CORRELATION BETWEEN THE MECHANICAL AND HISTOLOGICAL PROPERTIES OF LIVER TISSUE

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This is to certify that I have examined this copy of a master's thesis by

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TABLE OF CONTENTS

ABSTRACT

In order to gain further insight into the mechanisms of tissue damage during the progression of liver diseases as well as the liver preservation for transplantation, an improved understanding of the relation between the mechanical and histological properties of liver is necessary. We suggest that this relation can only be established truly if the changes in the states of those properties are investigated dynamically as a function of elapsed “time”. In this regard, we first perform mechanical characterization experiments on three bovine livers to investigate the changes in gross mechanical properties (stiffness, viscosity, and fracture toughness) for the preservation periods of 5, 11, 17, 29, 41 and 53 hours after the harvesting. Then, histological examination is performed on the samples taken from the same livers to investigate the changes in apoptotic cell count, collagen accumulation, sinusoidal dilatation, and glycogen deposition as a function of the same preservation periods. Finally, the correlation between the mechanical and histological properties is investigated via Spearman’s Rank-Order Correlation method. The results of our study show that stiffness, viscosity, fracture toughness of bovine liver increase as the preservation period is increased. These macroscopic changes are very strongly correlated with the increase in collagen accumulation and decrease in deposited glycogen level at the microscopic level. Also, we observe that the largest changes in mechanical and histological properties occur after the first 11-17 hours of preservation.

ÖZET

Karaciğer hastalıklarının ilerleyişi süresince oluşan doku hasarlarının mekanizmaları ve aynı zamanda karaciğerin nakil amaçlı saklanması konusunda daha çok fikir sahibi olmak için, karaciğerin mekanik ve histolojik özellikleri arasındaki bağlantıları anlamak önemlidir. Bu bağlantıların doğru kurulabilmesinin ancak bu özelliklerin durumlarının değişiminin, zamanın bir fonksiyonu olarak dinamik bir şekilde incelenmesiyle sağlanacağını düşünmekteyiz. Bu amaç doğrultusunda ilk olarak üç dana karaciğeri üzerinde, bütün mekanik özelliklerini (sertlik, akışkanlık ve yırtılma direnci) incelemek amacıyla mekanik karakterizasyon deneyleri yapıldı. Bu deneyler, organlar elde edildikten sonraki 5, 11, 17, 29 ve 53 saatlik muhafaza sürelerinde tekrarlandı. Ardından, aynı karaciğerlerden örnekler alınarak, histolojik inceleme ile apoptotik hücre sayımı, kollajen birikimi, sinüzoidal dilatasyon ve glikojen depolarındaki değişimler, aynı muhafaza sürelerinin bir fonksiyonu olarak araştırıldı. Son olarak mekanik ve histolojik özellikler arasındaki korelasyon, Spearman'ın sıralama korelasyon metodu ile incelendi. Çalışmalarımızın sonucunda, dana karaciğerinin sertliği, akışkanlığı ve yırtılma direncinin, muhafaza süresiyle doğru orantılı olarak arttığı gözlendi. Makroskobik seviyedeki bu değişimlerin, mikroskobik seviyede kollajen birikimindeki artış ve depolanmış glikojen seviyesindeki düşüş ile güçlü bir korelasyon gösterdiği belirlendi. Ayrıca, karaciğerin mekanik ve histolojik özelliklerindeki en büyük değişimlerin 11 ve 17 saatlik muhafaza süreleri içerisinde gerçekleştiği görüldü.

LIST OF SYMBOLS

W	Strain energy function
I	Principle invariant
E	Linear elastic modulus
E_0	Short term elastic modulus
α_j	Relative modulus
τ_j	Time constant
du	Change in displacement
J	Fracture toughness
dA	Change in crack area
$d\Delta$	Change in strain energy
P	Frictional force
p	Significance level
r_s	Correlation coefficient

LIST OF ABBREVIATIONS

ECM	Extracellular Matrix
ICM	Intracellular Matrix
MRE	Magnetic Resonance Elastography
FE	Finite Element
LRS	Lactated Ringer's Solution
GMS	Generalized Maxwell Solid
H&E	Hematoxylene and Eosin
PAS	Periodic-Acid Schiff
TUNEL	Terminal Deoxynucleotidyl Transferase Nick End Labeling
RE	Relative Error
PT	Preservation Time
SI	Statistically Insignificant
SSI	Supersonic Shear Imaging
ST	Settling Time
AC	Apoptotic Cell
FT	Fiber Tissue
SD	Sinusoidal Dilatation
GD	Glycogen Deposition
HCV	Hepatitis C Virus

LIST OF FIGURES

LIST OF TABLES

Chapter 1

1. INTRODUCTION

Liver plays a major role in metabolism and acts as a source of energy for the body by storing glycogen. In addition, working with other systems and organs, it is responsible for several important functions such as storing iron, detoxifying harmful substances, maintaining the hormonal balance, producing bile to help with the digestion, regulating blood clotting, and producing immune factors to fight infections. The liver is prone to many diseases such as hepatitis, fatty liver, cirrhosis, and cancer. Liver fibrosis (scarring) is associated with major alterations in both the quantity and composition of ECM (extracellular matrix). Fibrotic liver contains more ECM than a healthy one, including fibronectin, undulin, elastin, laminin, hyaluronan, proteoglycans, and especially collagen fibers. In fact, liver fibrosis is considered as the net result of the imbalance between the collagen fiber synthesis and decomposition. When fiber synthesis is very active and the decomposition is suppressed, then apoptosis is induced and liver fibrosis advances. If the progression of disease becomes severe, then the liver failure occurs [1, 2].

Currently, the progression of liver disease is quantified by a liver biopsy, followed by a histological examination under a light microscope. Specific staining of ECM fibers is used to quantify the degree of liver fibrosis using computer-guided morphometric analysis. The liver biopsy is an invasive procedure with many disadvantages including the possibility of causing bleeding, allergic reactions, and renal failure on the patient. Additionally, the operation can be risky for patients who have blood disorders and congestive heart failures. Therefore, non-invasive measurement and diagnosis of liver diseases is highly desirable. One of the challenges in this regard is to establish a correlation between the material properties of liver measured non-invasively

and its histological states. Medical imaging techniques based on transient ultrasound elastography, called FibroScan [3, 4] and Magnetic Resonance Elastography, MRE, [5, 6] have been developed to quantify liver fibrosis non-invasively. In both approaches, the material properties measured at a certain frequency of stimulation are correlated with the fibrosis scores based on the histological examination. FibroScan and MRE measurements have demonstrated the increase in elastic modulus of liver tissue with an increase in fibrosis level. These measurements are performed externally without having any direct contact with the actual liver tissue, but the liver elasticity values reported in FibroScan and MRE studies are typically measured at a certain frequency only. Ozcan et al. performed invasive experiments with an impact hammer on 15 human livers, harvested from the patients having some form of liver disease, to investigate the frequency-dependent dynamic material properties of liver tissue as a function of liver fibrosis. They also observed an increase in elastic (storage) modulus of human liver as a function of increase in fibrosis level, characterized by histological scoring [7]. Mazza et al. conducted in vivo and ex vivo experiments with 10 human subjects having some liver pathology. Static mechanical properties were measured invasively on diseased liver segments using an aspiration device and fibrotic tissue is found 3 times stiffer than the normal tissue [8]. Egena et al. correlated the stiffness and viscosity of the bovine liver cells with fibrosis level. Presence of live and dead cells, and size distributions were measured. They suggested that tuning in liver stiffness could play an essential role in the control of primary liver tumors [9]. Lake et al. investigated the collagen alignment on the mechanical and structural behavior of soft tissues subjected to indentation. They observed that there is no significant difference between the mechanical peak responses, but there is a significant difference between stress relaxation responses of samples with different alignments [10]. In the studies discussed above, the material properties have been correlated with fibrosis scores to diagnose a disease or its severity. However, the histological scores do not really represent measurements of a continuous variable, but rather a degree of severity. Moreover, the terminology used in histological examination is not precise; the scoring mostly relies on

qualitative descriptions rather than quantitative measurements. Finally, there are problems in obtaining reproducible scores, since the process heavily relies on the expertise of the examiner [11]. A quantitative correlation between mechanical and histological properties of liver (rather than individual scores) is necessary to better understand the link between them, and hence the progression of liver fibrosis. In fact, the mechanobiology, an emerging field of study, aims to establish such links to gain further insight into the mechanisms of diseases and its prevention.

Another area where this insight can be helpful is the liver transplantation. The transplantation is the only treatment available today for severe liver failure. In this process, the diseased liver is replaced with a healthy one harvested from a donor. The liver harvested from a donor must be well preserved and then transported to the recipient immediately. Along this process, tissue damage occurs in the liver due to the drop in its temperature (hypothermia) and insufficient supply of blood to its vessels (ischemia). In order to preserve the liver during transportation, it is placed in a bag containing a chemical solution covered with ice. While the chemical solutions suggested in the literature for preserving a liver differ in components, they all aim to delay cell death (apoptosis), which is inevitable [12]. During apoptosis, morphological changes in ECM structure and cell shape such as shrinkage and bulging are observed. Additionally, due to the ischemia, the endothelial cells start to die, triggering hepatic sinusoidal dilatation. The glycogen stored in the tissue is consumed by the living cells to obtain additional energy during this preservation period, resulting in a decrease in the glycogen level of tissue [13, 14, 15]. All these changes in histology of liver can be detected via specialized stains and quantified by image processing tools under light microscope. However, there is no consensus among the experts on how long the preservation period must be. Again, investigating the changes in mechanical and histological properties as a function of preservation time and the correlation between them can provide insight into how long a liver can be preserved before transplanted to a recipient and how to design the chemical solutions to elongate the preservation period.

In summary, although there are studies available about the mechanical and histological properties of liver separately, the number of studies in the literature investigating the correlation between them is very limited. Moreover, the material properties have been correlated with fibrosis scores only in the earlier studies without paying attention to the dynamical changes in these properties as a function of time. In this study, we investigate the correlation between the changes in gross mechanical and histological properties of liver tissue as a function of preservation "*time*". This approach is inspired by the dynamical systems theory, where the continuous behavior of complex dynamical systems is investigated as a function of time.

Mechanical characterization experiments and histological examination are performed on 3 bovine livers 5, 11, 17, 29, 41 and 53 hours after harvesting. First, static indentation and ramp-and-hold experiments are performed on each liver with a cylindrical probe having a hemispherical tip to estimate its hyper-viscoelastic material properties for different preservation periods. Then, needle insertion experiments are performed on the same liver with a sharp probe to estimate its fracture toughness. A finite element (FE) model of bovine liver developed in ANSYS and an inverse analysis are used to estimate the material properties of each liver [16, 17]. To investigate the histological properties of the same livers in tandem, the tissue samples taken from the livers at different preservation periods are stained with different methods. They are labeled with Tunel detection apoptosis kit to count the number of apoptotic cells. Masson`s trichrome stain is used to measure the amount of collagen accumulation. Glycogen deposition is demonstrated with Periodic Acid Schiff reagent. Sinusoidal dilatation, another histopathologic change in liver, is examined on tissue sections stained with Hematoxylene & Eosin. Following the measurement of mechanical and the histological properties as a function of the preservation time, the correlation between them is investigated using Spearman's Rank-Order correlation method. Moreover, a sensitivity analysis is performed on the mechanical and histological properties of the liver to determine the critical period of preservation based on the largest change in the properties.

Chapter 2

2. MATERIALS AND METHODS

2.1 Preparation of Livers for Mechanical Characterization

In this study, experiments are performed on 3 bovine livers to investigate the relation between the mechanical and the histological properties of liver tissue as a function of preservation period. The livers are preserved in Lactated Ringer's solution at +4^{o}C immediately after the harvesting. The right lobe of each liver is detached from the whole liver with the help of a sharp knife and all the experiments are performed on this lobe (Figure 1).

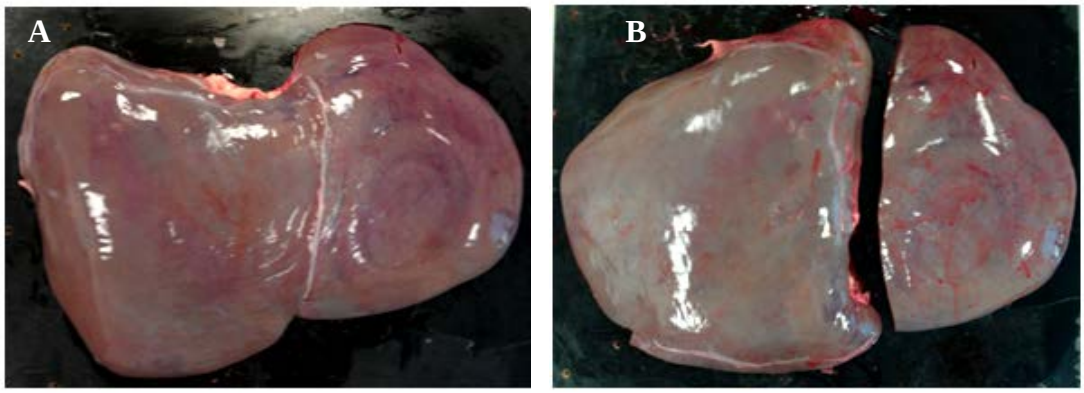

Figure 1 (A) The whole bovine liver and **(B)** its separated right lobe.

The transfer of the livers from the slaughterhouse to our laboratory took 4 hours and the preparation for the experiments took another hour following the transfer. Three

sets of mechanical experiments (static indentation, ramp-and-hold, and needle insertion) are performed on each liver 5, 11, 17, 29, 41 and 53 hours after harvesting using the experimental setup developed in our laboratory [18]. This setup consists of a step motor, a power screw, a moving shuttle on the power screw, a probe/needle on the shuttle, and a force sensor (Figure 2A). All experiments were performed in the central region of the right lobe of each liver. Attention is paid to stay away from the edges of the liver to reduce the boundary effects. Each experiment was repeated 5 times at different locations in close proximity.

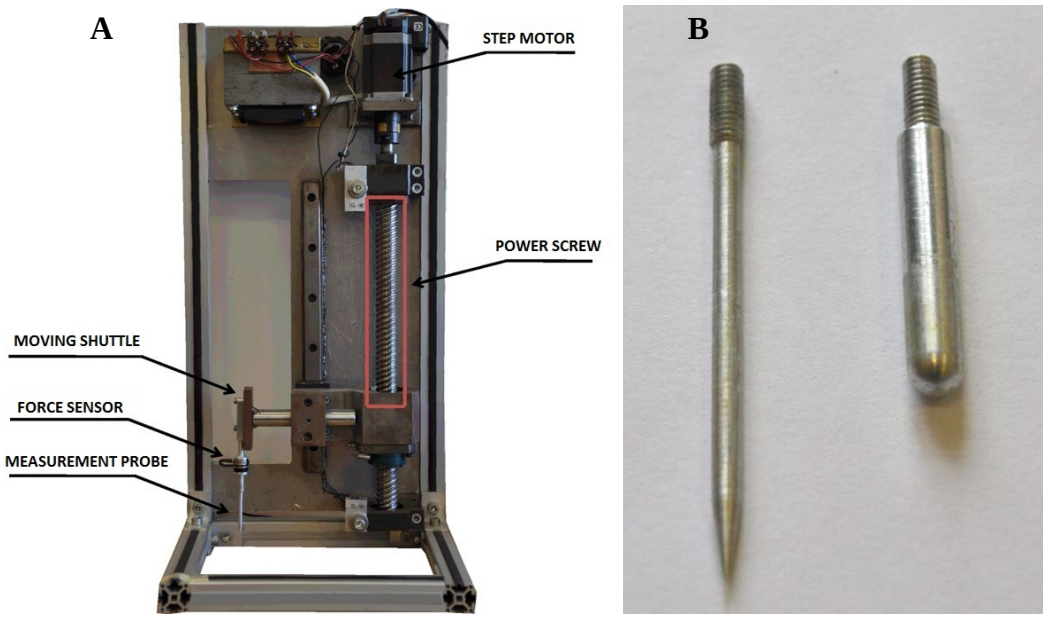

Figure 2 (A) Our set-up for conducting mechanical characterization experiments. **(B)** The needle (diameter = 3 mm) and the cylindrical probe having a round tip (diameter = 6 mm) used for the mechanical characterization experiments.

2.2 Static Indentation Experiments

Static indentation experiments are performed on the livers to investigate their strain-dependent hyperelastic material properties. Each liver is compressed to 20 mm depth with the aid of a cylindrical probe (Figure 2B) at a slow rate of 0.5 mm/s to minimize the dynamic effects. The force response of liver tissue is measured as a function of the compression depth (Figure 3).

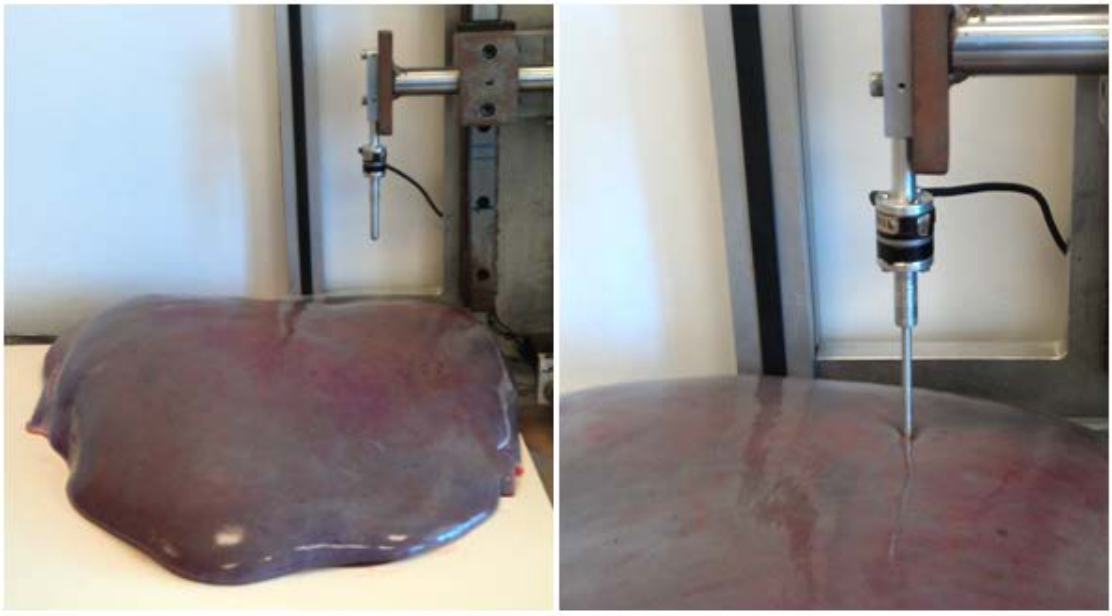

Figure 3 Scenes from the compression experiments performed on one of the livers tested in our study.

2.3 Ramp-and-Hold Experiments

Ramp-and-hold experiments are performed on the livers to investigate their time-dependent viscoelastic material properties. First, each liver is compressed to 20 mm depth at a rate of 48 mm/s using the cylindrical probe. Then, the probe is hold at that

position for 600 seconds and the force response of the liver tissue is measured as a function of relaxation time.

2.4 Needle Insertion Experiments

Needle insertion experiments are performed on the livers with a sharp needle to estimate their fracture toughness. The needle is penetrated into 20 mm depth with a rate of 3mm/s and the force response in normal direction is measured as a function of the penetration depth. Following a brief period of relaxation, the needle is retracted from the liver, only to be inserted once more into the same hole to measure the force response again. The fracture toughness is estimated by these two consecutive insertions using the energy-based fracture mechanics approach given in Gokgol et al. [17].

2.5 Characterization of Material Properties

Since the mechanical characterization experiments are performed with a cylindrical probe on the right lobe of the livers and not on samples with a fixed cross-section, it is not possible to obtain hyper-viscoelastic material properties directly from the measurements. For this reason, first, a FE model of liver is constructed in ANSYS from axisymmetric 2D elements having homogeneous, isotropic, hyper-viscoelastic, and nearly incompressible material properties and then, an inverse analysis is performed on the model to extract the material properties of the livers by inputting the measured experimental data [16, 17]. In order to reduce the number of FE computations, a two-dimensional FE model is preferred over a three-dimensional one and only the region around the contact is considered in the model, where the solution is assumed to be symmetric with respect to the axis of loading. The base of the FE mesh is constrained to have zero displacement (Figure 4).

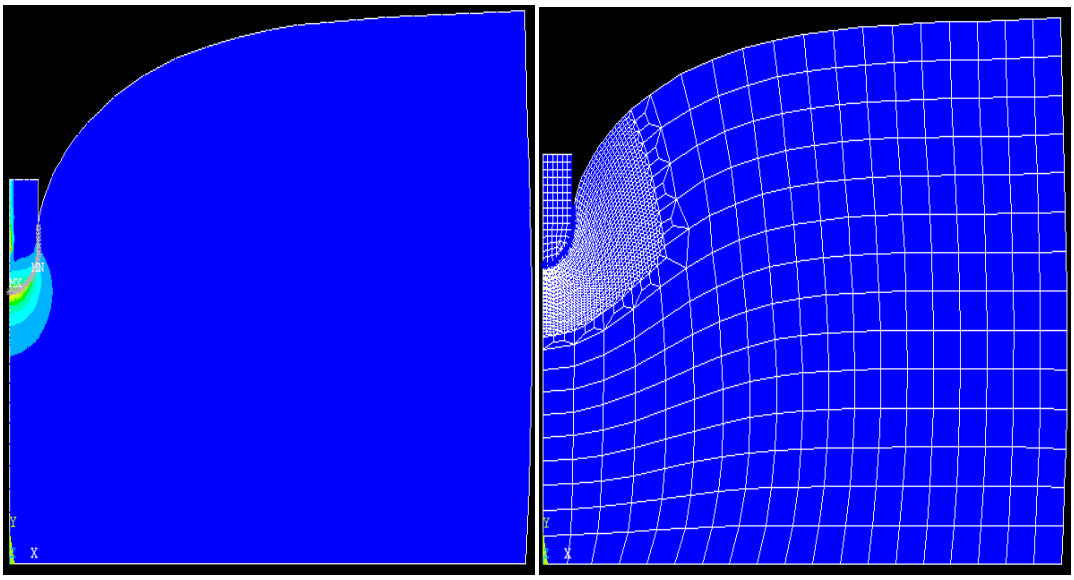

Figure 4 A finite element model of bovine liver deformed with a cylindrical probe.

The hyperelastic behavior of the livers is modeled using the polynomial strain energy function with N = 2:

$$W = C_{10}(I_1 - 3) + C_{01}(I_2 - 3) + C_{20}(I_1 - 3)^2 + C_{11}(I_1 - 3) + C_{02}(I_2 - 3)^2 \quad (1)$$

where, C_{10}, C_{01}, C_{20}, C_{11} and C_{02} are the material coefficients, and I_1 and I_2 are the invariants of the left Cauchy-Green deformation tensor.

A Generalized Maxwell Solid (GMS) is used to model the viscoelastic behavior of the livers [18]. Then, the time-dependent relaxation of the livers under ramp-and-hold strain input can be expressed analytically as:

$$E_R(t) = E_0\left[1 - \sum_{j=1}^{N} \alpha_j\right] + E_0 \sum_{j=1}^{N} \alpha_j e^{-t/\tau_j} \quad (2)$$

where, E_0 is the short-term elastic modulus, α_j represents the relative modulus, τ_j stands for the time constant, and N is the number of Maxwell arms used in the GMS model.

The hyperelastic material coefficients (C_{10}, C_{01}, C_{20}, C_{11} and C_{02}) and the viscoelastic material coefficients for N = 3 (α_1, τ_1, α_2, τ_2, α_3, τ_3) are determined via the inverse analysis through optimization iterations in ANSYS [16, 17]. The optimization algorithm minimizes the force error defined as:

$$Error = \sum_{j=1}^{M} \left(F_j^{EXP} - F_j^{FEM}\right)^2 \quad (3)$$

where, M represents the number of data samples used to represent the force curves, F_j^{EXP} is the experimental force value of the j^{th} sample, and F_j^{FEM} is the force value obtained from the FEM solution at the corresponding time step. The inverse solution is iterated until the total error in force is less than 0.1 N.

The fracture toughness of each bovine liver is calculated based on the energy-based fracture mechanics approach. The data (force versus penetration depth) collected via two consecutive needle insertions is used for the analysis [17]. The energy balance for the first insertion is:

$$F_1\, du = J\, dA + d\Delta + P\, du \quad (4)$$

where, F_1 is the force acting on the needle during the first insertion, du is the change in the needle displacement, J is the fracture toughness (material property), dA is the change in crack area, J dA is the fracture work, $d\Delta$ is the change in strain energy, P is the frictional force and Pdu is the work done by the friction. During the second insertion, the needle is inserted to the same spot and no rupture occurs. Hence, the energy balance for the second insertion is:

$$F_2\, du = d\Delta + P\, du \quad (5)$$

where, F_2 is the force acting on the needle during the second insertion, which is less than F_1. The change in strain energy, dΔ, and the work done by the friction, Pdu, are the same for the both insertions. The fracture toughness, J, is obtained by subtracting Equation 5 from Equation 4 as:

$$J = \int (F_1 - F_2) du \,/\, \int dA \qquad (6)$$

2.6 Histological Examination

Histological specimens are prepared from the parenchyma of the caudate lobe. Small tissue blocks, 3 mm in thickness, are obtained from each liver at different preservation periods for histological examination. The samples are fixed in 10% neutral buffered formalin for 24 hours at room temperature to preserve their structure. After the fixation, the samples are dehydrated by bathing them in a graded series of mixtures of ethanol and water. This is followed by a hydrophobic clearing agent (xylene) to remove the alcohol, and finally the infiltration agent (paraffin wax), which replaces the xylene. Then the samples in paraffin wax are heated in the oven at 60°C for 2 hours. Finally, tissue samples are embedded in paraffin. Samples from each specimen are sectioned at 7-10 μm in thickness by using Leica M72S microtome.

The sections are stained with three different stains for the histological examination: 1) Hematoxylene (Hematoxylene solution modified to Gill III, Merck) & Eosin (Eosin Y solution 0.5 % alcoholic, Merck) (H&E) is used for detection of the structural changes in sinusoids, 2) Masson`s Trichrome stain (Masson-Goldner Staining kit, Merck) is used to detect the changes in connective tissue and to demonstrate collagen accumulation, and 3) Periodic-Acid Schiff stain (PAS Staining kit, Merck) is used to visualize the changes in glycogen deposition in the cytoplasm of the liver cells.

Additionally, hepatocytes, which are undergoing apoptosis, are examined by using Apop Tag Plus Peroxidase Kit (Intergen S 7101, Millipore). This kit helps to

determine DNA fragmentation in the cells, which is known as TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) technique.

All treated sections are examined under Axiovision Zeiss light microscope. Microscopic images are captured from ten different areas on each section at 100X magnification (Carl Zeiss, Imager M1). Four measurement programs are designed by using AxioVision image analysis software (Zeiss). These programs help to count the nuclei of apoptotic cells (stained as brown/dark brown with Apo Tag Plus Peroxidase kit) and to measure the accumulation of collagen on the sections stained with Masson`s trichrome, the area of sinusoids on each section stained with H&E, as well as the amount of glycogen deposition in the cells on each section stained with PAS.

Chapter 3

3. RESULTS

3.1 Material Properties

The average force response of the three bovine livers measured for different preservation times as a function of compression depth are given in Figure 5A. The hyperelastic material coefficients of the livers (C_{10}, C_{01}, C_{20}, C_{11}, and C_{02}) estimated through the inverse FE solution are tabulated in Table 1. The linear elastic modulus (Young's modulus) of the livers for small strain (the last column in Table 1) is calculated from $E = 6\,(C_{10} + C_{01})$. The change in linear elastic modulus as a function of preservation time is shown in Figure 5B.

Table 1 The hyperelastic material coefficients and the linear elastic modulus of bovine liver (average of 3 animals) for different preservation periods.

Preservation Time (PT) [Hour]	C_{10}	C_{01}	C_{20}	C_{11}	C_{02}	Linear Elastic Modulus, E [kPa]
5	130.8 ± 65.5	130.2 ± 47.4	200.9 ± 78.6	387.1 ± 38.5	440.3 ± 148.7	1.57 ± 1.1
11	86.9 ± 39.1	196.4 ± 97	1063.3 ± 66.5	252.3 ± 194.3	584.6 ± 103	1.70 ± 1.1
17	447.7 ± 250.5	44.1 ± 22.1	1452.7 ± 312.7	413.8 ± 141.9	706.8 ± 155.9	2.95 ± 2.2
29	364.1 ± 78.4	953.5 ± 167.3	1001.8 ± 218.2	1004.0 ± 284.6	1061.0 ± 261.6	7.91 ± 1.8
41	2100.3 ± 92.1	1391.7 ± 206.9	725.8 ± 84	799.8 ± 125.1	579.1 ± 196.1	20.95 ± 2.9
53	4435.2 ± 263.3	1165.6 ± 488.5	72.2 ± 32.4	489.4 ± 92.6	606.0 ± 184.2	33.60 ± 3.7

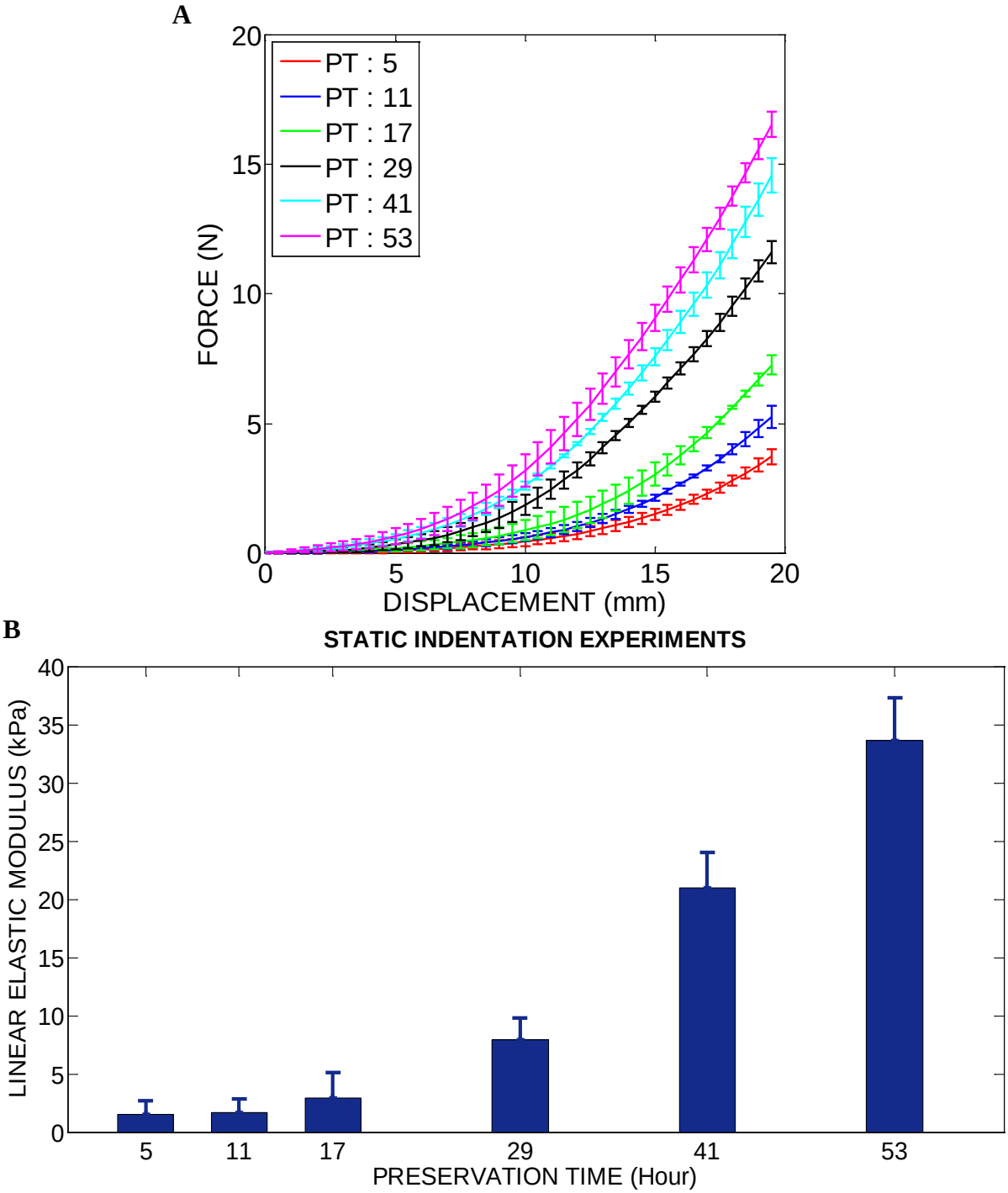

Figure 5 (A) The force response of bovine liver (average of 3 animals) as a function of compression depth for different preservation times. **(B)** The linear elastic modulus of bovine liver (average of 3 animals) as a function of preservation time.

The viscoelastic material coefficients of the livers (α_1, τ_1, α_2, τ_2, α_3, τ_3) estimated through the inverse FE solution are tabulated in Table 2. The settling time of the force response is estimated from the relaxation curve by defining a percent relative error as $RE = 100\,(F_R\,(t) - F_\infty)/\,F_\infty$. The relative error is chosen as RE = 5% in our estimation. The normalized force relaxation response of bovine livers and the change in settling time as a function of the preservation time are shown in Figure 6.

Table 2 The viscoelastic material coefficients and settling time of bovine liver (average of 3 animals) for different preservation times.

Preservation Time (PT) [Hour]	α_1	α_2	α_3	τ_1	τ_2	τ_3	Settling Time (ST) [Sec]
5	0.44 ± 0.03	0.07 ± 0.01	0.28 ± 0.02	6.65 ± 0.41	37.10 ± 3.75	58.80 ± 6.32	198 ± 3.8
11	0.42 ± 0.04	0.08 ± 0.02	0.36 ± 0.03	6.74 ± 0.77	46.65 ± 4.64	64.83 ± 1.50	261 ± 2.7
17	0.40 ± 0.02	0.08 ± 0.01	0.39 ± 0.02	6.75 ± 1.46	58.63 ± 5.08	66.39 ± 2.20	289 ± 4.6
29	0.37 ± 0.04	0.08 ± 0.02	0.37 ± 0.03	6.45 ± 1.22	65.29 ± 10.1	76.18 ± 5.87	295 ± 3.2
41	0.32 ± 0.01	0.06 ± 0.01	0.38 ± 0.09	6.95 ± 0.47	68.16 ± 7.8	84.23 ± 3.52	303 ± 5.1
53	0.30 ± 0.06	0.08 ± 0.01	0.37 ± 0.06	5.70 ± 0.62	54.87 ± 9.2	87.93 ± 12.53	309 ± 2.9

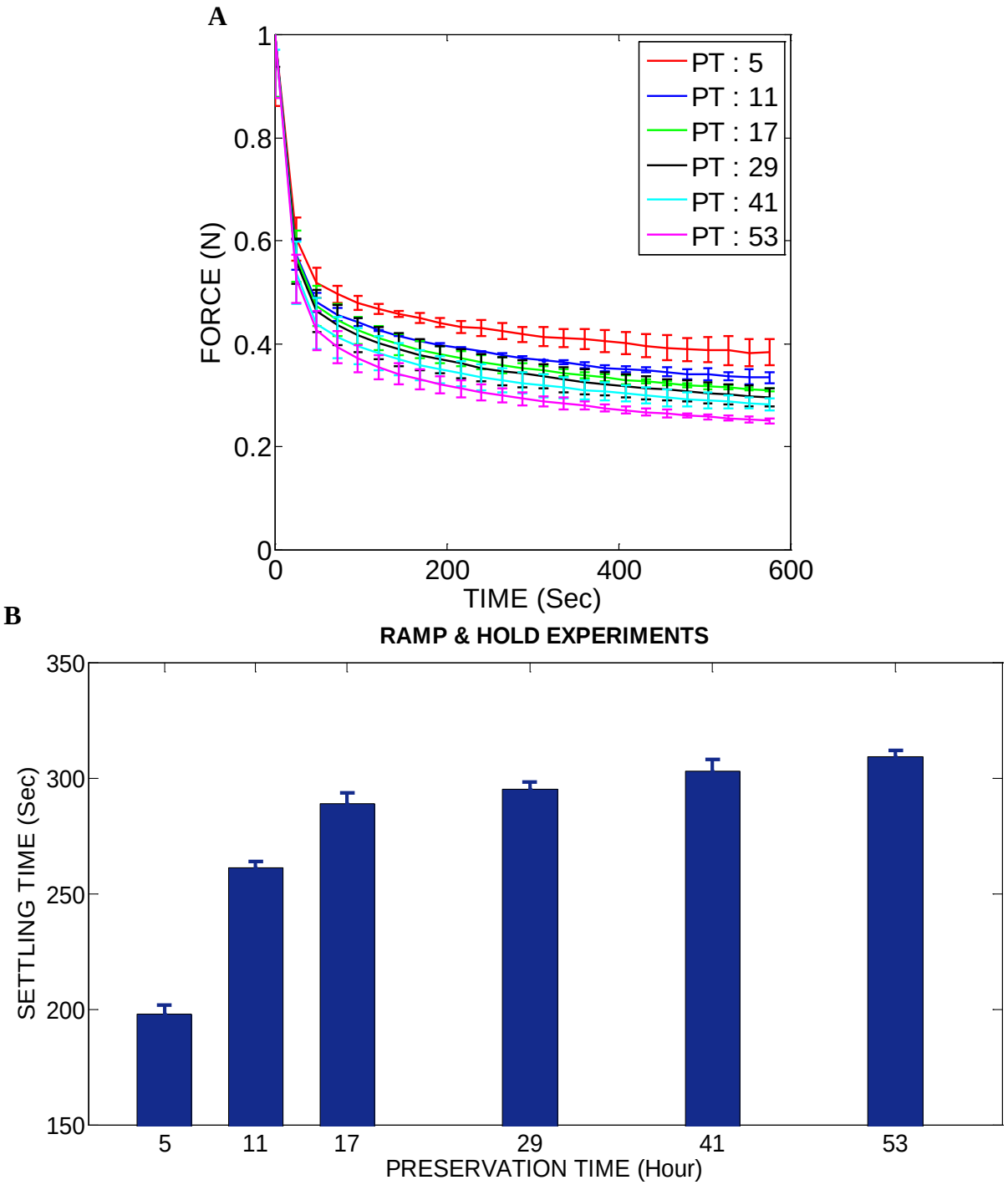

Figure 6 (A) The normalized force relaxation response of bovine liver (average of 3 animals) for different preservation periods. **(B)** The settling time of the relaxation response for different preservation periods.

The fracture toughness (J) of each liver is estimated from two consecutive needle insertions. The force displacement responses of the liver of Animal #1 during the first and second insertions are shown in Figure 7A. As shown in the figure, the curves are parallel following the initial rupture (see the sudden drop in force response in Figure 7A). The fracture toughness of the liver is estimated by first integrating this difference over the needle displacement and then dividing it by the crack area (the circumference of the needle times the penetration depth). The change in fracture toughness of bovine liver as a function of preservation time is plotted in Figure 7B.

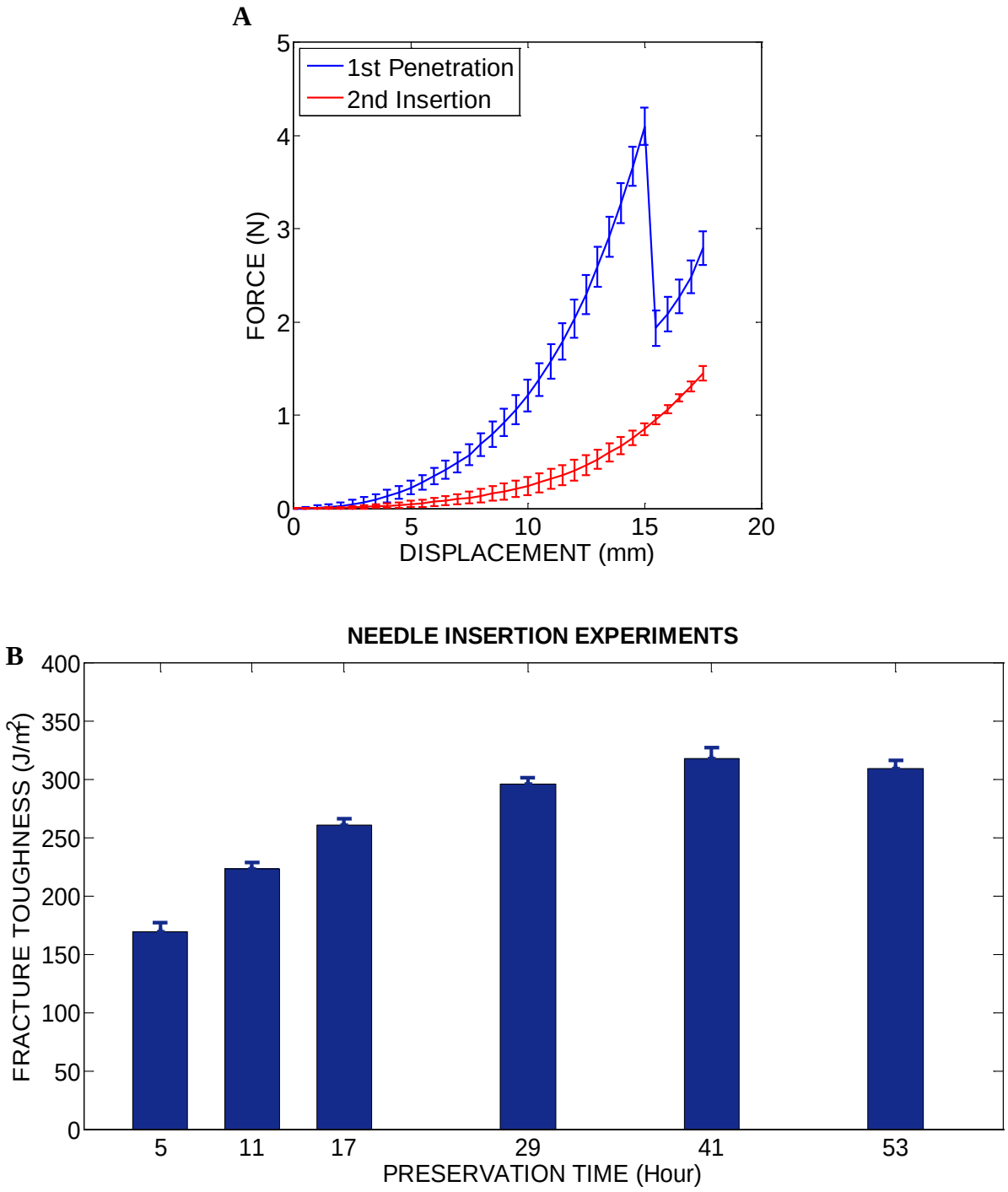

Figure 7 (A) The force displacement responses of the bovine liver of Animal #1 during the first and second needle insertions. **(B)** The change in fracture toughness of bovine liver (average of 3 animals) as a function of preservation time.

3.2 Histological Properties

The exemplar images of the sections stained as brown/dark brown with TUNEL technique at 5 and 53 hrs are showed in Figure 8A and 8C, and the images showing the cells marked on micrographs are given in Figure 8B and 8D. The changes in histological properties of bovine liver as a function of preservation period are tabulated in Table 3.

Apoptotic cells are counted in ten different areas. The change in the count of apoptotic cells of the bovine livers (average of 3 animals) as a function of preservation time is plotted in Figure 8E. The results show that apoptotic cell number increase significantly until 29 hour, and then remain similar.

Table 3 The histological properties of bovine liver (average of 3 animals) for different preservation times.

Preservation Time (PT) [Hour]	Apoptotic Cell (AC) [Count]	Fiber Tissue (FT) [%]	Sinusoidal Dilatation (SD) [%]	Glycogen Deposition (GD) [%]
5	12.93 ± 6	6.17 ± 2.8	28.34 ± 13.2	35.99 ± 7.9
11	25.5 ± 14.2	6.5 ± 2.9	30.82 ± 11	25.14 ± 9.8
17	36.96 ± 11	10.15 ± 4.5	30.34 ± 5.9	13.88 ± 9.4
29	47.53 ± 13.1	14.22 ± 6.7	37.1 ± 13.2	10.48 ± 8.4
41	44.43 ± 12.2	15.29 ± 7.6	32.33 ± 5.6	8.1 ± 7.63
53	43.96 ± 9.3	15.98 ± 6.3	38.92 ± 6.6	3.31 ± 1.9

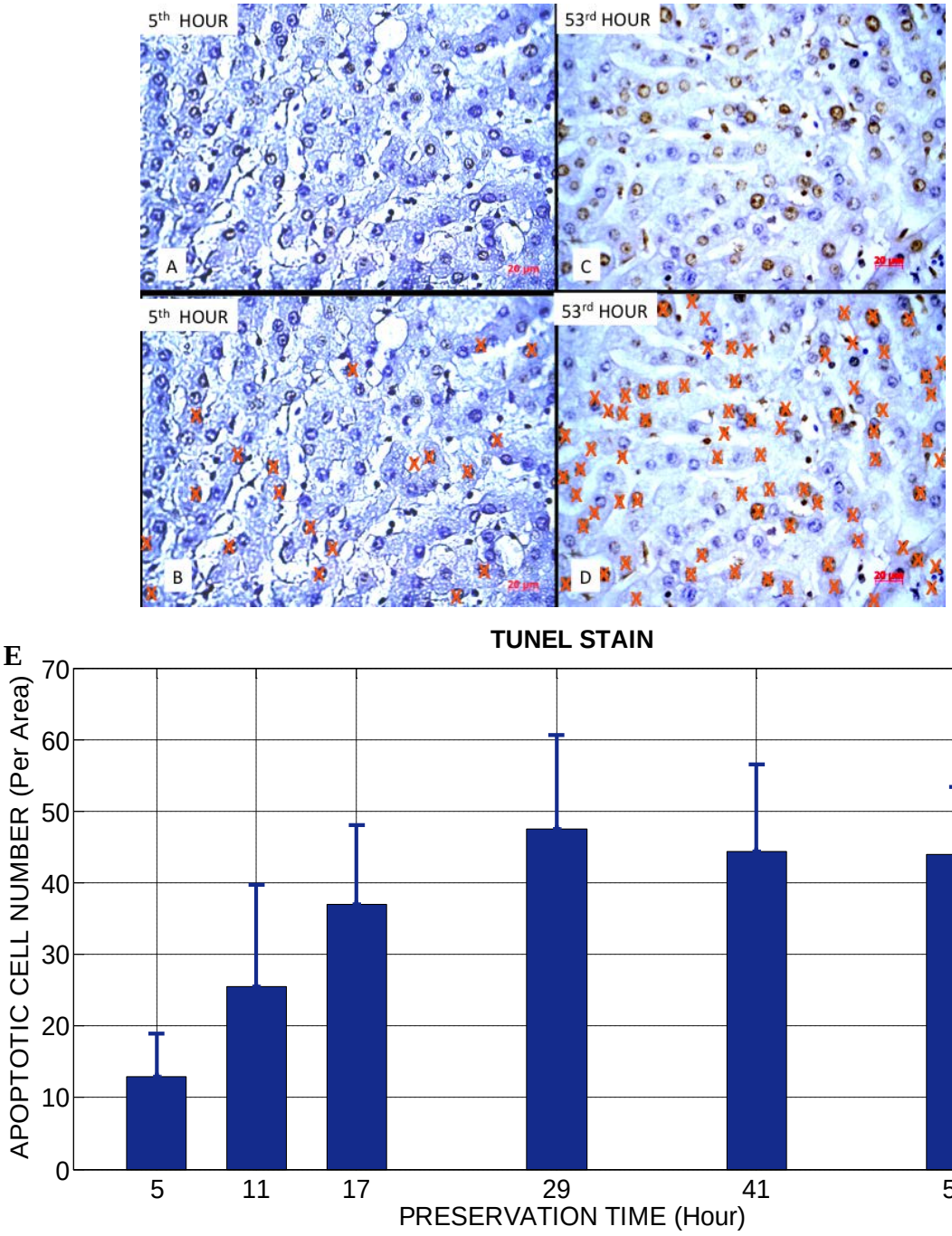

Figure 8 (A, C) The exemplar images of the sections labeled with TUNEL technique and preserved for PT = 5 hrs and PT = 53 hrs. The dark blue stained nuclei shows the healthy cells while the brown/dark brown stained nuclei shows the cells undergoing apoptosis. **(B, D)** These cells were marked on micrographs and then counted. **(E)** The change in the apoptotic cell count (average of 3 animals) as a function of preservation time.

Examination of Masson`s trichrome stained sections (green colored regions) reveal that connective tissue increases as a function of preservation time (Figure 9A and 9C). Using the image analysis software, the collagen accumulated regions are labeled first (Figure 9B and 9D) and their areas are measured quantitatively. The change in the connective tissue, especially accumulation of collagen, of the bovine livers (average of 3 animals) as a function of preservation time is plotted in Figure 9E.

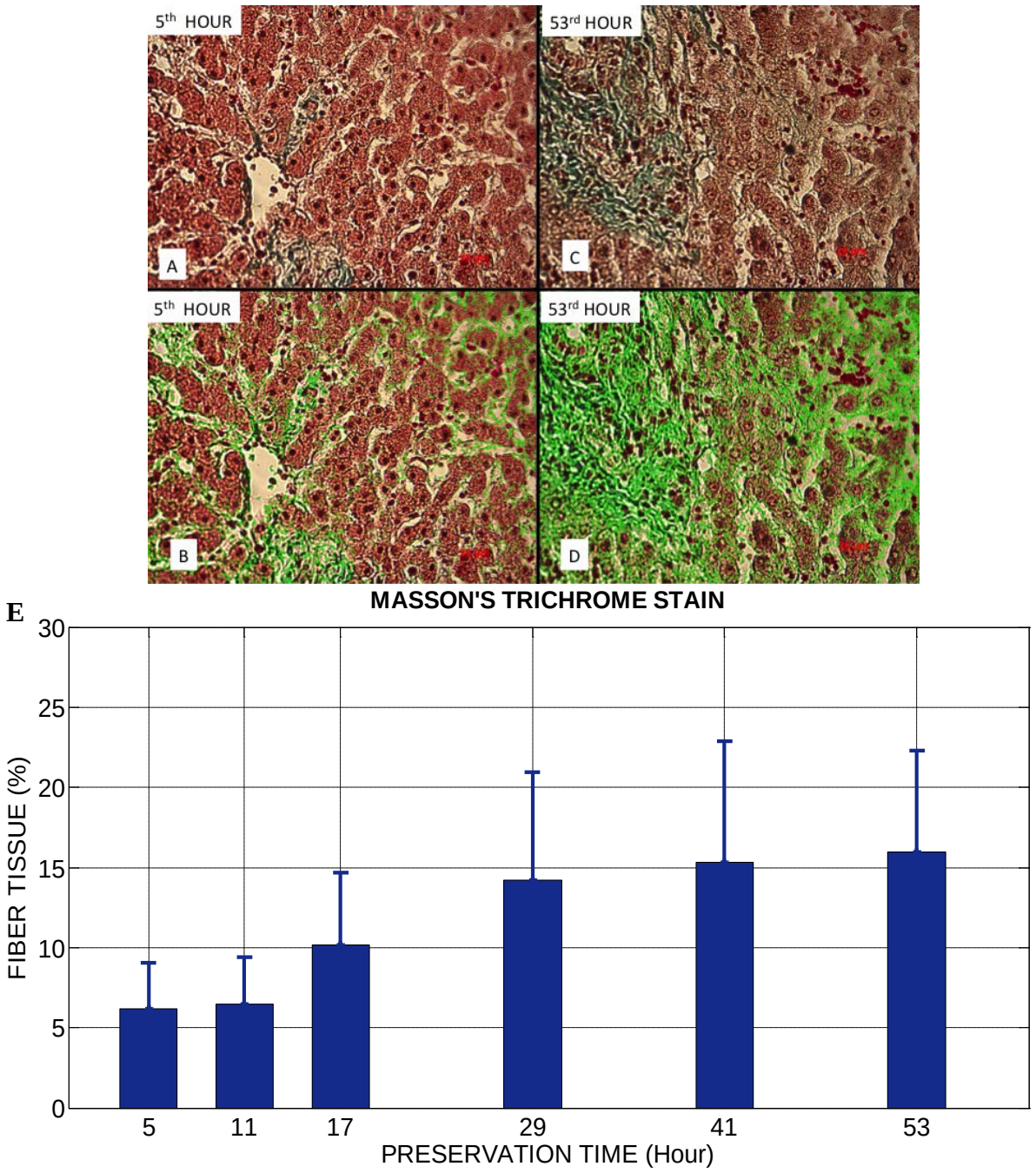

Figure 9 (A, C) The exemplar images of the sections treated by the Masson`s trichrome stain and preserved for PT = 5 hrs and PT = 53 hrs. **(B, D)** The software labels the green stained areas and then measures the accumulation of collagen. **(E)** The change in the connective tissue (average of 3 animals) as a function of preservation time.

The examination of 10 sections from each animal, which are stained with H&E (outlined with red lines), reveals that sinusoidal dilatation is observable in some areas (Figure 10A and 10C). Using the image analysis software, the borders of the sinusoids are outlined first, and then the areas enclosed by the borders (Figure 10B and 10D) are measured. The change in the sinusoidal dilatation of the bovine livers (average of 3 animals) as a function of preservation time is plotted in Figure 10E (the statistical analysis shows that the change is not statistically significant).

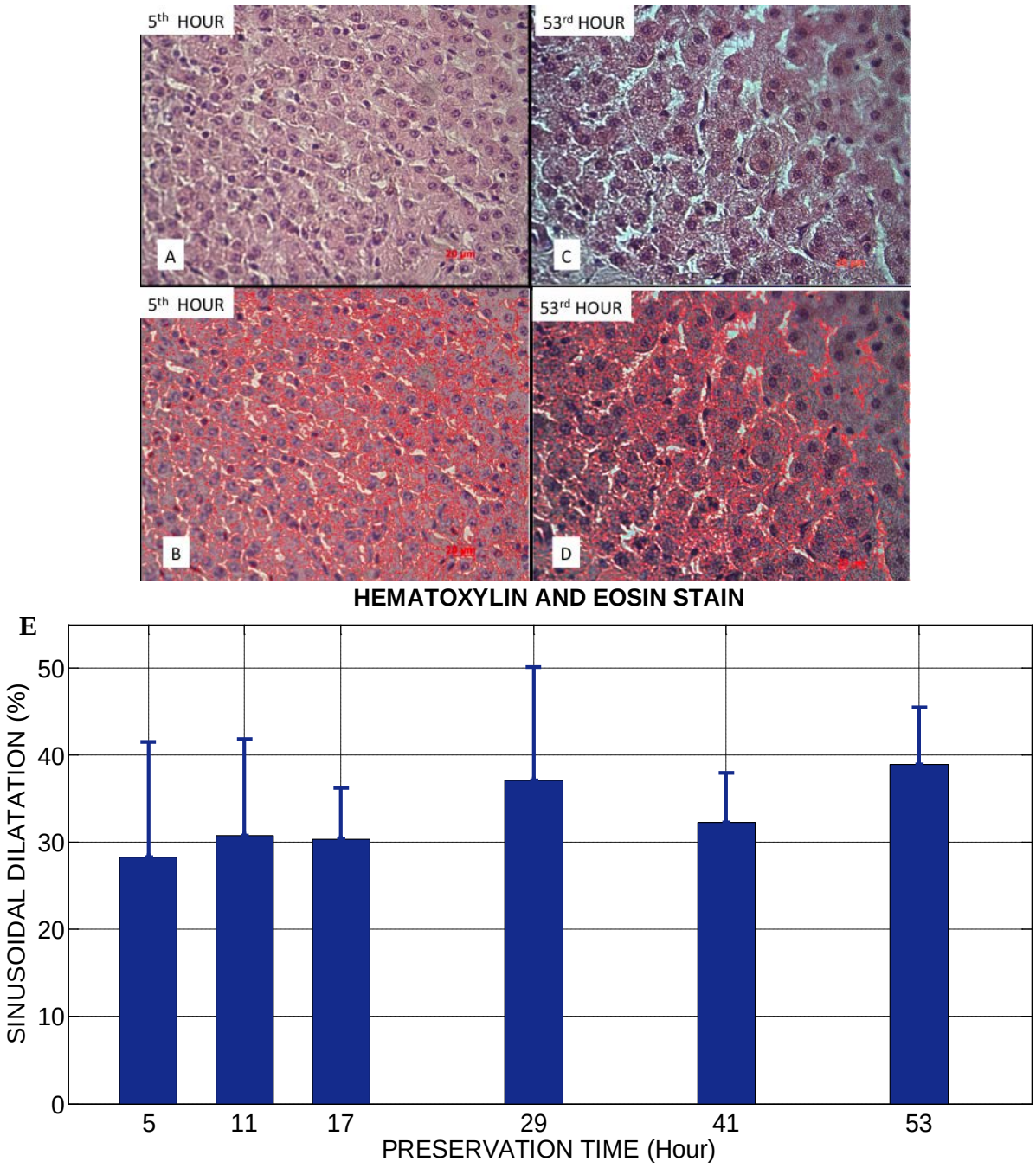

Figure 10 (A, C) The microphotographs show the sections stained with the Hematoxylene and Eosin and preserved for PT = 5 hrs and PT = 53 hrs. **(B, D)** The marked areas show the dilated sinusoids. **(E)** The change in the sinusoidal dilatation (average of 3 animals) as a function of preservation time.

Glycogen accumulation in cells has been demonstrated by using PAS stain (purple-magenta colored regions). It is clear that the glycogen accumulation in the cytoplasm of the cells preserved for PT = 53 hrs is significantly lower than that of PT = 5 hrs (Figure 11A and 11C). The image analysis software labels blue-magenta stained regions on these sections and returns these area (Figure 11B and 11D). The change in the deposited glycogen level in the cells of the bovine livers (average of 3 animals) as a function of preservation time is plotted in Figure 11E.

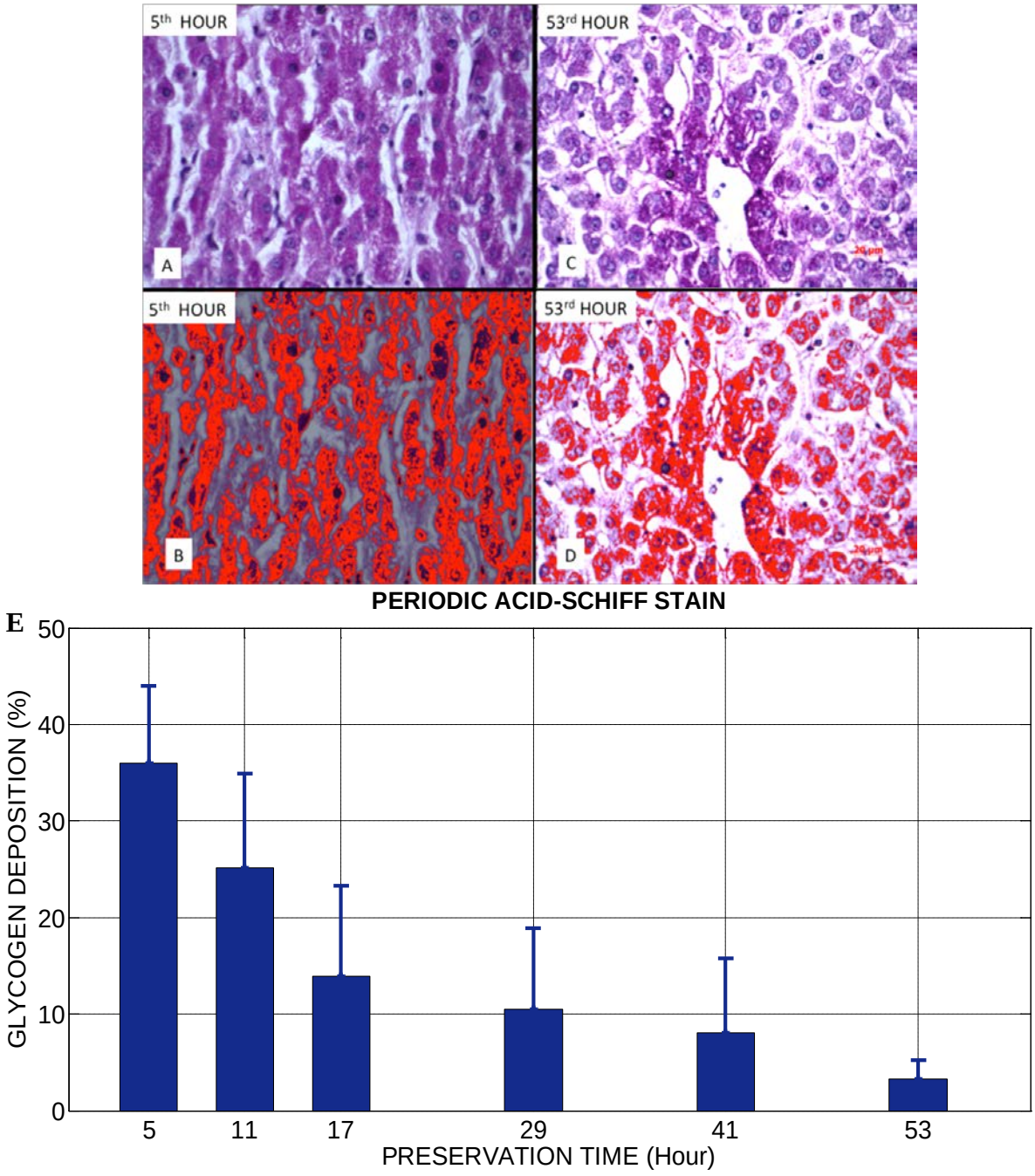

Figure 11 (A, C) The exemplar images of the sections stained with the Periodic Acid-Schiff and preserved for PT = 5 hrs and PT = 53 hrs. **(B, D)** The image analysis program labels the areas in the images and then measures the glycogen accumulation in the cells. **(E)** The change in the deposited glycogen level (average of 3 animals) as a function of preservation time shows dramatic change in glycogen deposition in the cells.

Following the characterization of material and histological properties, the correlation between them is investigated via the Spearman's Rank-Order Correlation method. Spearman's Rank-Order Correlation is a measure of a monotronic relationship between two data sets. The significance level is chosen as $p = 0.05$. The correlation coefficients, r_s, and the strength of correlation between mechanical and histological properties are tabulated in Table 4.

Table 4 The correlation coefficients, r_s, and the strength of correlation (0-0.19: 'very weak', 0.20-0.39: 'weak', 0.40-0.59: 'moderate', 0.60-0.79: 'strong', 0.80-1.0: 'very strong', SI: Statistically Insignificant) between the mechanical and histological properties of bovine liver.

Correlation Coefficient (r_s)	Apoptotic Cell Count	Connective Tissue	Sinusoidal Dilatation	Glycogen Deposition	Young's Modulus	Fracture Toughness	Settling Time
Apoptotic Cell Count	-	0.84	SI	-0.48	0.51	0.68	0.69
Connective Tissue	*Very Strong*	-	SI	-0.66	0.74	0.80	0.83
Sinusoidal Dilatation	SI	SI	-	-0.53	SI	SI	SI
Glycogen Deposition	*Moderate*	*Strong*	*Moderate*	-	-0.90	-0.92	-0.87
Young's Modulus	*Moderate*	*Strong*	SI	*Very Strong*	-	0.87	0.81
Fracture Toughness	*Strong*	*Very Strong*	SI	*Very Strong*	*Very Strong*	-	0.90
Settling Time	*Strong*	*Very Strong*	SI	*Very Strong*	*Very Strong*	*Very Strong*	-

Moreover, a sensitivity analysis is performed on the mechanical and histological properties of the liver to determine the critical period of preservation based on the largest change in the properties (Table 5). A normalized sensitivity measure is defined as the change in property per change in preservation time.

$$Sensitivity = \frac{(property[k+1] - property[k]) \,/\, property[k]}{(PT[k+1] - PT[k])} * 100 \quad (7)$$

where, "*property[k]*" is the k[th] value of a mechanical or histological property measured at "*PT[k]"*.

Table 5 The sensitivity values calculated for histological and mechanical properties.

Preservation Time (PT)	Apoptotic Cell Count	Connective Tissue	Sinusoidal Dilatation	Glycogen Deposition	Young's Modulus	Fracture Toughness	Settling Time
5	-	-	-	-	-	-	-
11	16.19 ± 1.75	0.87 ± 0.09	1.46 ± 0.16	-5.01 ± 0.55	1.49 ± 0.16	10.76 ± 1.19	5.30 ± 0.58
17	7.49 ± 0.83	9.35 ± 1.03	-0.26 ± 0.02	-7.46 ±0.82	12.26 ± 1.36	4.64 ± 0.51	1.78 ± 0.19
29	2.38 ± 0.26	3.34 ± 0.37	1.85 ± 0.2	-2.04 ± 0.22	13.98 ± 1.55	1.68 ± 0.18	0.14 ± 0.01
41	-0.54 ± 0.06	0.58 ± 0.06	-1.07 ± 0.11	-1.89 ± 0.21	13.75 ± 1.24	0.86 ± 0.09	0.25 ± 0.02
53	-0.08 ± 0	0.41 ± 0.04	1.7 ± 0.18	-4.92 ± 0.54	5.03 ± 0.55	-0.31 ± 0.03	0.16 ± 0.01

Chapter 4

4. DISCUSSION

The mechanical properties (stiffness, viscosity, and fracture resistance) of bovine liver increase as a function of preservation time and there is a '*very strong*' correlation among them (Table 4). The results of the static indentation experiments performed in this study show that the liver tissue becomes stiffer as it spends more time in the preservation fluid. The tangent elastic modulus of bovine liver varies between 1.6 kPa (PT = 5 hrs) and 33.6 kPa (PT = 53 hrs). Hence, our results suggest that bovine liver preserved in fluid for 53 hrs becomes almost 21 times stiffer than its initial state at 5 hrs. All these values are in line with the earlier findings in the literature. Chen et al. estimated the elastic modulus of bovine liver between 0.4-0.7 kPa using an ultrasound device and between 0.3-1.6 kPa using a mechanical tensile testing device [19]. Ocal et al. estimated the elastic modulus of fresh bovine liver as 4.1 kPa [18]. Liu and Bilston performed rheological experiments and reported the shear modulus of bovine liver as 0.6 kPa [20]. Brosses et al. examined the material properties of bovine liver using the SSI (Supersonic Shear Imaging) technique and estimated its shear modulus as 3.4 kPa [21]. The linear elastic modulus of porcine liver was estimated as ~10 kPa in the earlier studies [22, 23, 24, 25]. The elastic modulus of human liver was estimated as ~20 kPa by Nava et al. [26] and between 10-20 kPa by Ozcan et al. [7].

The results of our ramp and hold experiments show that the liver tissue becomes more viscous with time, which is in agreement with the results of the earlier studies [18, 27]. As the tissue becomes more viscous, it takes longer for it to relax and reach steady state, as shown in Figure 6A. Our results suggest that bovine tissue preserved in fluid for 53 hrs becomes 1.6 times more viscous than its state at 5 hrs (Figure 6B).

The results of the needle insertion experiments show that the fracture toughness of the liver increases up to PT = 29 hrs, and after that no significant change is observed. We found that the toughness values vary between 169 ± 7 J/m^2 (PT = 5 hrs) and 308 ± 6 J/m^2 (PT = 53 hrs). Hence, our results suggest that it is 1.8 times more difficult to cut or tear bovine liver tissue preserved in fluid for 53 hrs, when it is compared to its state at 5 hrs. These results are in good agreement with the earlier findings. Gokgol et al. estimated the fracture toughness of bovine liver as 164 ± 6 J/m^2 [17]. The fracture toughness of porcine liver was estimated to vary between 75.8 J/m^2 and 185.6 J/m^2 in Azar and Hayvard [28] and between 186.9 and 224.8 J/m^2 in Chanthasopeephan et al. [29].

In the histological examination, the changes in apoptotic cell count, collagen accumulation, sinusoidal dilatation and glycogen deposition in hepatocytes are investigated as a function of preservation period. Our results show ~4-folds increase in apoptotic cell count at PT = 29 hrs compared to PT = 5 hrs ($p < 0.05$), but no significant change is observed after PT = 29 hrs ($p > 0.05$). There are two experimentally distinguishable mechanisms of cell death: Apoptosis and necrosis. Apoptosis is the mechanism which affects individual cells Induced by physiological stimuli like environmental changes. Necrosis happens when cells are exposed to a more serious or sudden physical/chemical insult. Immersion of liver tissue in ringer lactate at cold temperature was used for preservation of the tissue before transplantation for many years. The main aim for usage of this solution was to cause minimum environmental changes for the cells. However, it is clear that every method, besides in vivo conditions, cause environmental changes at some degree and finally certain cells undergo apoptosis. We wanted to examine the dynamic changes in the tissue from the beginning and make quantitative analysis of the cells undergoing apoptosis because of these environmental changes. The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability. In current study, TUNEL has been chosen as a method since it is a highly sensitive labeling technique for demonstration of the

cells with DNA fragmentation. As a summary, the labeled cells with TUNEL are the cells undergoing apoptosis and finally would be dead. The number of these cells increases until 29th hours and then remains steady during the experimental period. It is clear that the liver cells would continue to die the longer the liver remains in cold storage. But this time, the main mechanism of cell death would be necrosis and number of necrotic cells would increase dramatically since the cells are facing with more serious conditions. TUNEL preferentially labels apoptosis in comparison to necrosis. We preferred to demonstrate cells undergoing apoptosis instead of necrotic cells to provide a more precise comparative dynamic study and did not show necrotic cells, which mostly appears at the end of the experiment. Natori et al. investigated the apoptosis of sinusoidal endothelial cells during cold preservation of liver. The results showed that the number of apoptotic cells have increased 6-folds after 24 hrs preservation [15]. Toom et al. investigated the effects of preservation solutions on the morphology of rat liver. They observed a significant morphological damage in sample cells preserved for 42 hrs compared to 18 hrs [30]. Malhi et al. suggested that apoptosis is a prominent factor of chronic liver diseases. It has also been reported that apoptosis stimulates inflammatory and fibrotic changes [31, 32]. Calabrese et al., investigated the apoptotic cell levels in hepatitis C virus (HCV) infection, and they found that the apoptotic cell index varies between 0.01% and 0.54% and the index increases with the level of infection [33].

Our results show that the accumulation of fibrous tissue at PT = 29 hrs is ~2.5 times higher than the value measured at PT = 5 hrs *($p < 0.05$)*, but no significant change is observed after PT = 29 hrs ($p > 0.05$). There is *'very strong'* correlation (Table 4) between the apoptotic cell count and the accumulation of fibrous tissue since the decrease in blood supply naturally triggers the programmed cell death and this controlled mechanism also involves the cells which produce connective tissue.

Our results show no significant change in sinusoidal dilatation as a function of preservation time. Jain et al. and Puhl et al. investigated the change in sinusoidal dilatation levels and modifications in endothelial cell structures during cold storage and

perfusion. They observed an increase in sinusoidal dilatation as a function of preservation time due to perfusion damage [14, 34].

Our results show a significant decrease (10 folds) in the glycogen level when PT = 5 hrs and PT = 53 hrs are compared ($p < 0.05$), which has a '*moderate*' negative correlation with the apoptotic cell count and a '*strong*' negative correlation with the accumulated connective tissue (Table 4). The glycogen is made and stored primarily in the healthy liver cells, and functions as the secondary energy storage. This result shows that certain percentage of hepatocytes could not synthesize and store glycogen since the deposited glycogen was consumed for the survival and then the cells underwent to apoptosis. Corps et al. investigated the cell viability in rat livers with different preservation solutions during cold ischemia. ATP, ADP and AMP degraded in 4 hours and the results showed a significant decrease in the glycogen levels [13]. Nowak et al. and Zaouali et al. investigated energy kinetics and glycogen-ATP contents and observed a decrease in glycogen levels as a function of preservation time [35, 36].

Table 4 shows that there is a strong relation between the mechanical and histological properties of the liver. The mechanical properties (stiffness, viscosity, and fracture resistance) are strongly correlated with apoptotic cell count (positive), very strongly with the connective tissue (positive) and the glycogen level (negative), and not correlated at all to the sinusoidal dilation. These results are also in agreement with the results of the earlier studies, which are limited in number. The correlation between the fibrous tissue and liver stiffness has been already utilized for the diagnosis of liver diseases [3, 4, 5, 6]. Mori et al. investigated the correlation between liver stiffness and collagen deposition levels with patients who have non-alcoholic fatty liver disease. They observed that not only the increase in the collagen level, but also the presence of myofibroblasts triggers the chronic liver diseases [37]. Leal-Egaña et al. investigated the effect of fibrous structure on the spread of liver cancer, and the mechanical property of liver. They suggest that the progression of liver cancer can be prevented by tuning the stiffness of liver by increasing the fibrous tissue in the organ [9]. Lake et al. investigated the effect of the initial collagen alignment on the mechanical properties of

soft tissues. The results showed that different initial alignments do not directly affect the strain dependent elastic response, but have an influence on the time-dependent relaxation response [10].

Investigating the correlation between mechanical and histological properties not only provides insight into liver damage during disease progression but also during liver preservation for transplantation. During the liver transplantation, the donor and the recipient are mostly in different locations, so the preservation conditions and the transportation duration are both very important. Preservation solutions are designed to inhibit the negative effects of ischemia and to continue the tissue viability. However, even the most effective solutions can preserve the organ up to certain duration only, though there is no consensus among the physicians on how long this period should be. In this study, we investigated the change in material and histological properties of bovine liver during cold storage. The sensitivity analysis performed based on the mechanical properties showed that the largest change in property occurs between 11-17 hrs. The sensitivity analysis performed based on the histological properties also supports this observation. Although the largest change in liver stiffness was observed after 17 hrs of preservation, the largest changes in viscosity and fracture toughness occurred after 11 hrs of preservation. The largest change in apoptotic cell count occurred after 11 hrs, and for collagen accumulation and glycogen deposition, the largest change was observed after the first 17 hrs.

Chapter 5

5. CONCLUSION AND FUTURE WORK

In order to better understand the damage occurring in liver tissue during disease progression and preservation, the relation between the mechanical and histological properties of liver must be investigated in depth. However, the number of studies in the literature investigating the relationship between the mechanical and histological properties of liver is very limited. Most of the earlier studies in this area have focused on the measurement of one mechanical property (elastic modulus) at a certain frequency, and then finding its correlation with semi-quantitative histological scores. We suggest that the liver damage during disease progression (or during liver preservation) can only be understood truly if the relation between the states of mechanical and histological properties is investigated as a function of time as it is done in dynamical systems theory to investigate the behavior of complex systems. Our results show that stiffness, viscosity and fracture toughness of the bovine livers increase as a function of preservation time. Moreover, the number of apoptotic cells and the connective tissue around cells increase while the deposited glycogen level decrease as a function of preservation time. Finally, the changes in stiffness, viscosity and fracture toughness of the livers are strongly correlated with the changes in the accumulated connective tissue (positive) and the deposited glycogen level (negative).

Our study, in addition to investigating the changes in mechanical and histological properties and the correlations between them, also provided insight into how long the liver should be preserved in a Lactated Ringer solution. Our results show that the largest changes in mechanical and histological properties occur after the first 11-17 hrs of preservation. With the insight gained in this study, we plan to further

investigate the effect of most commonly used preservation solutions (University of Wisconsin and HTK) on the mechanical and histological properties of liver and the correlations between them. Additionally, we plan to examine the tissue samples under an electron microscope. Through this examination, more insight can be gained not only for the changes in ECM, but also for the changes occurring at molecular level in intracellular matrix (ICM).

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