure 4.3 and more specifically for the frequencies of ω=0.1 Hz, 1 Hz and 10 Hz in Figure 4.4. Also, the average and standard deviation values of storage and loss moduli for ω=0.1 Hz, 1 Hz and 10 Hz are tabulated in Table 4.1.

The storage and loss moduli of the tissue samples increase as a function of frequency. The storage and loss moduli of the tissue samples stored in Ringer and UW solutions increase as the preservation period is longer. However, the storage and loss moduli of the samples stored in HTK solution show relatively small change. For example, at a low frequency (ω=0.1 Hz), the storage modulus of the samples stored in Ringer, HTK and UW solutions are between 361-911 Pa, 262-338 Pa and 266-829 Pa, respectively. Also, the loss modulus of the same samples are between 78-247 Pa, 51-67 Pa and 64-256 Pa, respectively. When the frequency is increased to 10 Hz, the storage modulus of the samples stored in Ringer, UW and HTK solutions varies between 801-2084 Pa, 622-2073 Pa and 547-701 Pa, respectively. In addition, the loss modulus of the same samples are between 228-675 Pa, 217-815 Pa and 137-187 Pa, respectively. In order to investigate the impact of the preservation solution and period on the storage and loss moduli of the liver tissue, two-way ANOVA is performed. The results show that the preservation period, solution and their interaction have significant effects on the storage and loss moduli of the livers (p<0.05). Furthermore, paired t-tests are performed to evaluate the statistical difference between the measurements performed at different preservation periods. The results suggest that the storage modulus of the liver samples preserved in Ringer, UW and HTK solutions change significantly at the 11^{th} h (p<0.05), 29^{th} h (p<0.001) and 11^{th} h (p<0.001), respectively. On the other hand, the loss modulus of the same samples preserved in Ringer, UW and HTK solutions change significantly (p<0.001) at the 17^{th} h, 29^{th} h and 29^{th} h, respectively. In particular, the storage moduli of the samples preserved in Ringer and UW solutions increase almost 2-folds at the 41^{st} h (p<0.001) and almost

4-folds at the 53^{rd} h ($p<0.001$) compared to the measurements obtained at the 5^{th} h. However, the storage and loss moduli values of the samples preserved in HTK solution show relatively small changes (1.1 folds, $p<0.001$) throughout the whole testing period.

Table 4.1: The average and standard deviation of the storage and loss moduli of bovine liver samples at 0.1 Hz, 1 Hz and 10 Hz with respect to the preservation solution and period.

Preservation Period [h]	Frequency [Hz]	Ringer		HTK		UW	
		Storage Modulus [Pa]	Loss Modulus [Pa]	Storage Modulus [Pa]	Loss Modulus [Pa]	Storage Modulus [Pa]	Loss Modulus [Pa]
5	0.1	361 ± 31	78 ± 7	295 ± 47	61 ± 6	271 ± 45	64 ± 9
	1	520 ± 40	128 ± 12	414 ± 60	98 ± 12	392 ± 61	109 ± 16
	10	850 ± 132	230 ± 26	632 ± 84	179 ± 25	632 ± 71	217 ± 34
11	0.1	385 ± 22	81 ± 4	338 ± 26	67 ± 3	269 ± 19	65 ± 4
	1	539 ± 24	128 ± 8	465 ± 30	106 ± 6	389 ± 22	110 ± 6
	10	801 ± 40	228 ± 16	701 ± 37	187 ± 16	630 ± 46	217 ± 13
17	0.1	431 ± 25	89 ± 9	305 ± 20	61 ± 6	266 ± 12	65 ± 4
	1	605 ± 32	142 ± 11	426 ± 34	95 ± 6	386 ± 12	109 ± 12
	10	944 ± 30	245 ± 17	628 ± 44	168 ± 8	622 ± 47	217 ± 38
29	0.1	409 ± 5	87 ± 2	278 ± 38	54 ± 4	296 ± 90	74 ± 18
	1	578 ± 4	138 ± 3	385 ± 45	86 ± 8	433 ± 122	126 ± 31
	10	894 ± 26	235 ± 6	585 ± 51	147 ± 19	707 ± 182	252 ± 61
41	0.1	544 ± 148	128 ± 34	262 ± 63	51 ± 9	452 ± 83	128 ± 28
	1	776 ± 207	203 ± 52	360 ± 82	79 ± 16	676 ± 134	216 ± 46
	10	1189 ± 305	358 ± 88	547 ± 123	137 ± 29	1122 ± 216	430 ± 85
53	0.1	911 ± 318	247 ± 109	314 ± 91	61 ± 12	829 ± 32	256 ± 7
	1	1338 ± 496	390 ± 174	432 ± 110	95 ± 17	1261 ± 37	425 ± 7
	10	2084 ± 773	675 ± 306	658 ± 142	171 ± 36	2073 ± 55	815 ± 20



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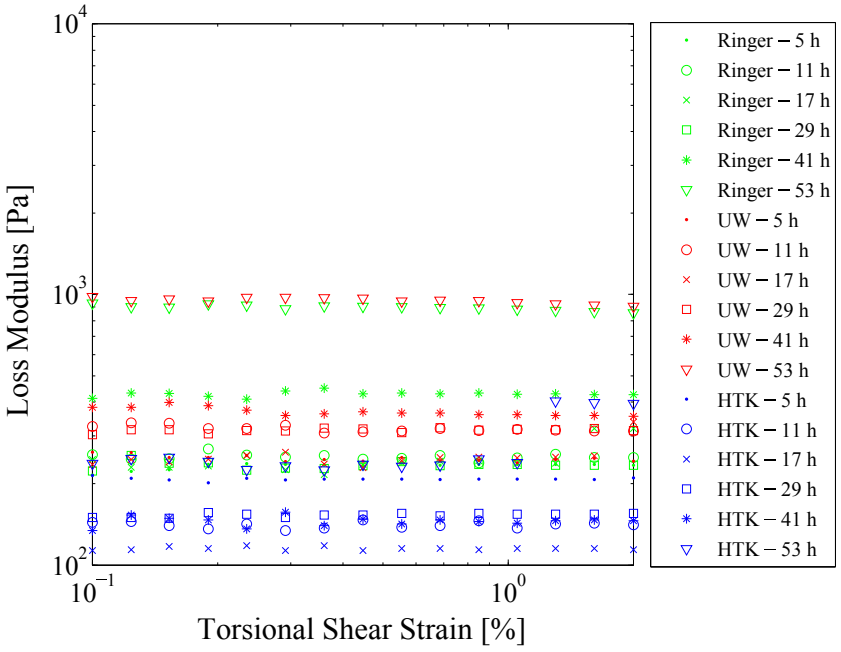

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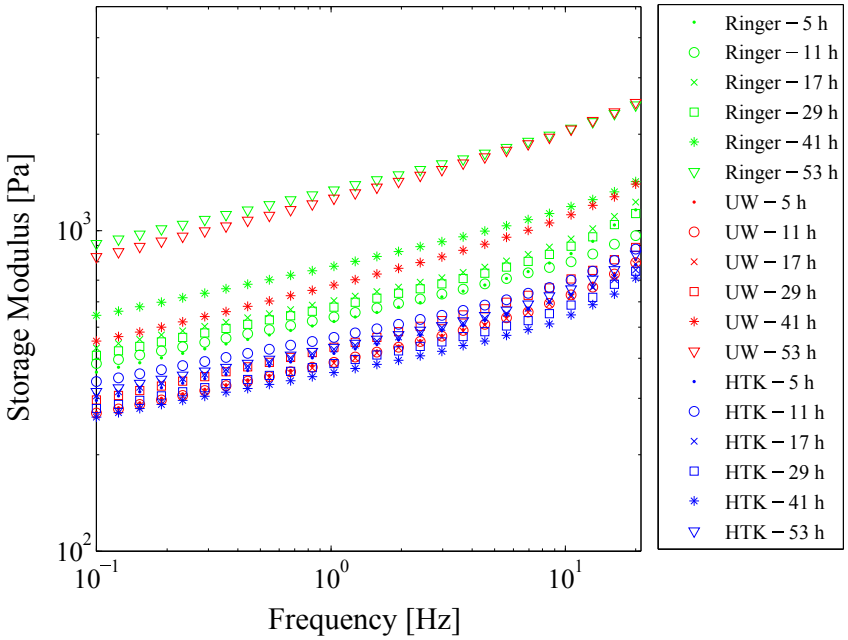
Figure 4.2: (a) The storage and (b) loss moduli of bovine liver samples as a function of preservation period, solution and torsional shear strain.



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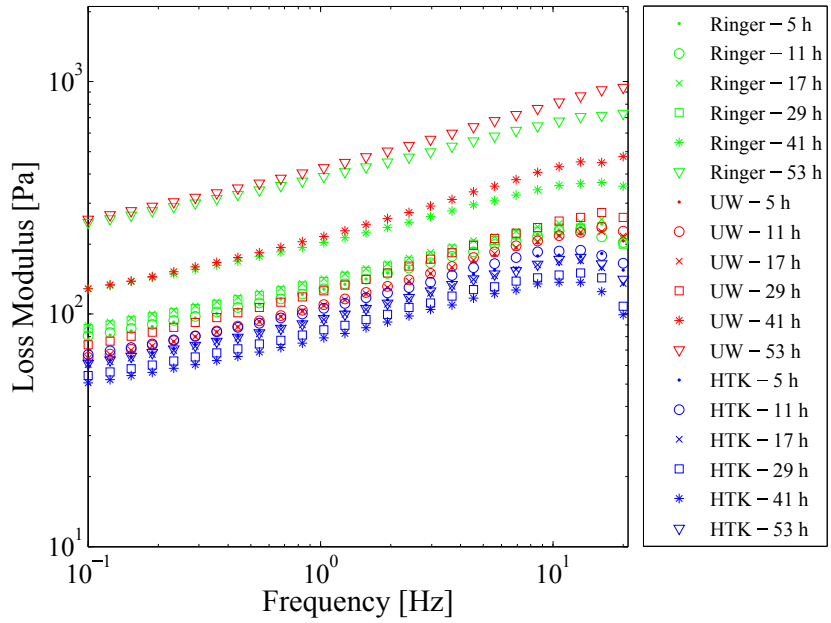

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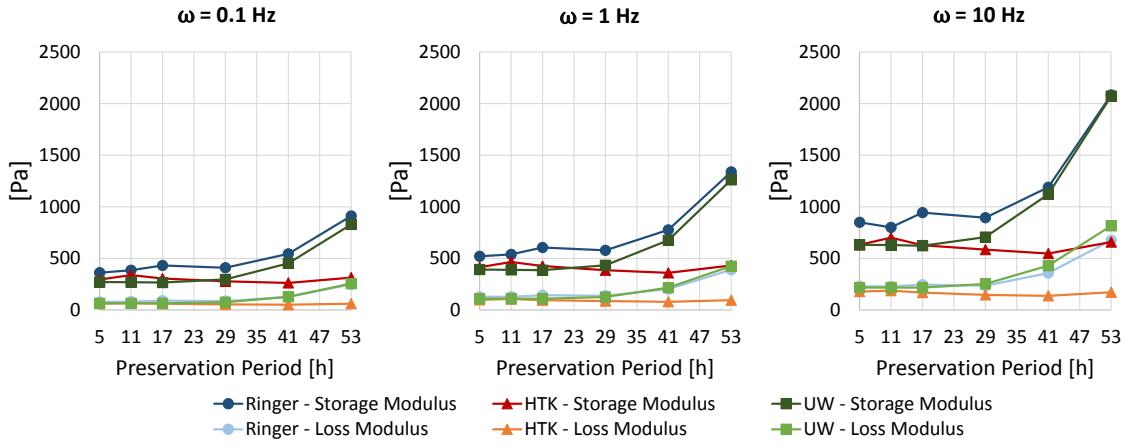
Figure 4.3: (a) The storage and (b) loss moduli of bovine liver samples as a function of preservation period, solution and oscillation frequency.



(a) (b) (c)

Figure 4.4: The storage and loss moduli of the liver tissue as a function of preservation solution and period for the frequency of stimulation at (a) 0.1 Hz, (b) 1 Hz, and (c) 10 Hz.

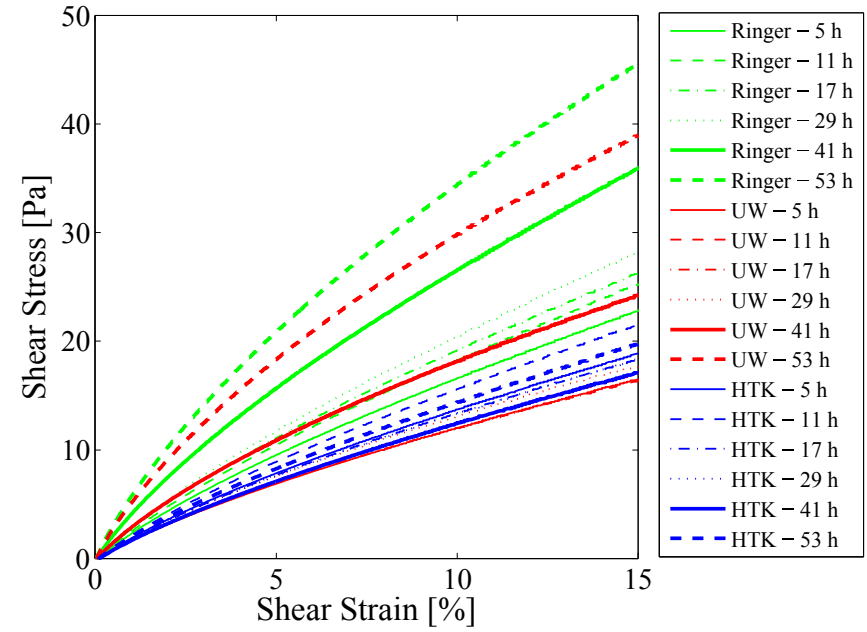
4.2.2 Quasi-Static Shear Loading Experiment

The average torsional shear stress and tangent shear modulus of bovine liver samples stored in Ringer, UW and HTK solutions for 5-53 h are given as a function of torsional shear strain in Figure 4.5. Moreover, the average and standard deviation of the tangent shear modulus values at 0.1%, 5% and 15% are presented in Table 4.2. While, the torsional shear stress of the tissue samples increase with respect to the torsional shear strain, the tangent shear modulus of the samples decrease to a steady state value regardless of the preservation solution and period. Also, it is seen that the samples stored in the Ringer solution have the highest tangent shear modulus; on the other hand, the samples stored in the HTK solution have the lowest tangent shear modulus. Similar to the results of the frequency sweep experiments, the torsional shear stress and tangent elastic modulus values of the samples stored in Ringer and UW solution increase with respect to the preservation period. However for those

stored in HTK solution, the torsional shear stress and tangent elastic modulus values fluctuate in a narrow range. At a small torsional shear strain (0.1%), the tangent elastic modulus of the samples stored in Ringer, UW and HTK solutions are between 265-631 Pa, 190-443 Pa, and 224-261 Pa, respectively. At a large torsional shear strain (15%), the tangent shear modulus of the samples stored in Ringer, UW and HTK solutions vary between 123-242 Pa, 84-166 Pa, and 103-130 Pa, respectively. Moreover, two-way ANOVA tests demonstrate that the preservation period, solution and their interaction have significant effects on the torsional shear stress and tangent shear modulus of the livers ($p<0.05$). Additionally, Bonferroni corrected paired t-tests show that the torsional shear stress and tangent shear modulus of the liver samples preserved in Ringer, UW and HTK solutions change significantly at 11^{th} h ($p<0.001$). At 0.1% torsional shear strain, the tangent shear modulus of the samples stored in Ringer and UW solutions from 5 h to 53 h increases 2.38 fold ($p<0.001$) and 2.24 fold ($p<0.001$), respectively. However, at 0.1% torsional shear strain; the tangent shear modulus increases 1.17 fold ($p<0.001$) for samples stored in HTK solution throughout the whole preservation period.

4.3 Discussion

We conducted dynamic and static mechanical experiments on bovine liver tissue using an oscillatory shear rheometer. We measured storage modulus of liver samples stored in Ringer, UW and HTK solutions 361-2084 Pa, 266-2073 Pa and 262-701 Pa at the frequency range of 0.1-10 Hz, shear strain of 0.5% and preservation period range of 5-53 h, respectively. Also, the loss moduli of the samples preserved in Ringer, UW and HTK solutions are measured 78-675 Pa, 64-815 Pa and 51-187 Pa at the frequency range of 0.1-10 Hz, shear strain of 0.5% and preservation period range of 5-53 h, respectively. In general, the results of our DSL experiments show good agreement with the early findings reported in the literature. Liu and Bilston [31] performed oscillation experiments at 37 °C on bovine liver tissue under a torsional shear strain of 0.11%



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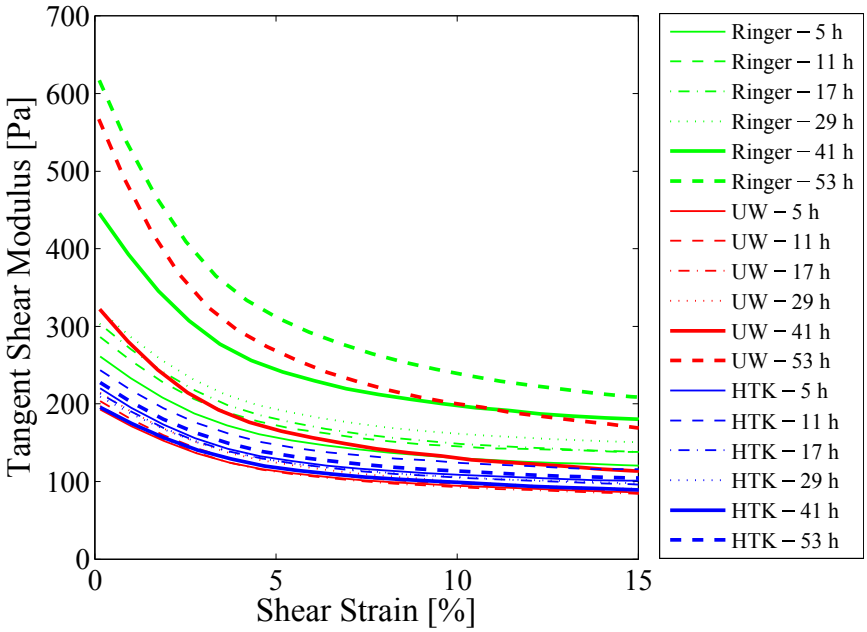


(b)

Figure 4.5: (a) The average torsional shear stress and (b) tangent shear modulus of the bovine liver samples as a function of preservation period, solution and oscillation frequency.

Table 4.2: The average and standard deviation of the storage and loss moduli of bovine liver samples at 0.1 Hz, 1 Hz and 10 Hz with respect to the preservation solution and period.

Preservation Period [h]	Torsional Shear Strain [%]	Ringer	HTK	UW
		Tangent Shear Modulus [Pa]	Tangent shear Modulus [Pa]	Tangent Shear Modulus [Pa]
5	0.1	265 ± 22	224 ± 38	198 ± 26
	5	156 ± 24	129 ± 30	114 ± 22
	15	123 ± 22	103 ± 26	88 ± 19
11	0.1	292 ± 16	261 ± 23	214 ± 26
	5	174 ± 18	157 ± 14	123 ± 18
	15	141 ± 18	130 ± 12	95 ± 14
17	0.1	322 ± 22	241 ± 31	190 ± 19
	5	195 ± 18	142 ± 20	108 ± 9
	15	161 ± 21	115 ± 15	84 ± 7
29	0.1	328 ± 7	229 ± 20	219 ± 51
	5	196 ± 16	135 ± 17	124 ± 36
	15	160 ± 15	107 ± 13	94 ± 28
41	0.1	467 ± 93	235 ± 25	322 ± 28
	5	265 ± 68	139 ± 17	175 ± 19
	15	204 ± 59	111 ± 15	124 ± 16
53	0.1	631 ± 40	235 ± 58	443 ± 64
	5	340 ± 28	136 ± 44	244 ± 39
	15	242 ± 16	108 ± 40	166 ± 21

and for the frequency range of 0.006-20 Hz. The shear storage and loss moduli of the bovine liver were varied between G_S=1000-7000 Pa and G_L=300-1000 Pa, respectively. Klatt et al. [29] conducted rheological experiments with bovine liver samples in the frequency range of 2.5-62.5 Hz frequency and at 0.3% torsional shear strain and 1 °C temperature. The storage and loss moduli were varied between G_S=1000-3000 Pa and G_L=400-1270 Pa, respectively. Nicolle et al. [43] performed rheological experiments on porcine liver samples at 37 °C in the frequency range of 0.1-4 Hz under a strain rate of 0.0151 s^{-1}, 0.133 s^{-1} and 0.7 s^{-1} and for the post-mortem period of 0-24 h. The storage and loss moduli of the samples were measured as G_S=800-1100 Pa and G_L=150-300 Pa, respectively. Tan et al. [62] investigated the effect of the pre-strain on the viscoelastic behavior of the liver tissue at 2-6 h post mortem time. The authors conducted amplitude sweep experiments at a frequency of 1 Hz, for shear strain value varying between 0.005-2%. The storage and loss moduli of liver tissue were reported to vary between G_S=350-450 Pa and G_L=70-90 Pa, respectively for the pre-strain value in the normal direction varying between 1-20%. Wex et al. [70] performed strain (1 Hz, 0.0001-1% strain), stress (1 Hz, 0.1-100 Pa), and frequency sweeps (0.1-10 Hz, 0.001% shear strain) and relaxation experiments (1 Hz, 1 Pa, 600 s) on porcine liver using a rheometer. The magnitude of the complex shear modulus of porcine liver was reported to vary between $|G^*|$=636-668 Pa under strain sweep for the post-mortem period of 1 h and as $|G^*|$=443-538 Pa for the post-mortem period of 20-27 h. The magnitude of the complex shear modulus was reported to vary between $|G^*|$=564-1004 Pa and $|G^*|$=470-578 Pa under frequency sweep for the post-mortem period of 1 h and 20-27 h, respectively. Valtorta and Mazza [67] used a torsional resonator to characterize the complex shear modulus of bovine liver (few hours after harvesting) for the frequency range of 110 kHz at the ambient temperature. The results of the in-vitro experiments suggested that the magnitude of the complex shear modulus of the liver varies between $|G^*|$=5000-20000 Pa. Sapin-de Brosses et al. [53] measured the magnitude of complex shear modulus of bovine liver as $|G^*|$=3400±500

Pa by Supersonic shear imaging technique for the frequency range of 150 - 200 Hz at a temperature of 25 °C and for post-mortem period of 0-48 h. Riek et al. [49] used MRE technique to examine the dynamic mechanical properties of bovine liver tissue at a temperature of 17 °C-19 °C for the frequency range of 100-800 Hz and post-mortem period of 0-2 h. The authors reported the storage and loss moduli of bovine liver tissue as G_S=1420-4910 Pa and G_L=740-1620 Pa, respectively. Kruse et al. [30] measured the magnitude of the complex shear modulus of the porcine liver at 37 °C as $|G^*|$=3000 Pa at 100 Hz and as $|G^*|$=5000 Pa at 300 Hz by using the MRE method.

There are also studies in the literature reporting the dynamic elastic modulus of animal and human livers (Note that the linear elastic and shear moduli are related through for homogenous and isotropic materials. If the Poissons ratio is taken as ν=0.5, then this relation reduces to). DeWall et al. [12] used dynamic compression testing to quantify the viscoelastic properties of 16 human liver samples for a frequency range of 1-30 Hz under a compression strain of 2%, a pre-compression strain of 1-6%, and for a post-mortem period of 0-2 h. The elastic storage and loss moduli of the healthy human liver were measured as E_S=2000-6000 Pa and E_L=500-2000 Pa, respectively. Kiss et al. [28] applied cyclic stimuli (0.1 Hz to 400 Hz) to the canine liver tissue in the normal direction within 72 h of post-mortem time at 21 °C and estimated the storage and loss modulus as E_S=4000-10000 Pa and E_L=800-10000 Pa, respectively. Ocal et al. [45] performed impact hammer experiments on bovine liver for the frequency range of 0-100 Hz at the room temperature and for post-mortem period of 1-48 h. They measured the elastic storage and loss moduli of the bovine liver as E_S=5000-80000 Pa and E_L=1000-13000 Pa, respectively.

Furthermore, we calculated tangent shear modulus of liver samples stored in Ringer, UW and HTK solutions 123-631 Pa, 84-443 Pa and 103-261 Pa up to a shear strain of 15%, at a loading velocity of 0.0005 s^{-1} and preservation period range of 5-53 h, respectively. The results of the previous studies examining the normal elas-

tic modulus (E) and tangent shear modulus (G_T) of the liver tissue are inspected by using the relation (ν=0.49: Poissons ratio for the liver tissue). Our results are in an agreement with the measurements performed by Yeh et al. [73], Chen et al. [7], and Garo et al. [19]. Yeh et al. [73] measured the elastic modulus of the normal human liver with a mechanical measurement device as E=640 Pa at 5% strain and the elastic modulus of the human liver with cirrhosis (score: 5) E=1650 Pa at 5% strain. Chen et al. [7] calculated the elastic modulus of the bovine liver E=620240 Pa using an ultrasound device and E=940650 Pa using a mechanical tensile testing device at up to 5% strain. Garo et al. [19] reported the moduli of the porcine brain tissue at 4.5% shear strain as G=195 Pa at post-mortem times between 0-6 h and G=305 Pa at the post-mortem time of 10 h. However, our results are not comparable with some of the studies reported in the literature (Kruse et al. [30], Ottensmeyer [46], Saraf et al. [54], Samur et al. [51], and Nava et al. [40]). The reason for the discrepancy might be due to many possible factors such as the differences in the measurement devices and samples, experimental techniques and procedures. Kruse et al. [30] reported the complex shear stiffness of the porcine liver $|G^*|$=3000 Pa at 100 Hz and $|G^*|$=5000 Pa at 300 Hz by using the MRE method at 37 °C. Ottensmeyer [46], estimated the elastic modulus of the porcine liver E=10000 Pa. Saraf et al. [54], used the Kolsky bar technique and estimated the shear modulus of the human liver G=37000-340000 Pa at a shear rate of 280-1800 s^{-1}. Samur et al. [51] found the elastic modulus of porcine liver in the range of E=10000-17000 Pa with a robotic indenter device. Nava et al. [40] conducted experiments on healthy human liver with an aspiration device. They estimated the long term and instantaneous linear elastic modulus of human liver as E=20000-60000 Pa, respectively.

There are several reasons for the discrepancy between the findings of the above studies. Obviously, the differences in the measurement devices, measurement methods, and the measured subjects (human, porcine, bovine, canine) contribute significantly to this discrepancy. Also, there are differences in the frequency of stimulation

and several studies in the literature have already shown that the storage and loss moduli of the liver tissue are frequency-dependent. Another reason for the discrepancy could be the effect of temperature. The storage and loss moduli of the liver tissue have been shown to change with temperature as well. Furthermore, in our experiments, we observed the effect of pre-stress in the normal direction on the torsional shear stress measured by the rheometer (note that a certain amount of pre-stress in normal direction is necessary to hold the tissue sample between the parallel plates of the rheometer). While the effect of normal stress on rheological shear measurements has been mentioned briefly in a few studies and most authors report the magnitude of the normal force applied to the samples, we are not aware of any systematic study investigating the effect of this normal force on the torsional shear stress measurements. This topic needs a further investigation. Also, in our experiments, the tissue samples were prepared from parenchyma of each liver after removing its Glisson's capsule. We note that the Glisson's capsule is about three times stiffer than parenchyma and removing Glisson's capsule may lead to underestimation of the mechanical properties by 2-3 folds as pointed in Hollenstein et al. [22]. Moreover, the fluid motility inside the samples were minimized through the pre-conditioning experiments which ensured the extraction of preservation solution before the actual experiments.

In this study, we show that the preservation solution and period significantly affect the material properties of liver tissue. For example, we found that the change in the storage and loss moduli of the samples stored in HTK solution is far more less (about 1.1 folds, $p<0.001$) than those of the samples stored in UW solution (about 4 folds, $p<0.001$) at 53^{rd} h. Our results show that the storage and loss moduli of liver tissue increase as it spends more time in the preservation solution. Moreover, based on the torsional shear stress and tangent shear modulus, we found that the static mechanical properties of the liver samples preserved in Ringer, HTK and UW solutions change significantly at the 11^{th} h ($p<0.001$). At a small torsional shear strain, the tangent shear modulus of the samples stored in Ringer, UW and HTK solutions increases

2.38, 2.24 and 1.17 folds from 5 h to 53 h preservation period, respectively. Garo et al. [19], Kerdok et al. [27], Ocal et al. [45], and Yarpuzlu et al. [72] also observed that liver tissue becomes stiffer and more viscous as the post-mortem time increases. On the other hand, Wex et al. [70] have recently reported that the complex shear modulus of liver tissue decreases with increasing post-mortem times.

Chapter 5

EFFECT OF PRESERVATION SOLUTION AND PERIOD ON HISTOLOGICAL PROPERTIES OF LIVER

5.1 Materials and Methods

5.1.1 Preparation of Tissue Samples

For the histological analyses, small tissue blocks having a thickness of 3 mm are obtained from the parenchyma of the left lobe of each liver and put into Ringer, HTK and UW solutions at the 5^{th} preservation period. These small samples are fixed in 10% neutral buffered formalin at 11 h, 17 h, 29 h, 41 h, and 53 h preservation periods for 24 h at room temperature and dehydrated by bathing in a graded series of mixtures of ethanol and water. Also, it should be emphasized that for the control purposes, the same procedure is followed for the small samples preserved in Ringer solution at the 5^{th} preservation period. Afterwards, hydrophobic clearing agent (xylene) is used to remove the alcohol and also, an infiltration agent (paraffin wax) is used to replace xylene. Then, the samples in paraffin wax are heated in the oven at 60 °C for 2 h. Finally, these samples are sectioned at 7-10 μm in thickness using a microtome and stained with different kits to analyze the histological properties under a light microscope.

5.1.2 Examination of Histological Properties

The tissue sections are colored with four different stains for histological examination: (1) Apop-Tag Plus Peroxidase kit (Intergen S7101, Merck Millipore Inc.), also known as Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) technique, is used to examine the hepatocytes undergoing apoptosis, (2) Masson's Trichrome stain (Masson-Goldner Staining kit, Merck Millipore Inc.) is used to detect the changes in connective tissue, (3) Hematoxylene (Hematoxylene solution modified to Gill III, Merck) & Eosin (Eosin Y solution 0.5% alcoholic, Merck Millipore Inc.) H&E is used to detect structural changes in sinusoids, and (4) Periodic-Acid Schiff stain (PAS Staining kit, Merck) is used to detect the changes in glycogen level in the liver cells.

All treated sections are examined under a light microscope (Axio Imager, Carl Zeiss Inc.). The microscopic images, captured from ten different areas on each section, are examined at 100X magnification by Axio Vision image analysis software package (Carl Zeiss Inc.). Four software scripts are developed (1) to count the nuclei of apoptotic cells labeled by Apop-Tag Plus Peroxidase kit, (2) to measure the collagen accumulation on each section stained with Masson's trichrome, (3) to calculate the area of sinusoids on each section stained with H&E, and (4) to measure the glycogen level in the cells on each section stained with PAS.

5.2 Results

We analyze the changes in the number of the apoptotic cells, the connective tissue accumulation, the sinusoidal dilatation, and the level of glycogen deposition of the tissue blocks fixed at the 11^{th} h, 17^{th} h, 29^{th} h, 41^{st} h and 53^{rd} h of cold ischemic preservation (10 micrographs $\times$ 3 preservation solutions $\times$ 5 preservation periods = a total of 150 micrographs are analyzed). Also, the histological properties of the tissue blocks stored in Ringer solution and fixed at the 5^{th} h preservation period are

examined for the control purposes (10 micrographs × 1 preservation solution × 1 preservation period = a total of 10 micrographs are analyzed). The results of the histological analyses are summarized in Table 5.1.

In order to investigate the initial histological state of the liver tissue, the tissue blocks preserved in Ringer solution for 5 h are also fixated and colored with TUNEL, Masson's trichrome, H&E, and PAS stains. The stained sections are further processed for control purposes using the software scripts developed for the histological examination (Figure 5.2).

The apoptotic cells in each tissue section are identified by Apop Tag Plus Peroxidase kit. The brown/dark brown cells on the micrographs are marked and counted to determine the number of apoptotic cells. The change in the number of apoptotic cells as a function of preservation solution and period is given in Figure 5.1a. The exemplar micrographs showing the apoptotic cells of the liver tissue preserved for 11 h and 53 h are shown in Figure 5.3. The results show that the number of apoptotic cells increase as a function of preservation period. For the samples stored in Ringer and HTK solutions, the apoptotic cell count increase significantly after the 11^{th} h, while those preserved in UW solution increases after the 29^{th} h compared to the control group ($p<0.05$). The samples stored in HTK solution have the highest number of apoptotic cells, on the other hand the samples stored in UW solution have the lowest number of apoptotic cells at the end of the preservation period.

The accumulation of connective tissue in the preserved liver tissues is analyzed by the Masson's trichrome stain. The connective tissue is colored as green in the micrographs and its area is measured. The change in the accumulated connective tissue as a function of preservation solution and period is plotted in Figure 5.1b. The exemplar micrographs showing the connective tissue accumulation for the preservation periods of 11 h and 53 h are given in Figure 5.4. For the samples stored in Ringer and UW solutions, the connective tissue accumulation increase significantly after the 17^{th} h compared to the control group ($p<0.05$). Nevertheless, for the samples preserved in

HTK solution, the connective tissue accumulation increases significantly only after the 53^{rd} h compared to the control group ($p<0.05$). The results suggest that the accumulation of the connective tissue increases as a function of preservation period regardless of the preservation solution. At the end of the preservation period, the highest and the lowest accumulation is observed in the samples stored in UW and HTK solutions, relatively.

The tissue sections are stained with H&E to quantify the amount of sinusoidal dilatation. The borders of the sinusoids are outlined with red lines and the areas enclosed by these borders are measured. The change in the sinusoidal dilatation of the bovine liver as a function of preservation solution and period is plotted in Figure 5.1c. The exemplar micrographs showing the sinusoidal dilatation of the liver tissue preserved for 11 h and 53 h are shown in Figure 5.5. The results suggest that the amount of sinusoidal dilatation increase as a function of preservation period. For the samples preserved in Ringer, UW and HTK solutions, the sinusoidal dilatation increase significantly after the 29^{th} h, 53^{rd} h and 17^{th} h compared to the control group, respectively ($p<0.05$). The largest and lowest dilatation is detected in the samples preserved in HTK and Ringer solutions at the 53^{rd} h, respectively.

The glycogen level in the cells of the bovine liver is examined with the PAS stain. The magenta colored areas in the tissue sections are detected and measured by the image analysis software. The change in the deposited glycogen level is plotted as a function of preservation solution and period in Figure 5.1d. The exemplar micrographs showing the glycogen accumulation of the liver tissue preserved for 11 h and 53 h are given in Figure 5.6. It is clear that the glycogen level in the cytoplasm of the cells preserved in all solutions decrease as a function of preservation period. The glycogen level in the tissue sections preserved in Ringer and HTK solutions decrease significantly after the 11^{th} h ($p<0.05$) while, those preserved in UW solution decrease significantly after the 29^{th} h compared to the control group ($p<0.05$). When the glycogen accumulation levels are inspected at the 53^{rd} h for each solution, it is

observed that the sections stored in UW solution has the highest level and the sample sections preserved in Ringer solution has the lowest level of glycogen.

Table 5.1: The histological properties of bovine liver (average of 3 animals).

Preservation Period [h]	Apoptotic Cell [count]			Connective tissue [%]			Sinusoidal dilatation [%]			Glycogen level [%]		
	Ringer	HTK	UW	Ringer	HTK	UW	Ringer	HTK	UW	Ringer	HTK	UW
5	13 ± 6			6 ± 3			28 ± 13			36 ± 8		
11	26 ± 14	42 ± 9	6 ± 5	7 ± 3	7 ± 4	7 ± 5	31 ± 11	31 ± 7	22 ± 6	25 ± 10	25 ± 10	34 ± 13
17	37 ± 11	43 ± 9	8 ± 6	10 ± 5	8 ± 4	11 ± 6	30 ± 6	36 ± 12	28 ± 7	14 ± 9	14 ± 9	36 ± 15
29	48 ± 13	59 ± 16	19 ± 13	14 ± 7	8 ± 5	15 ± 6	37 ± 13	35 ± 11	32 ± 8	10 ± 8	17 ± 10	20 ± 10
41	44 ± 12	58 ± 17	31 ± 20	15 ± 8	8 ± 4	15 ± 8	31 ± 5	42 ± 10	30 ± 6	8 ± 8	12 ± 10	12 ± 8
53	44 ± 9	57 ± 13	34 ± 21	16 ± 6	13 ± 8	17 ± 8	39 ± 7	45 ± 10	43 ± 7	3 ± 2	6 ± 5	9 ± 8

5.3 Discussion

We examined histological properties of bovine liver via various staining kits as a function of preservation solution and period. The results suggest that the number of apoptotic cells, connective tissue, and sinusoidal dilatation increase while the glycogen level deceases as a function of preservation period. We observed less number of apoptotic cells in the samples stored in UW solution and less connective tissue (the main component of the ECM) accumulation in the samples preserved in HTK solution. These findings are compatible with the pharmacology of the preservation solutions. UW is known to be an intracellular type preservation solution and effective in protecting the integrity of the cell structure. On the other hand, HTK is an extracellular type preservation solution and effective in preserving the integrity of the ECM structure [16]. Also, our results are in agreement with the findings reported in the literature. Straatsburg et al. [61] analyzed the apoptosis in cold-preserved rat livers stored in HTK, UW, and Celsior solutions under a light microscope. The authors reported that the median number of apoptotic cells stored in UW solution is lower than those stored in HTK solution. Natori et al. [39] examined the level of apoptosis during cold preservation of liver in UW solution. The authors observed that the number of apoptotic cells have increased 6-folds after 24 h preservation. Abrahamse et al. [1] studied cell necrosis and apoptosis after 24 h and 48 h preservation of porcine liver in UW, HTK and Celsior solutions. The authors reported that UW and Celsior solutions are superior to HTK solution in terms of hepatocyte (liver cell) preservation. Lately, Budzinski et al. [6] assessed the cell apoptosis in porcine livers preserved for 12 h in UW and HTK solutions. For an apoptosis scale ranging from 0 to +3, the authors reported the level of apoptotic bodies as +3 in both UW and HTK solutions. However, the accumulated connective tissue was higher for the samples preserved in UW solution. Puhl et al. [48] reported that the sinusoidal dilation is higher in rat liver cells preserved in HTK solution than those preserved in UW

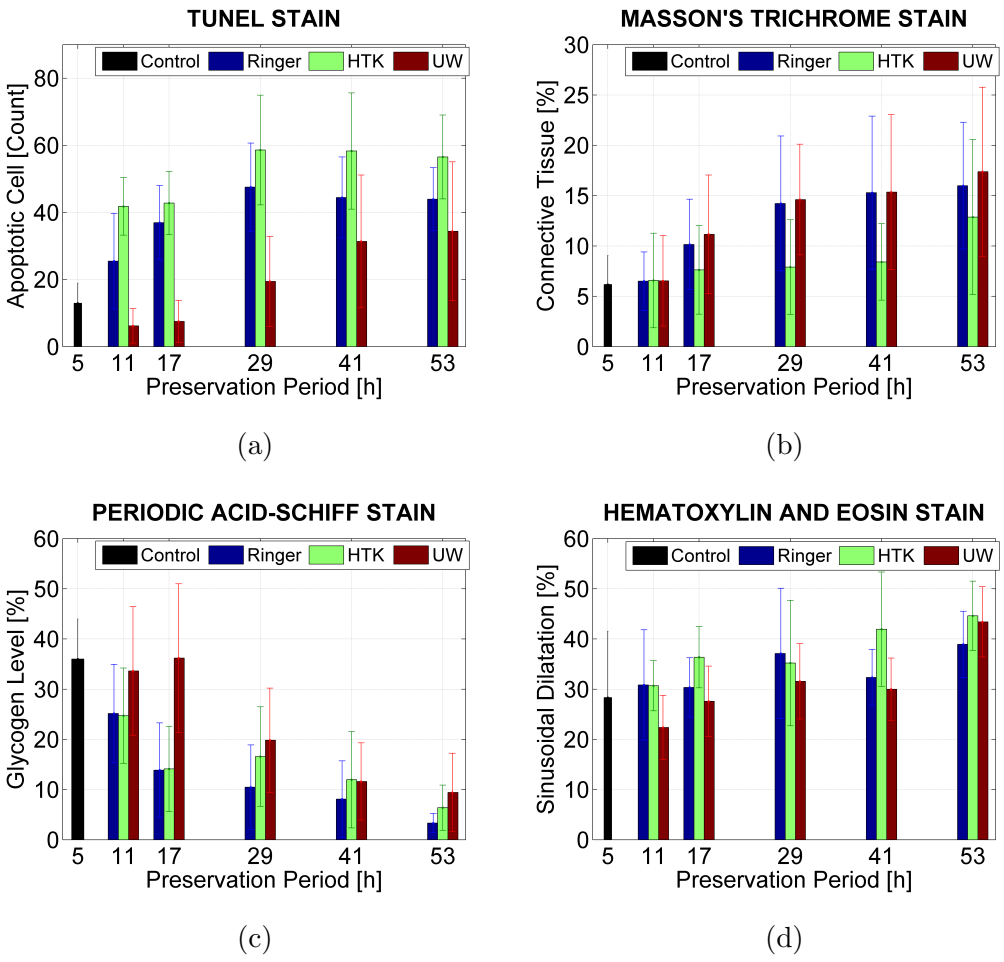


Figure 5.1: The change in the (a) apoptotic cell count, (b) connective tissue, (c) sinusoidal dilatation, and (d) glycogen level as a function of preservation solution and period (average of 3 animals).

solution. Jain et al. [25] investigated the flow dynamics of sinusoidal perfusion during 24 h hypothermic machine perfusion of human livers preserved in UW solution. The authors observed an increase in sinusoidal dilatation as a function of preservation period due to perfusion damage. Furthermore, in our histological measurements, the glycogen level decreases as a function of increasing preservation period. This result suggests that certain percentage of hepatocytes could not synthesize and store glycogen since the deposited glycogen was consumed for the survival. In our study, the glycogen level was the highest in the samples stored in UW solution and lowest in the samples preserved in Ringer solution. Corps et al. [9] analyzed the adenine triphosphate and metabolites of the perfused rat livers preserved in HTK and UW solutions. The authors reported that livers perfused with UW solution do not consume glycogen as much as HTK-perfused livers due to the presence of alpha-ketoglutarate in HTK solution.

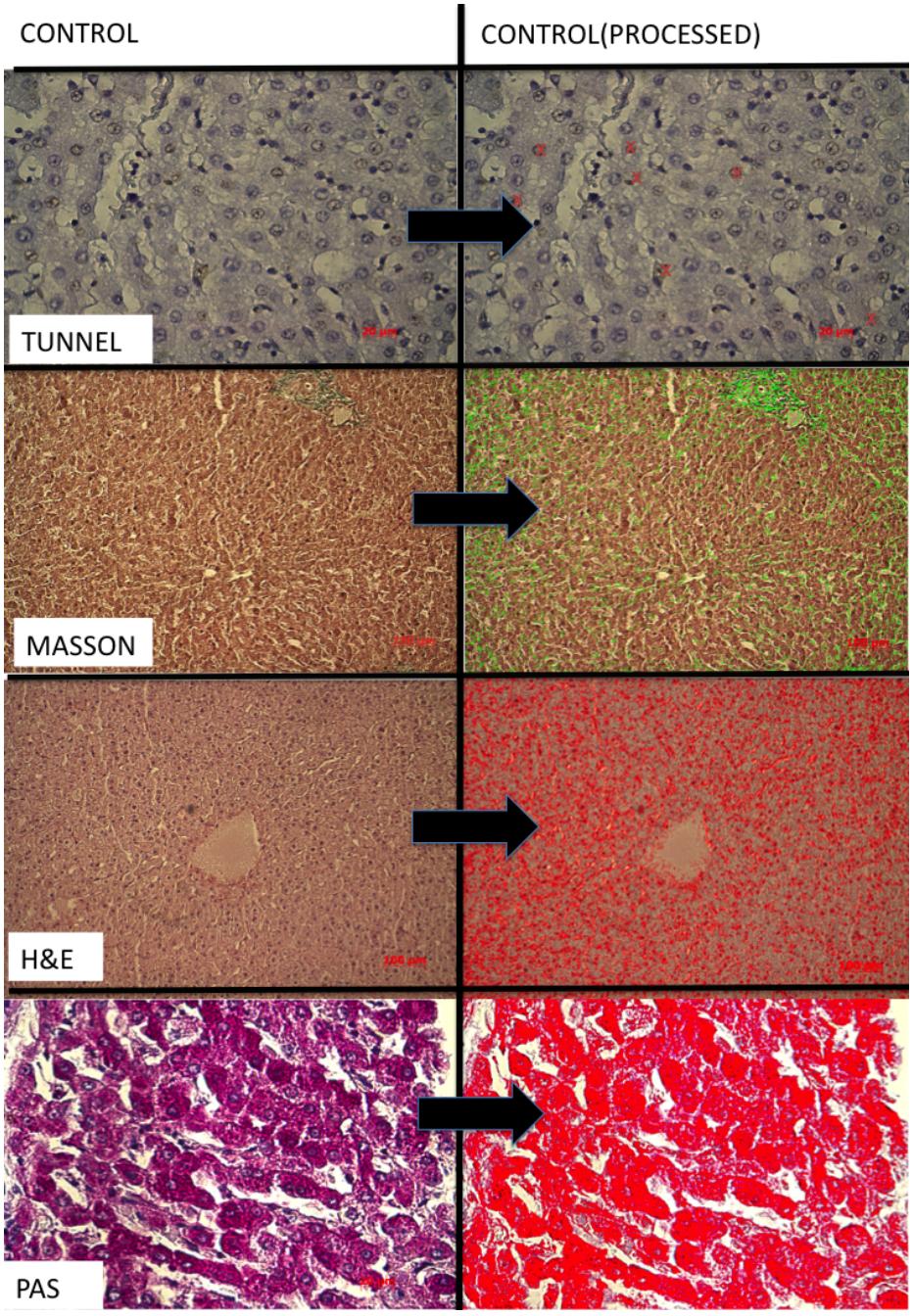


Figure 5.2: The change in the apoptotic cell count, connective tissue, sinusoidal dilatation, and glycogen level as a function of preservation solution and period (average of 3 animals).

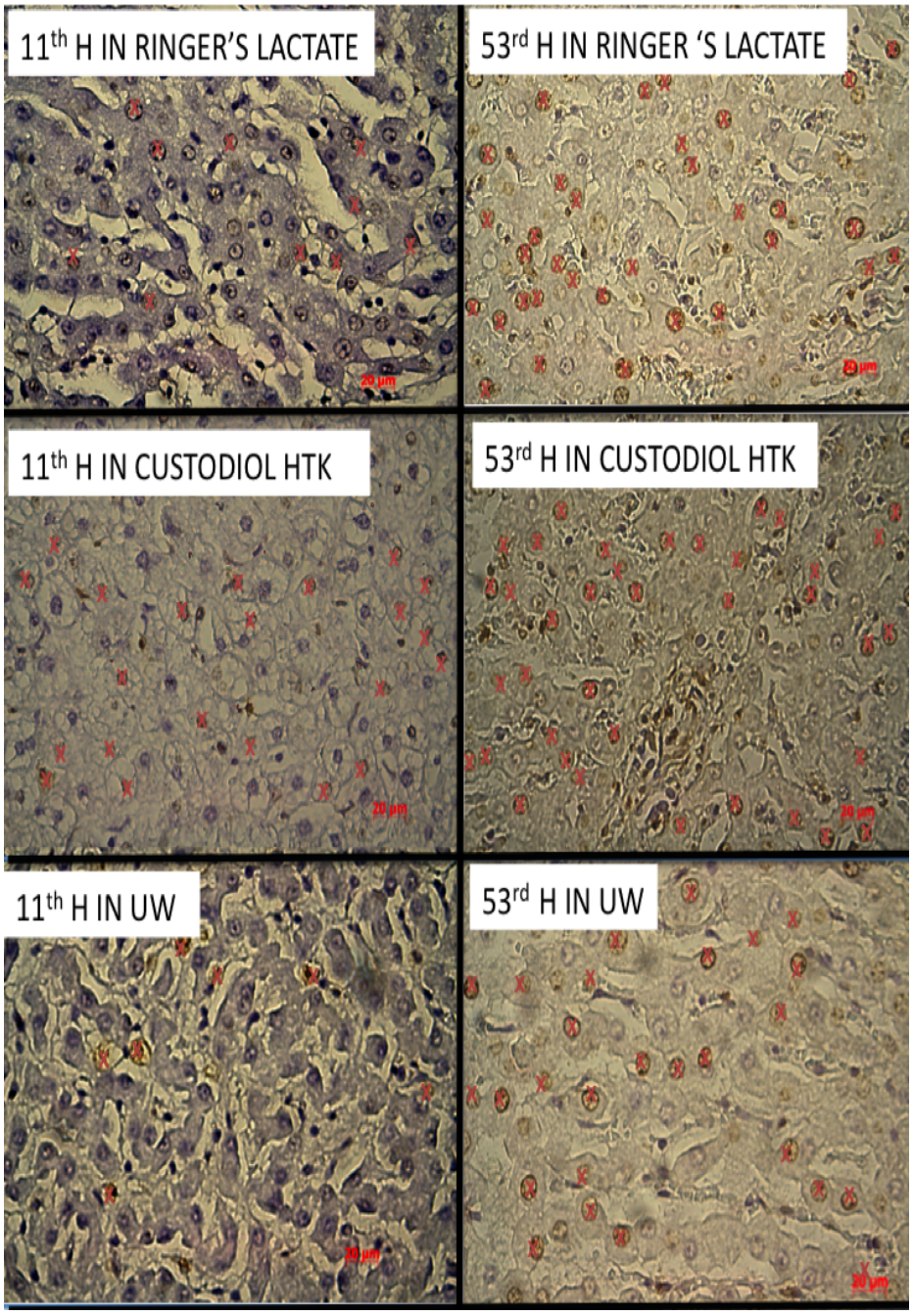


Figure 5.3: The exemplar images of the sections labeled with TUNEL technique and preserved for 11 h and 53 h in Ringer, HTK and, UW solutions. The dark blue stained nuclei shows the healthy cells while the brown/dark brown stained nuclei shows the cells undergoing apoptosis.

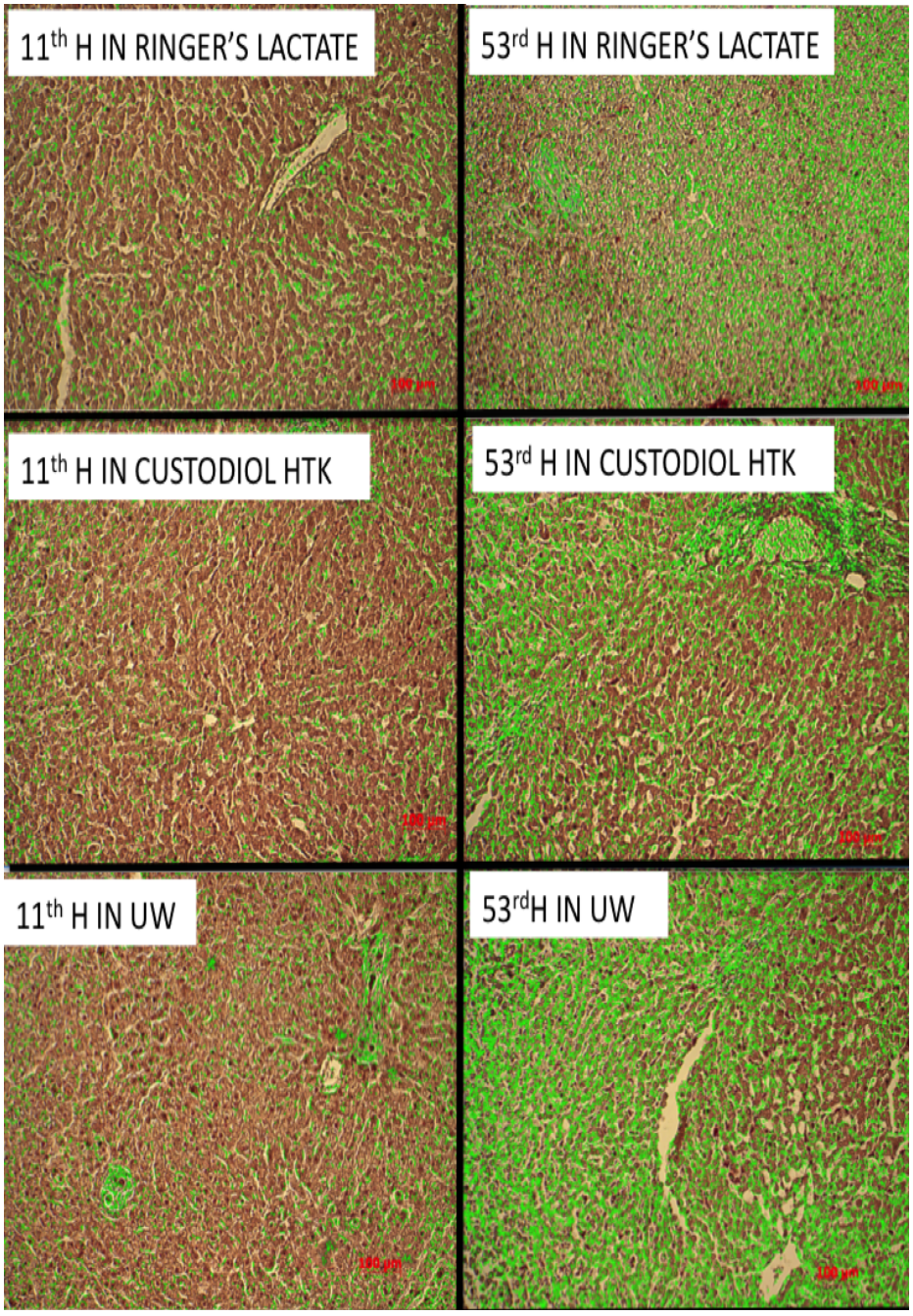


Figure 5.4: The exemplar images of the sections treated by the Masson's trichrome stain and preserved for 11 h and 53 h in Ringer, HTK and, UW solutions. The software labels the green stained areas as connective tissue and then measures these areas.

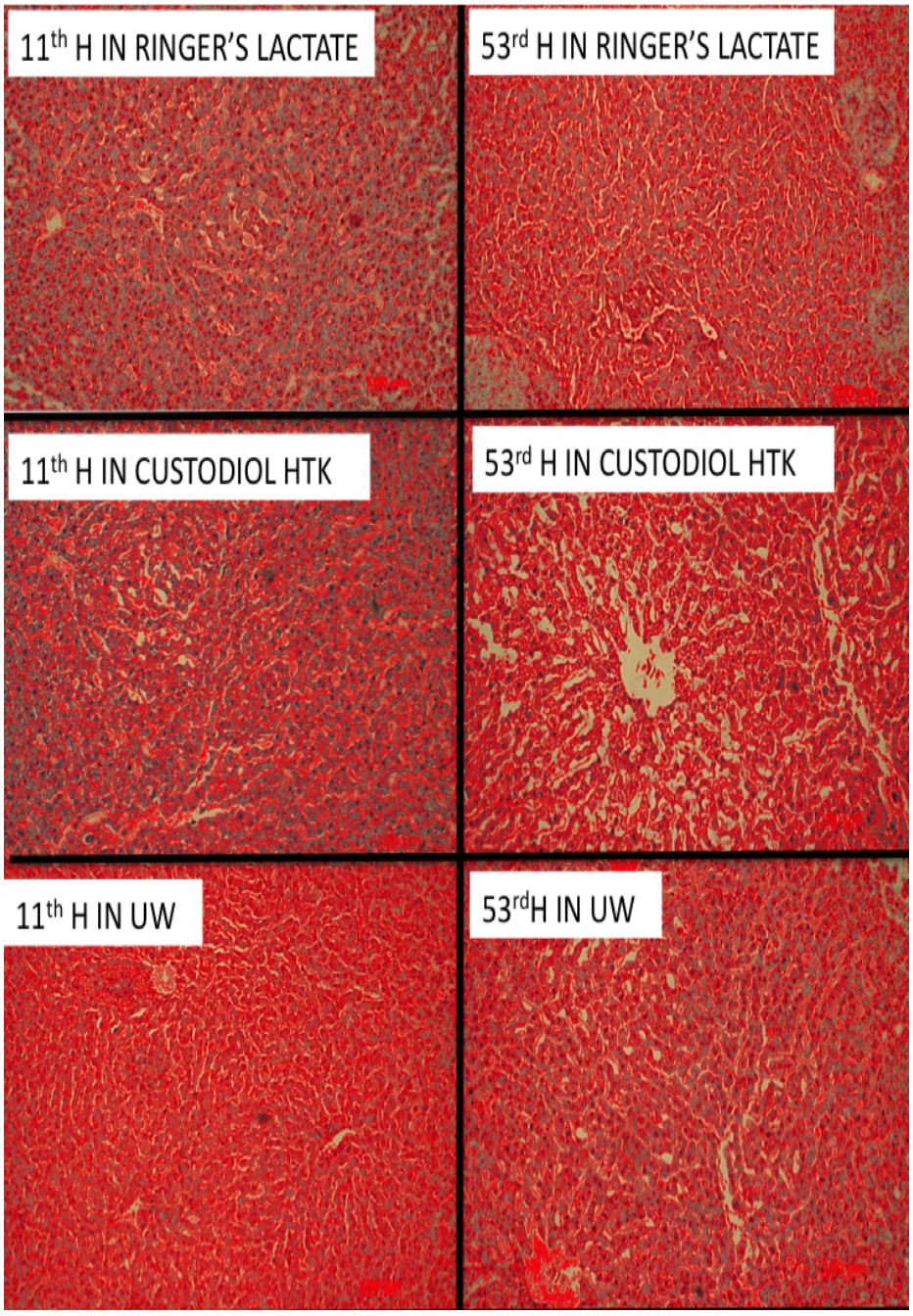


Figure 5.5: The microphotographs show the sections stained with the Hematoxylene and Eosin and preserved for 11 h and 53 h in Ringer, UW and HTK solutions. The borders outlined with red lines and the areas enclosed by these borders show the dilated sinusoids.

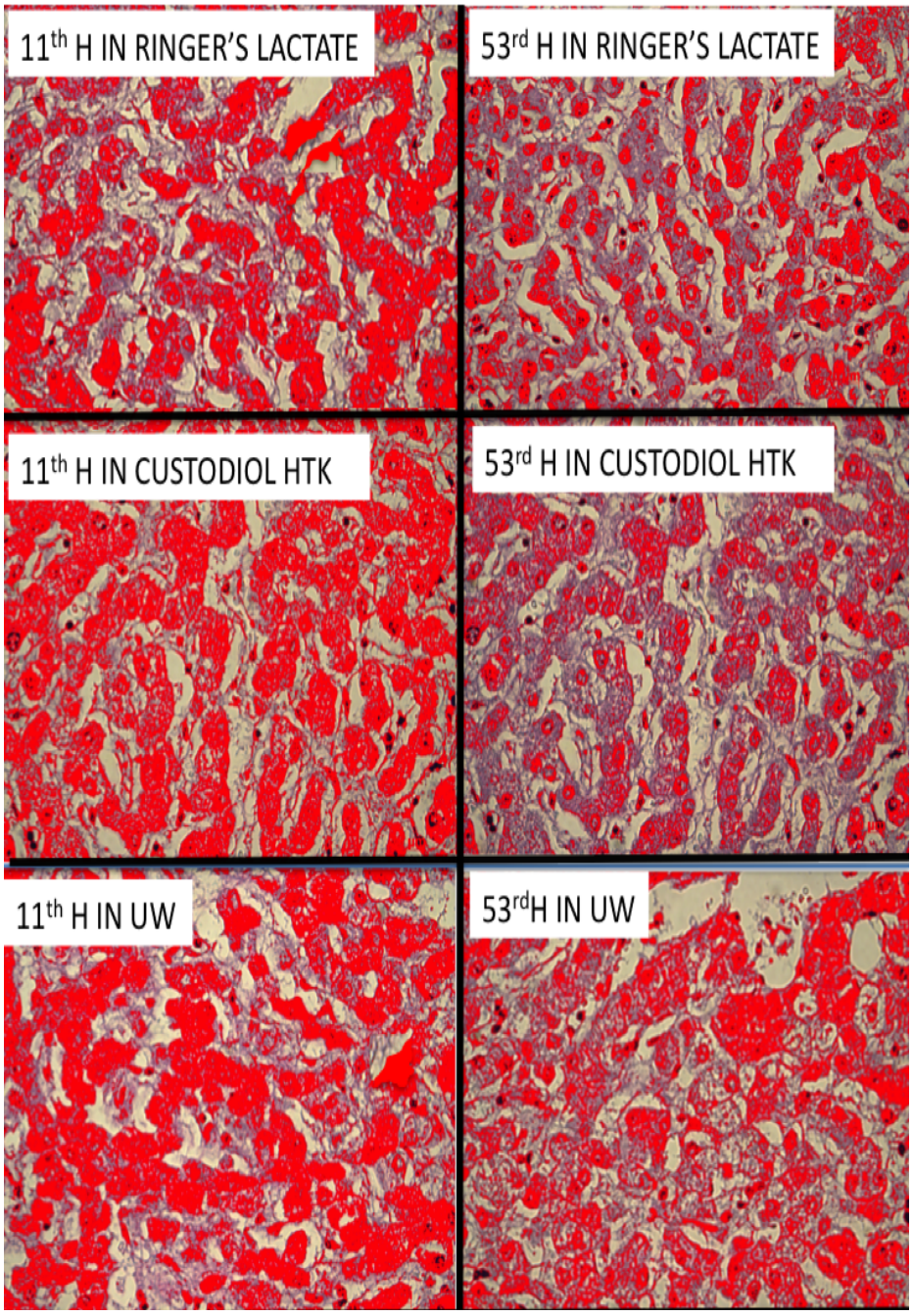


Figure 5.6: he exemplar images of the sections stained with the Periodic Acid-Schiff and preserved for 11 h and 53 h in Ringer, UW and HTK solutions. The image analysis program labels the magenta colored regions in the images and then measures the glycogen level in the cells of the tissue.

Chapter 6

CORRELATION BETWEEN THE MECHANICAL AND HISTOLOGICAL PROPERTIES OF LIVER

6.1 Materials and Methods

One of the goals of this study is to provide an insight into the relation between the mechanical and histological properties of the liver tissue. For this purpose, the changes in gross mechanical properties of the liver tissues are correlated with the histological properties in micro scale using the Spearmans Rank-Order Correlation method. Before the analyses, the histological data collected for each preservation period from ten different areas of each tissue section is averaged for three animals (the average of ten histological measurements $\times$ 5 preservation periods $\times$ 3 livers = 15 data points for each histological property). Also, the mechanical data collected from three tissue samples for each preservation period is averaged for three animals (the average of three mechanical measurements $\times$ 5 preservation periods $\times$ 3 livers = 15 data points for each mechanical property). In the correlation analyses, the significance level is chosen as p=0.05 and the strength of the association between two data sets is evaluated based on the correlation coefficient (r_S) as very strong (VS, 1.0 $\geq |r_S| \geq$ 0.8), strong (S, 0.8 $> |r_S| \geq$ 0.6), moderate (M, 0.6 $> |r_S| \geq$ 0.4), weak (W, 0.4$> |r_S| \geq$ 0.2) or very weak (VW, 0.2 $> |r_S| \geq$ 0). Additionally, two-way ANOVA test is used to investigate the effect of the preservation period, solution, and

their interaction on the mechanical properties of the liver. Then, Bonferroni corrected paired t-tests are performed to further assess the statistical differences between the individual groups.

6.2 Results

The correlation coefficients and the strengths of the correlations between the mechanical and histological properties of the liver samples preserved for 11 h - 53 h in Ringer, HTK and UW solutions are tabulated in Table 6.1, respectively. The results of the correlation analyses show that the apoptotic cell count is strongly correlated with the amount of connective tissue, but not correlated with the other parameters for the samples stored in Ringer and HTK solutions. Also, the apoptotic cell count is moderately correlated with the dynamic mechanical properties at low frequencies, strongly correlated with the dynamic mechanical properties at high frequencies, and moderately correlated with the quasi-static mechanical properties for the samples stored in UW solution. The connective tissue accumulation is strongly/very strongly correlated with the quasi-static and dynamic mechanical properties for the samples preserved in Ringer and UW solutions. But, there is no correlation between these parameters for the samples stored in HTK solution. The sinusoidal dilatation is moderately/strongly correlated (negative) with the glycogen level for the samples stored in all preservation solutions. The sinusoidal dilatation is not correlated with the quasi-static and dynamic mechanical properties of the samples stored in Ringer solution. However, it is correlated with the loss modulus at low frequency and tangent shear modulus at high strain for the samples stored in HTK solution. Also, it is moderately/strongly correlated with the mechanical properties stored in UW solution. The glycogen level is moderately/very strongly and strong/very strongly correlated (negative) with the mechanical properties for the samples stored in Ringer and UW solutions, respectively. Nevertheless, it is not correlated with the mechanical properties of the samples stored in HTK solution. Generally speaking, the quasi-static and

dynamic mechanical properties are very strongly correlated with each other for the samples stored in all solutions.

6.3 Discussion

We investigated the relation between the mechanical and histological properties of liver tissue as a function of preservation period and solution. The previous studies investigating the relation between the mechanical and histological properties of the liver tissue have mainly focused on the correlation between the stiffness of the diseased liver tissue at a certain frequency and its fibrosis score. Ozcan et al. [47], Mazza et al. [35], Sandrin et al. [52], Yeh et al. [73], Manduca et al. [32], Huwart et al. [24], Mori et al. [38], and Yarpuzlu et al. [72] have already observed a positive correlation between the accumulated connective tissue and an increase in liver stiffness. We performed a more in-depth study and investigated the correlation between histological properties (number of apoptotic cells, amount of connective tissue accumulation, degree of sinusoidal dilatation, and level of glycogen) and mechanical properties (storage and loss moduli, and the tangent shear modulus) as a function of both preservation solution and period. Our results show that the apoptotic cell count, which is directly related to the grafts survival, is correlated with the mechanical properties of the liver tissue stored in UW solution. The depletion of the glycogen depositions, which is an early indicator for future cell deaths, is correlated with the mechanical properties of the liver tissue stored in Ringer and UW solutions. Also, the accumulated connective tissue, which is used to determine the stage of liver fibrosis, is solely correlated with the mechanical properties of the liver tissue stored in Ringer and UW solutions. Furthermore, the sinusoidal dilatation is moderately correlated with the dynamic mechanical properties, and strongly correlated with the quasi-static mechanical properties of the samples stored in UW solution. The results of our analyses demonstrated that the correlation between the inspected properties is solution-dependent. For instance, for the liver samples preserved in UW solution, both the histological and the mechanical

properties change with respect to the preservation period. Therefore, the correlations between the histological and the mechanical properties are strong. However, when the liver tissue is preserved in HTK solution, the mechanical properties are preserved well and do not alter much, but the histological properties (except the connective tissue) alter significantly as a function of preservation period. Hence, the correlations between the mechanical and histological properties are mostly weak. In summary, these results suggest that the preservation solution must be taken into consideration while developing a diagnostic technique for the examination of the transplant suitability based on the correlation between the material and pathological properties.

Finally, we suggest an upper limit for the preservation period based on the statistical analyses performed on the mechanical and histological properties. For each preservation solution, we examined the statistical differences between the measurements performed at different preservation periods. The results reveal that UW solution preserves the histological properties of the liver tissue effectively for 29 h considering the apoptotic cell death and glycogen storage, 17 h considering the amount of the connective tissue and 53 h considering the sinusoidal dilatation. HTK solution preserves the histological properties of the liver tissue about 11 h considering the level of apoptosis and glycogen deposition, 53 h considering the connective tissue and 17 h considering the amount of sinusoidal dilatation. Also, it is seen that Ringer solution preserves the histological properties of the liver tissue about 11 h considering the apoptotic cell death and glycogen storage, 17 h considering the connective tissue, and 29 h considering the amount of sinusoidal dilatation. Also, based on the statistical analyses, the mechanical properties of the liver tissue are most effectively preserved in UW solution in the short run (about 29 h considering the storage and loss moduli and about 11 h considering the tangent shear modulus). In contrast, the mechanical properties of liver tissue are most successfully preserved in HTK solution in the long run (between 29-53 h considering the storage, loss, and tangent shear moduli). Moreover, these results also suggest that Ringer solution is the least effective solution

for preserving the mechanical properties of the liver tissue (11 h considering the dynamic and quasi-static mechanical properties). To sum up, our results suggest that if the preservation time is expected to be shorter than 11 h, any of the three solutions (Ringer, HTK or UW) can be used safely. If the preservation period is expected to be shorter than 29 h, the use of UW solution is suggested. However, if the preservation period is expected to be longer than 29 h (but shorter than 53 h), HTK solution is a better option owing to its superiority in preserving the mechanical properties of the liver samples. The last observation was also supported by the small change in the connective tissue accumulation of the same samples preserved in HTK solution. In general, our results are in good agreement with the results of the earlier studies. Several studies have reported that there are no major differences between the solutions when the preservation period is short ([17] within 10 h; [15] within 15 h; and [61] within 16 h). However, if the preservation period is longer, UW solution has shown to be superior to the other preservation solutions ([61] after 16 h; [11] between 18-42 h; [65] after 24 h; [60] after 24 h; [5] after 24 h; [50] after 24 h; and [13] after 24 h).

Table 6.1: The correlation coefficients and the strength of correlation ($1.0 \geq |r_S| \geq 0.8$: "Very Strong (VS)"; $0.8 > |r_S| \geq 0.6$: "Strong (S)"; $0.6 > |r_S| \geq 0.4$: "Moderate (M)"; $0.4 > |r_S| \geq 0.2$: "Weak (W)", $0.2 > |r_S| \geq 0$: "Very Weak (VW)"; $p < 0.05$: "SI: Statistically Insignificant") between the mechanical and histological properties of bovine liver samples preserved for 5 h - 53 h in (Ringer, HTK, and UW) solutions.

Parameters		Apoptotic Cell [Count]	Connective Tissue [%]	Sinusoidal Dilatation [%]	Glycogen Level [%]	Storage Modulus [Pa]		Loss Modulus [Pa]		Tangent Shear Modulus [Pa]	
						ω=0.1 Hz	ω=10 Hz	ω=0.1 Hz	ω=10 Hz	γ =0.1%	γ=15%
Apoptotic Cell [Count]		-	0.75,0.62,SI	SI,SI,SI	SI,SI,SI	SI,SI,0.52	SI,SI,0.61	SI,SI,0.58	SI,SI,0.62	SI,SI,0.52	SI,SI,0.57
Connective Tissue [%]		S,S,SI	-	SI,0.58,0.77	SI,SI,-0.88	0.61,SI,0.69	0.60,SI,0.75	0.75,SI,0.73	0.61,SI,0.81	0.72,SI,0.83	0.59,SI,0.80
Sinusoidal Dilatation [%]		SI,SI,SI	SI,M,S	-	-0.6,-0.6,-0.7	SI,SI,0.51	SI,SI,0.56	SI,-0.53,0.52	SI,SI,0.61	SI,SI,0.69	SI,-0.61,0.65
Glycogen Level [%]		SI,SI,SI	SI,SI,VS	S,M,S	-	-0.77,SI,-0.74	-0.88,SI,-0.81	-0.76,SI,-0.80	-0.64,SI,-0.84	-0.58,SI,-0.84	-0.60,SI,-0.86
Storage	ω=0.1 Hz	SI,SI,M	S,SI,S	SI,SI,M	S,SI,S	-	0.88,0.71,0.93	0.93,0.91,0.95	0.89,0.91,0.93	0.77,SI,0.91	0.90,0.74,0.88
Modulus [Pa]	ω=10 Hz	SI,SI,S	S,SI,S	SI,SI,M	VS,SI,VS	VS,S,VS	-	0.9,0.72,0.99	0.81,0.71,0.99	0.73,SI,0.91	0.76,0.52,0.88
Loss	ω=0.1 Hz	SI,SI,M	S,SI,S	SI,M,M	S,SI,S	VS,VS,VS	VS,S,VS	-	0.95,0.95,0.98	0.80,SI,0.91	0.83,0.71,0.90
Modulus [Pa]	ω=10 Hz	SI,SI,S	S,SI,VS	SI,SI,S	S,SI,VS	VS,VS,VS	VS,S,VS	VS,VS,VS	-	0.80,SI,0.91	0.81,0.7,0.91
Tangent Shear	γ=0.1%	SI,SI,M	S,SI,VS	SI,SI,S	M,SI,VS	S,SI,VS	S,SI,VS	VS,SI,VS	VS,SI,VS	-	0.85,SI,0.91
Modulus [Pa]	γ = 15%	SI,SI,M	M,SI,S	SI,S,S	M,SI,VS	VS,S,VS	S,M,VS	VS,S,VS	VS,S,VS	VS,SI,VS	-

Chapter 7

CHARACTERIZATION OF THE VISCOELASTIC MATERIAL PROPERTIES OF LIVER

7.1 Materials and Methods

A Kelvin-Voigt fractional derivative model KVFDM is used to estimate the optimum viscoelastic material coefficients of liver tissue by minimizing the error between the experimental data and the corresponding values generated by the model using a non-linear least square fit algorithm. Since we measure the torsional shear stress, our KVFDM consists of a torsional spring and a torsional spring-pot element connected in parallel. It is capable of representing both time- (relaxation, creep) and frequency-(dynamic oscillation) dependent behavior of liver tissue. This model has been successfully utilized by other researchers in the literature for representing the viscoelastic behavior of soft tissues [28, 63, 70]. The constitutive equations for KVFDM in time and frequency domains are given in Equation 7.1 and 7.2. Here, V [Pa$\cdot$s$^{\alpha}$] is a unique quasi-property of the material, which shows the magnitude of the material response, $D^{\alpha}[\gamma_A(t)]$ is the fractional derivative of shear strain with the order of, where. Since, V contains a non-integer power of time, it is not a true material property, but a quasi-property [4, 26]. Quasi-properties are used to compactly describe the viscoelastic relaxation spectrum via only few parameters. Also, the spring-pot element can be described as the interpolation between a spring ($\alpha = 0$, $G = V$ $[Pa]$) and a dash-pot

($\alpha = 1$, $V = \eta$ [Pa $\cdot$ s$^{\alpha}$]). The meaning of the spring-pot element is better appreciated in vectorial representation as suggested in [26]. If both sides of the Equation 7.2 are divided by the shear strain $\gamma_A(\omega)$, the complex shear modulus $G^*(\omega)$, is obtained (Equation 7.3), which can be separated into real (storage modulus,) and imaginary (loss modulus, G_L) parts (Equation 7.4). Then, the tangent of the phase angle between stress and strain, δ, is calculated using Equation 7.5.

$$\tau_A(t) = G\gamma_A(t) + VD^{\alpha}[\gamma_A(t)] \tag{7.1}$$

$$\tau_A(\omega) = G\gamma_A(\omega) + V(i\omega)^{\alpha}\gamma_A(\omega) \tag{7.2}$$

$$G^*(\omega) = G + V(i\omega)^{\alpha} \tag{7.3}$$

$$G^*(\omega) = [G + V\cos(\frac{\pi\alpha}{2})\omega^{\alpha}] + i[V\sin(\frac{\pi\alpha}{2})\omega^{\alpha}] \tag{7.4}$$

$$\tan(\delta) = \frac{V\sin(\frac{\pi\alpha}{2})\omega^{\alpha}}{\mathrm{G{+}V}\cos(\frac{\pi\alpha}{2})\omega^{\alpha}} \tag{7.5}$$

In our approach, we utilize the storage (G_S) and loss (G_L) moduli data recorded by the DSL experiments to estimate the viscoelastic material coefficients of the KVFDM (G, V, and α) for each preservation solution and period.

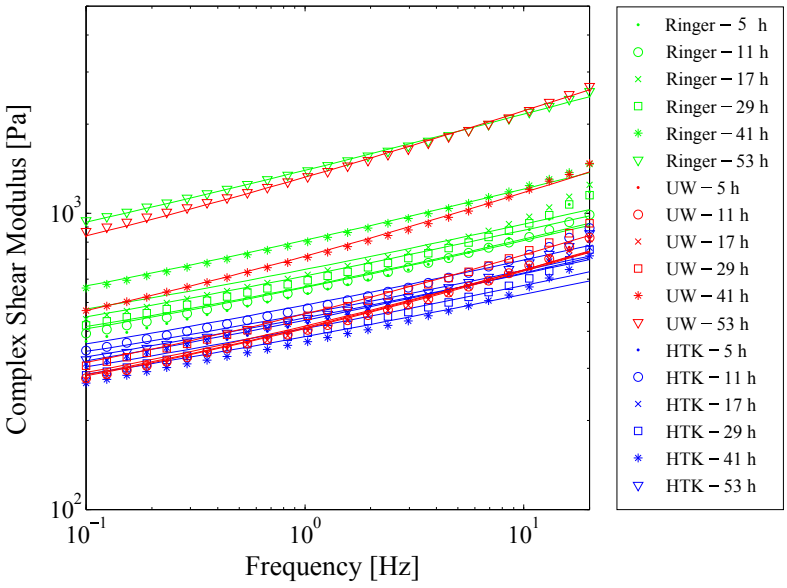
7.2 Results

We fit the KVFDM to the complex shear modulus data collected from the DSL experiments as a function of preservation solution and period (Figure 7.1a). The

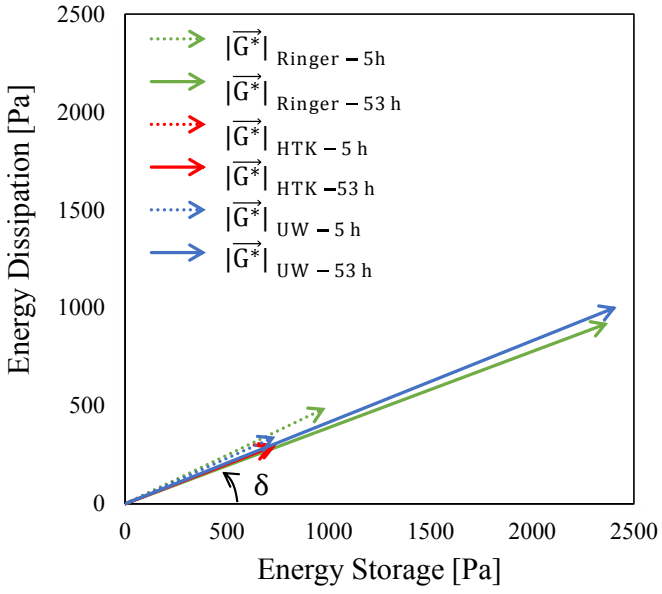
estimated viscoelastic coefficients (G, V, and α) and the coefficient of determination (R^2) for G^*, G_S, G_L are tabulated in Table 7.1. The vectorial representations of the curve-fit results for the liver samples preserved for 5 h and 53 h are given in Figure 7.1b. In this figure, the x- and y- axes represent the energy storage and dissipation capabilities of the liver samples, respectively. It is observed that as the preservation period increases from 5 h to 53 h, the magnitude of the complex shear modulus of the liver samples preserved in Ringer and UW solutions increase about 3-folds. However, those preserved in HTK does not change significantly. Figure 7.2 presents the change in the viscoelastic material coefficients of liver tissue as a function of preservation solution. Moreover, Figure 7.3 shows the phase angle as a function of frequency, preservation solution and period. The phase angle of the liver samples preserved in Ringer and UW solutions increase as a function of frequency and preservation solution, but those preserved in HTK solution show a small change.

Table 7.1: The viscoelastic material coefficients of liver tissue as a function of preservation period and solution (R^2 is the coefficient of determination).

Preservation Period [h]	Viscoelastic Parameter	Ringer	HTK	UW
5	G [Pa]	193	137	134
	V [Pa $\cdot$ s$^\alpha$]	253	205	182
	α	0.22	0.21	0.25
	R^2 (for G^*, G_S, G_L)	0.98, 0.97, 0.99	0.99, 0.99, 0.97	0.99, 0.99, 0.99
11	G [Pa]	154	153	138
	V [Pa $\cdot$ s$^\alpha$]	284	235	174
	α	0.20	0.20	0.26
	R^2 (for G^*, G_S, G_L)	1.00, 1.00, 0.98	0.99, 0.99, 0.97	0.99, 0.99, 0.99
17	G [Pa]	188	130	131
	V [Pa $\cdot$ s$^\alpha$]	321	221	177
	α	0.20	0.20	0.26
	R^2 (for G^*, G_S, G_L)	0.99, 0.98, 0.97	1.00, 0.99, 0.96	1.00, 0.99, 0.99
29	G [Pa]	153	112	148
	V [Pa $\cdot$ s$^\alpha$]	327	212	195
	α	0.19	0.19	0.27
	R^2 (for G^*, G_S, G_L)	0.99, 0.99, 0.97	0.99, 0.98, 0.94	1.00, 0.99, 0.99
41	G [Pa]	194	103	186
	V [Pa $\cdot$ s$^\alpha$]	422	202	333
	α	0.21	0.18	0.27
	R^2 (for G^*, G_S, G_L)	1.00, 1.00, 0.99	0.98, 0.98, 0.93	1.00, 1.00, 1.00,
53	G [Pa]	234	148	273
	V [Pa $\cdot$ s$^\alpha$]	786	217	658
	α	0.22	0.20	0.26
	R^2 (for G^*, G_S, G_L)	1.00, 1.00, 1.00	0.99, 0.98, 0.97	1.00, 1.00, 1.00,

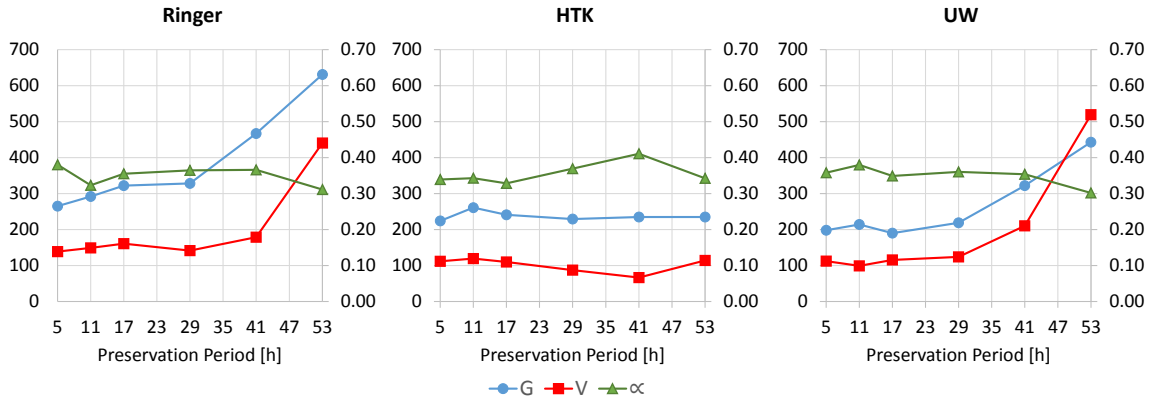


(a)



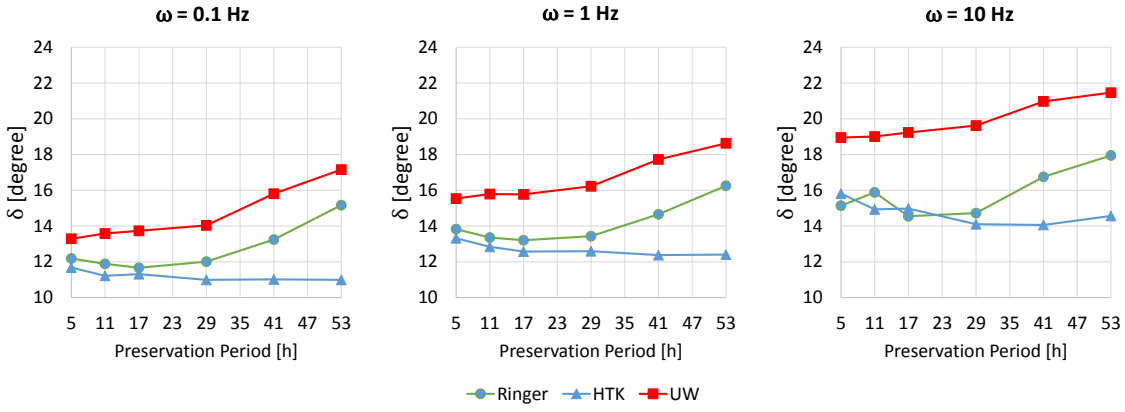
(b)

Figure 7.1: (a) The complex shear modulus of the bovine liver samples as a function of preservation period, solution and frequency. KVFDM is fit to the experimental data (solid lines). (b) The vectorial representation of curve-fit results for the liver samples preserved for 5 and 53 h. The y-axis represents the energy dissipation capability, while the x-axis shows the energy storage capability of the liver tissue.



(a) (b) (c)

Figure 7.2: The viscoelastic material coefficients of liver tissue preserved in (a) Ringer, (b) HTK, and (c) UW solutions.



(a) (b) (c)

Figure 7.3: The phase angle, as a function of preservation solution and period for the frequency of (a) 0.1 Hz, (b) 1 Hz, and (c) 10 Hz.

Chapter 8

CONCLUSION

8.1 Summary

We measured the mechanical properties of the bovine liver tissue stored in Ringer, UW and HTK solutions as function of preservation period using a shear rheometer. We found that the storage and loss moduli of liver tissue increase as it spends more time in the preservation solution. We observed that the largest increase in mechanical properties occur in the liver samples preserved in Ringer solution, while the smallest change occur in those stored in HTK solution. Also, the ratio between the loss and storage modulus (phase angle) increase for the bovine livers preserved in Ringer and UW solutions, while it stays almost constant for the liver samples preserved in HTK solution. In addition, we examined the histological properties of the same tissue samples as a function of preservation solution and period. We found that the number of apoptotic cells, the amount of connective tissue, and the amount of sinusoidal dilatation increase while the amount of glycogen deposition deceases as a function of increasing preservation period. These results provide an insight into the mechanism of tissue injury during cold ischemic preservation period. Since the blood is not supplied to the liver vessels during the cold ischemic preservation, the apoptosis is triggered, the connective tissue is produced as a control mechanism against the apoptosis, the sinusoids are dilated due to the venous flow impairment, and the glycogen supplies

are depleted due to the energy consumption.

We also investigated the relation between the mechanical and histological properties of the liver tissue. Our statistical analyses show that the preservation solution and period have significant effects on the material and histological properties of the liver tissue. The earlier studies have only investigated the histological properties of the liver tissue to investigate the preservation solutions and period. In our study, we analyzed both the mechanical and histological properties of the liver tissue simultaneously to investigate the extent of safe preservation in Ringer, UW and HTK solutions. Based on our statistical analyses, we conclude that the bovine liver tissue is preserved well in Ringer, UW and HTK solutions up to 11 h. After then, UW solution provides the best preservation up to 29 h. If a preservation period longer than 29 h is necessary, HTK solution is a better alternative since the material properties of the liver samples stored in UW solution changed drastically after 29 h.

8.2 Future Works

This work can be extended by investigating the following topics:

- Understanding the link between the mechanical and histological properties of human liver is the first step towards to the development of new protocols and medical devices for assessing the injury and suitability of the liver during the cold ischemic preservation. However, in this study, mechanical and histological properties of bovine liver were investigated. For practical purposes, the methods introduced in this thesis should be tested with human liver. In other words, the mechanical and histological state of the liver of transplant patients should be also investigated to check the feasibility of transferring the results obtained with bovine liver to human liver.

- The proposed methodology must be improved by further investigating the effect of preservation method (cold ischemic preservation vs. warm machine perfu-

sion), physical condition of liver (in vivo, ex vivo, and in vitro), and environmental conditions (preservation temperature, humidity, and hydration) on the measurements.

- The effect of measurement devices and methods on the measurements should be well-understood to collect reliable data. For example, in rheological experiments, some amount of compression is applied to ensure the full contact between the sample and the plates and hence to reduce the slippage. However, the effect of normal compression on the shear material properties of the viscoelastic solids (such as soft organ tissue) has not been investigated in detail.

- The proposed methodology can be improved by using a rheometer with an advanced imaging module. The imaging module can provide extra quantitative data to establish more accurate correlations between the mechanical and histological properties of liver. For example, Anton Paar, a leading manufacturer of rheological devices, has combined a rheometer with a light microscope (Rheo-Microscope) to allow the online visualization of the influence of shear deformation forces on the sample structure.

- The methodology proposed in this study can be utilized to investigate liver fibrosis. The evaluation of liver fibrosis is of great clinical interest. The early detection of liver fibrosis may lead to a more successful treatment. In fact, some cases of early fibrosis may be reversible with the elimination of the cause. Today, microstructural changes in the intracellular matrix (ICM) and extracellular matrix (ECM) of liver tissue during the initiation and progression of the liver fibrosis and cancer are still poorly understood. The correlations between the mechanical and pathological properties of human liver can be used to develop a mechanics based characterization of human liver for the evaluation of liver diseases. Hence, the proposed methods in this study are not only important

for the assessment of liver transplant but also for the evaluation of the liver diseases.

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