INJECTION OF RAT MESENCHYMAL STEM CELLS LEADS TO HOMING AND DIFFERENTIATION IN THE LIVER IN A BLUNT LIVER TRAUMA MODEL

Mostafa Alhabboubi

Department of Experimental Surgery,

McGill University, Montreal, Canada

Submission date: July 2020

"A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Experimental Surgery"

Table of Contents

Abstract

Background:

The liver heals remarkably after different forms of injuries. However, healing time can be lengthy following high-grade blunt injuries. We hypothesize that injected bone marrow derived mesenchymal stem cells (MSC) could locate and differentiate to hepatocytes after blunt trauma using a rat liver trauma model.

Methods:

Blunt liver trauma was induced to Lewis rats. MSC were extracted from Lewis rats' femurs and transfected with LacZ retrovirus so that they express B-galactosidase enzyme, giving their nuclei a blue color on light microscopy. Each rat received a single dose of BMDS ($n=6 \times 10_6$) within 24 hours of trauma. Through different steps of the experiment, route of injection was the tail vein (TV) in 9 rats, the portal vein (PV) in 19 rats and directly to the injured liver (DI) in 6 rats. Rats were euthanized at 2, 7 days after injection of MSC. Livers were harvested and examined under light microscopy to identify the MSC.

Results:

Liver sections showed localization and active migration of MSC to trauma sites in the PV group euthanized at 48 hours (3/10 rats). Furthermore, some stem cells differentiated to hepatocytes. Although with fewer cells, similar findings were present in 1/9 rats euthanized at 7 days in the PV group. There was no evidence of MSC localization in TV and DI groups.

Conclusions:

MSC can locate and differentiate to hepatocytes at blunt trauma site and may contribute to liver regeneration process. Portal vein injection of MSC has emerged as the most effective method of delivery to the liver following trauma among different delivery methods studied. This technique has the potential to become an effective therapeutic strategy to improve liver regeneration after severe blunt trauma. Methods of optimizing homing to injured tissue and evaluation of differentiated stem cell functionality are future areas of research.

Résumé

Contexte :

Le foie guérit parfaitement bien après différentes formes de lésions. Cependant, le temps de guérison peut être long à la suite des lésions contondantes plus sérieuses. Nous émettons l'hypothèse que la moelle osseuse injectée dérivée des cellules souches mésenchymateuses (CSM) pourrait se localiser dans le foie et s'y différencier en hépatocytes à la suite d'un traumatisme contondant à l'exemple du modèle de traumatisme hépatique du rat.

Les méthodes :

Un traumatisme hépatique a été provoqué chez le rat Lewis. Les CSM ont été extraites des fémurs de rats Lewis et transfectées avec le rétrovirus LacZ afin qu'elles expriment l'enzyme B-galactosidase, conférant à leurs noyaux une couleur bleue en microscopie optique. Chaque rat a reçu une dose unique de BMDS (n = 6 x 106) dans les 24 heures qui suivent le traumatisme. Au cours des différentes étapes de l'expérience, les voies d'injections ont été la veine de la queue (TV) chez 9 rats, la veine porte (PV) chez 19 rats et directement le foie lésé (DI) chez 6 rats. Les rats ont été euthanasiés après 2 jours et après 7 jours à la suite de l'injection des CSM. Les foies ont été prélevés et examinés au microscope optique pour identifier les CSM.

Résultats :

Des coupes de foie ont montré une localisation et une migration active des CSM vers les foyers de traumatismes dans le groupe PV euthanasié après 48 heures (3 rats sur 10). De plus, l'observation révèle que certaines cellules souches se sont différenciées en hépatocytes. Bien qu'avec moins de cellules, des résultats similaires ont été observés chez 1 rat sur 9, euthanasiés après 7 jours, dans le groupe PV. Il n'y a eu aucune preuve de localisation des CSM dans les groupes TV et DI.

Conclusions :

Les CSM peuvent se localiser et se différencier en hépatocytes dans le foyer du traumatisme contondant et contribuer ainsi au processus de régénération du foie. Parmi les différents modes d'administration étudiés, l'injection des CSM dans la veine porte s'est révélée être le mode d'administration au foie le plus efficace en cas de traumatisme. Cette technique pourrait devenir une approche thérapeutique efficace pour améliorer la régénération du foie à la suite d'un traumatisme contondant. Les méthodes d'optimisation de l'accès au tissu lésé et d'évaluation de la capacité de différenciation des cellules souches sont des domaines de recherche que les prochaines études pourront explorer.

Acknowledgements

First and foremost, I would never have been able to finish my thesis without the guidance of my supervisor, Dr. Kosar Khwaja, who taught me how to conduct research with excellent guidance, care, patience, while providing me with an excellent atmosphere for doing research. Besides my supervisor, I would like to thank my thesis committee: Prof. Maria Petropavlovskaya, thesis chair, Dr. Tarek Razek and Dr. Dominic Shum-Tim, thesis committee members for their insightful comments, and excellent questions. I would like to express my thanks and gratitude to Dr.Zu-Hua Gao for his guidance and support throughout the project. I am deeply grateful to our laboratory technician, Ms. Minh Doung, who provided her support during the whole project. I would like to express the deepest appreciation to the staff at the animal laboratory at the Montreal General Hospital for their continued support and excellent and humane care of the experimented animals through the project.

I would also like to thank my parents, Abdulelah Alhabboubi and Zaineb Alhabboubi, for their continuous support, believing in me during hard times. The values they taught me help me to face every day's challenges. I would to thank my two brothers, Mohammad and Ali and three sisters, Manal, Majedah and Marwah. They were always supporting me and encouraging me with their best wishes.

To my caring, loving, and supportive wife, Taghreed Alhabboubi: my deepest heartfelt. Your inspiration when the times got rough are much appreciated and duly noted. It was a great luxury and reprieve to know that you

were willing to provide excellent care and management of our household activities while I completed my work. My heartfelt thanks. My deepest heartfelt appreciation also goes to my kids, Taliah and Abdulelah, who without a doubt make my life happier and expressive. They always engage me with love, smiles and big hugs.

Finally, I offer my regards and blessings to anyone who supported me in any respect during the completion of my Master degree.

Contribution of authors

Dr.Tarek Razek

Contribution: Helped with the original concept, development of methodology and discussion of the results

Dr.Dominique Shum Tim

Contribution: Provided expertise from previous research on using stem cells for cardiac injuries in animal models. Provided advice for research design and

Dr.Zu Ha Gao

Contribution: Provided advice on methodology. Helped with interpreting histological studies

Dr.Kosar Khwaja

Contribution: Helped with generating the research question, designing the study, interpreting the results, providing the logistic support for the research and reviewing the manuscript

Introduction

Epidemiology and impact of liver trauma:

The liver is a commonly injured organ in both blunt and penetrating abdominal trauma, and a consistent increase in the rates of blunt liver injuries has been observed throughout the last 25 years[1]. While most liver injuries are minor and can heal spontaneously with non-operative management [2, 3], some injuries can lead to devastating consequences, especially severe liver parenchymal and venous injuries as well as those involving the portal triad [4]. Mortality from high-grade liver traumas has been estimated to reach 5-57%[5] [6]. Furthermore, complete liver healing can take a long time, which adds to the morbidity resulting from these injuries. A retrospective review of prospectively collected database of 44 patients with blunt liver trauma who were managed non-operatively has shown a median healing time of 62 days and 118 days on Organ Injury Scales (OIS) IV and V, respectively[7]. The authors followed up the patients using conventional ultrasonography. They found that 75% of the patients recovered after 60, 80 and 98 days for OIS grades II, III and IV, respectively[7].

The most common mechanism of blunt liver injury is motor vehicle collision[8]. This is followed by pedestrian/car collisions, falls, assaults, and motorcycle crashes [4].

Anatomy of the liver:

The liver is situated in the right upper quadrant of the abdomen. The liver's size and anatomic location, directly under the right costal margin, makes it a susceptible organ for injury in blunt trauma and a frequently involved organ in penetrating trauma [4]. The liver is divided into two major lobes by Cantlie's line, which is an imaginary line from the gallbladder fossa to the inferior vena cava. It is further subdivided into eight lobes based on the distribution of the hepatic veins [4].

The liver's arterial supply comes from the common hepatic artery, which branches from the celiac artery. It provides about 25% of the hepatic blood flow and 50% of hepatic oxygenation. The artery then branches into the gastroduodenal, right gastric, and proper hepatic. The proper hepatic is found in the porta hepatis usually to the left of the common bile duct and anterior to the portal vein. At the hilum of the liver, the artery bifurcates into a right and a left hepatic artery [4].

The hepatic veins develop from within the hepatocytes' central lobar veins. The retrohepatic vena cava is about 8–10 cm in length. It receives the blood of the hepatic veins as well as multiple small direct hepatic vessels[4].

The portal vein is formed from the confluence of the splenic and superior mesenteric veins directly behind the pancreatic head. It provides about 75% of hepatic blood flow and 50% of hepatic oxygen. The portal vein lies posteriorly to the hepatic artery and bile ducts as it ascends toward the liver[4].

The primary functional unit of the liver is the hepatic lobule, which consists of one central vein and six surrounding portal triads [9]. A portal triad is formed of a small portal vein, hepatic artery, and bile duct. Blood enters the liver from the portal vein and hepatic artery, and it flows through liver sinusoids toward the central vein [9].

Physiology of the liver:

The liver serves as the regulatory site for energy metabolism by coordinating the uptake, processing, and distribution of nutrients and their subsequent energy products. The liver also synthesizes a large number of proteins, enzymes, and vitamins that participate in a broad range of bodily functions. Finally, the liver detoxifies and eliminates many exogenous and endogenous substances, serving as the major filter of the human body [10].

The most notable characteristic of the liver is its regenerative ability. Investigations have shown that the liver can regenerate to its full size even after a 67% hepatectomy [11] . Liver regeneration is a hyperplastic response of all cell types of the liver, in which the microscopic anatomy of the functional liver is maintained [10]. Division of mature hepatocytes usually carries out this response. However, recent findings support a dual view in which mature differentiated epithelial liver cells and facultative stem cells mediate liver maintenance and growth [9]. These stem cells are thought to be recruited when the liver's response to injury is impaired.

Diagnosis of liver trauma:

Technological advances over the recent decades allowed the diagnosis of liver injuries with a high degree of accuracy. Liver trauma is diagnosed by using the Helical Computed Tomography (CT) scan. CT can help accurately diagnose parenchymal injuries and exclude surgical lesions such as bowel or pancreatic injuries. The introduction of multi– detector row CT has greatly enhanced image resolution and markedly decreased the time required for scanning, thereby allowing examination of the whole body within a few minutes [12]. The major CT features of blunt liver trauma are lacerations, subcapsular and parenchymal hematomas, active hemorrhage, and juxtahepatic venous injuries. [12]. Hepatic lacerations are the most common type of parenchymal liver injury and appear as irregular linear or branching low-attenuation areas at contrast-enhanced CT. Subcapsular hematomas appear as elliptic collections of low-attenuating blood between the liver capsule and enhancing liver parenchyma on contrast-enhanced CT. Parenchymal hematomas or contusions are characterized by focal low-attenuation areas with poorly defined irregular margins in the liver parenchyma on contrast-enhanced CT. Active hemorrhage following blunt liver trauma is typically identified at early phase contrast-enhanced CT as focal high-attenuation areas that represent a collection of extravasated contrast material secondary to arterial bleeding[12].

The most widely accepted grading system for liver traumas is the American Association for the Surgery of Trauma (AAST) classification system. It scores the injury from I to VI based on the size of lacerations and/or hematomas found on CT scan [8, 13]. This grading system can predict clinical outcomes, where

higher grades of injury lead to worse outcomes. Several studies have shown increased complication rates with increased grades of injury ranging from 1% for those with Grade III injuries, to 21% for Grade IV and 63% for Grade V injuries[5, 14, 15].

Management of blunt liver trauma

The approach to manage blunt liver trauma has changed gradually from observation to operative intervention to the current approach of selective operative or non-operative intervention[16]. The principles of the Advanced Trauma Life Support Protocol (ATLS) are followed in the management of liver trauma. Regardless of their injury grade, patients who are hemodynamically normal and do not have signs of abdominal peritonitis are considered for non-operative management[16]. Non-operative management of liver injuries consists of a period of in-hospital/ICU observation/monitoring, serial abdominal examinations, serial hematocrit measurements, a period of post discharge restricted activity[16] with the possible adjunct use of interventional radiology. With high success rates (82%-100%)[8, 17-19], non-operative management of blunt hepatic injuries has become the standard of care at most American trauma centers[16].

Outcomes of blunt liver trauma:

The outcomes of liver trauma have improved over the years [20]. However, blunt liver trauma can still lead to considerable morbidity[5]. Complications include hepatic bleeding, biliary complications, abdominal compartment syndrome and

infectious complications [5]. A multicenter study reported complications rate of 14% and increased rates of hepatic complications with high-grade injuries. They reported a rate of 5% of hepatic complications in patients with grade 3 injuries, 22% in patients with grade 4 injuries, and 52% in patients with grade 5 injuries [5]. Furthermore, mortality rates from blunt hepatic trauma can reach up 57% as mentioned earlier.

Healing time for liver injuries can vary depending on injury type and severity. The median healing time for hepatic sub-capsular hematomas was found to be different from hepatic lacerations in a study by Tiberio et al [21]. The median healing time for a Grade I hematoma was found to be 6 days, while it was 16 days for Grade II and 108 days for Grade III hematoma. For lacerations, the median healing time was found to be 29 days for Grade II, 34 days for Grade III, and 78 days for Grade IV. Although there is a lack of concrete evidence of the time during which the physical activities should be limited for such a population[16], involved patients are recommended to limit their activities until full healing, which can be up to four months depending on the severity of trauma[22]. This restriction of activity can be limiting for patients especially if their professions require a high degree of physical activity.

Stem cells:

Stem cells are defined as cells that have the ability to self-replicate and divide asymmetrically, producing one daughter cell that is a stem cell and one daughter cell that is either a progenitor cell or a specialized cell[23, 24]. By definition, stem cells can self-renew and give rise indefinitely to more cells of

the same type. Progenitor cells, also known as transit-amplifying cells, are more lineage committed than stem cells, giving rise to specialized cells, and can only replicate a limited number of times [24].

Stem cells have been studied extensively as a potential source for tissue regeneration in different organs and have shown promising results. One of the well-studied cell lines that showed promising results are the mesenchymal stem cells (MSCs).

MSCs are a type of stem cells that can be found in different types of tissues such as bone marrow [25], muscle [11] and amniotic tissues [26].

They are convenient to use because they are readily accessible [27] and can be easily cultured [28]. Moreover, their use is not associated with the ethical concerns associated with the use of other potential sources such as fetal tissue[27]. They can differentiate to multiple mesenchymal cell lines including hepatocytes [28], osteoblasts [25, 29], chondrocytes[25], adipocytes and myocytes [25, 30]. They can also transdifferentiate to non-mesenchymal cell lines such as neurons under experimental conditions [27].

MSCs have been investigated extensively and have shown distinctive qualities that make them a plausible option for the treatment of various liver diseases. MSCs can transdifferentiate to liver cells [11, 31] , can enhance tissue repair by secreting trophic molecules [32], have anti-fibrotic properties [31] and inhibit the activation of hepatic stellate cells [31]

The use of stem cells to treat different forms of liver injuries:

Several animal studies showed encouraging results for using stem cells to treat various liver injuries. The investigations incorporated using different lines of stem cells to treat several forms of liver injuries, including acute and chronic induced injuries.

Acute injuries:

The potential beneficial effects of using stem cells were demonstrated in different forms of acute liver pathologies. Multiple animal models were developed and showed promising results. Yue Yu et al used an ischemia/reperfusion model in the setting of a small-for-size (30%) liver transplantation rat model to demonstrate the efficacy MSCs. They injected the stem cells through the portal vein in a group of rats that underwent 30% liver transplantation. The rats that received the MSCs had reduced mortality rates and exhibited improved liver function and liver weight recovery during the early post-transplantation period[33]. A liver resection model was developed by Bellayr et al to test the ability of muscle-derived MSCs to localize and differentiate to the liver after 50% resection. They have shown successful translocation and transdifferentiation to the remaining tissue of the liver [11]. They even demonstrated long-term survival of these cells in the liver three months after the injection. Another experiment involved intraperitoneal injection of biencapsulated bone marrow cells in 90% hepatectomized rats. The rats that received the stem cells showed better survival and blood biochemistry results than the rats that did not receive the stem cells [34].

Chronic injuries:

Multiple investigations showed the ability of stem cells to provide favorable outcomes in chemically induced liver cirrhosis animal models. M.T Abdel Aziz et al studied the effect of using MSCs in a rat CCL4 induced liver fibrosis model. The rats that received the MSCs showed markedly decreased liver fibrosis and significant improvement of serum albumin compared with the rats that did not receive the MSCS. They concluded that MSCs have a potential therapeutic effect against the fibrotic process through their effect in minimizing collagen deposition in addition to their capacity to differentiate into hepatocytes [31].

The use of stem cells in lung trauma:

Few studies investigated the effects of using stem cells on mechanically injured organs such as the lungs and brain. A study compared the effect of using MSCs vs bone marrow stem cells (BMSC) in a rat blunt lung trauma model. Rats that were subjected to trauma were grouped based on receiving either one type of the aforementioned cells or not receiving any. Both types of the stem cells homed to the injured lungs and exerted healing effects, which were comparable between the two types of cells [35]. However, it seems that the literature is lacking in studies examining the effects of using stem cells on liver blunt trauma.

The above examples illustrate the huge potential for exploiting the properties of stem cells in treating different forms of liver diseases and injuries. Thus, we aim to explore the effects of the administration MSC to a blunt trauma liver injury animal model, envisioning the possibility of translating these effects to treat liver trauma patients in the future.

Methodology:

Animals:

Immunocompetent male Sprague Dawley (SD) and Lewis rats (275-350 g) were purchased from Charles Rivers Laboratory, USA and used for this study. All of procedures in this study were approved by the Montreal General Hospital Facility Animal Care Committee of the Research Institute of the McGill University Health Center. Most of the procedures complied with McGill University's Standard Operating Procedures (SOPs). Any deviation from SOPs was approved by the aforementioned committee.

The experiments took place from January 2013 to March 2015.

Trauma models:

A-Weight drop model:

In the initial phase of the project, we adopted two trauma models developed by Cox et al[36] and Karamercan et al[37] and added a few modifications to develop our own model. Both models applied a weight drop method in order to induce mechanical trauma. Cox et al developed a gross liver trauma scoring system adopted from the AAST scoring system. They scored liver trauma based on the gross appearance of the liver after the trauma where the presence and sizes of

hematoma and/or laceration and their respective sizes denoted an increasing score from I-IV (Table 1). On the other hand, Karamercan et al focused on histological appearance of the liver where they denoted a score from 0 to 4 based on the severity of the liver's vascular and parenchymal cellular damage (Table 2). Anesthetized rats were positioned underneath a hollow 3⁄4 in. PVC tube 40-50 cm in length, so that the end of the tube is positioned at the abdomen directly below the xiphoid process and costal cartilage. Metal plates (each weighting, 73.6 g) with a flat surface were lumped together and dropped through the tube onto the anaesthetized subject animal (Figure 1). The law E = M x H x G was applied to calculate the force of impact where, M stands for mass in Kilograms, H for height in Meters and G is for gravitational acceleration (9.8 m/s^2)[37] .The goal of the trauma model was to achieve a grade 3 or 4 trauma based on Cox model (Figure 2) and grade 3 or 4 trauma based on Karamercan model.

All of the rats were subjected to general anesthesia using isoflurane before inducing the trauma. To induce anesthesia, the rat was placed in the induction chamber. The oxygen flowmeter was adjusted between 0.8 and 1.5 L/min. The isoflurane vaporizer was adjusted to between 3% and 5%. For maintenance, a mask connected to the brain circuit was used. The flow meter was adjusted to between 400 and 800mL/min. The isoflurane vaporizer was adjusted to between 2 and 2.5%. Depth of anesthesia was verified by the loss of animal's pedal withdrawal (toe pinch) reflex. Ophthalmic ointment was applied to both eyes to prevent corneal desiccation. An analgesic was administered by using Carprofen

5-10 mg/kg subcutaneous. Warmed isotonic fluids (0.2-0.5 ml/10g) were injected subcutaneously. The hair was removed over the surgical area with a clipper allowing a perimeter of at least 1cm around the surgical site. Loose hair was removed with gauzes.

After ensuring that the rat is fully anesthetized, trauma was induced as described above. Next, the abdominal cavity was accessed by a midline incision. The liver was examined to identify the degree of trauma. After achieving hemostasis, injection of stem cells or normal saline was done through the portal vein or the iliocecal vein. The abdomen was closed using size 2-0 proline for the fascia and size 3-0 vicryl for the skin. All surgeries were done using aseptic techniques.

Post-operative animals were observed until regaining righting reflexes. Analgesics were repeated post surgically and for the next 72 hours according to the specified doses mentioned previously. The wounds were examined daily for signs of inflammation or infection such as redness, swelling or purulent discharge for at least 5 days.

B-Kelly clamp crush model:

Due to the high mortality rate faced with the application of the weight drop model, an alternative Kelly clamp crush model was adopted. Briefly, laparotomy was done on anaesthetized rats as described previously. Then, the left lateral lobe of the liver was identified. Subsequently, a straight Kelly clamp was applied to crush the left liver lobe one fingerbreadth to the left of the

falciform ligament, using all the teeth of the clamp, with one click of the clamp, for one second (Figure 3). Stem cells were injected after achieving hemostasis (Figure 4).

Stem cells were injected through the portal vein, tail vein or directly into the liver.

Livers and lungs were collected initially for the rats that underwent the tail vein injection in order to identify the engraftment site of the stem cells. Livers only were collected for the rats that underwent portal vein injection and direct injection to the liver.

Experimental Groups:

After establishing the trauma model, the rats were divided into three groups:

- A trauma group in which the rats in this group received trauma only with 1 ml of normal saline
- A trauma and stem cells group in which the rats received trauma and stem cells
- A stem cells group in which the rats in this group received stem cells only.

Isolation and preparation of stem cells from rat's bone marrow:

Isolation and primary culture of MSCs from the femur and tibial bones of donor rats was performed using Caplan's method. The bones were harvested from adult Lewis rats and both ends of the long bones were cut from the diaphysis. The bone marrow

was flushed out from the bone with complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and antibiotics: 100 U/ml penicillin G, 100 mg/mg streptomycin, (Gibco laboratories). The marrow plugs were then dissociated and the dispersed cells were centrifuged and re-suspended twice in complete medium. These cells were then implanted into tissue culture dishes and cultured in an incubator (Forma Scientific, Inc.) with 5% CO_2. The culture medium was replaced every three days and the non-adherent cells were discarded. Following two passages, the attached cells were divided into three new flasks and cultured until the cell density of the colonies grew to approximately 90% confluence. They were then transfected with reporter gene.

After two passages, the MSCs were transfected *in vitro* by a replication defective retrovirus containing the reporter Lac-Z gene that encodes for the bacterial-galactosidase enzyme produced by Lac-Z-GP AM12 amphotropic retrovirus producer cells. MSCs were cultured with the supernatant from the Lac-Z-GP AM12 cells culture flasks with hexadimethrine bromide 4ug/ml. The transfection medium was removed 24 hours after the last transfection. MSCs were maintained in complete medium containing 10% fetal bovine serum. Transfection efficiency was monitored in a subset of plates with X-gal staining to determine the percentage of cells expressing ß-gal activity. The cells were rinsed with phosphate buffered saline (PBS) and fixed with 1% glutaraldehyde at room temperature for 5 minutes. After replacing PBS with the staining solution (containing 1 mg/ml 5-bromo-4-chloro-3-indoyl-b-D-galactoside (X-gal), 2% dimethylsulfoxide, 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe\ (CN)_6 \cdot 3H_2O$

and 2 mM magnesium chloride), cells were incubated at 37°C, pH 7.8 to 8 and protected from light for 16 hours to detect the cells with ß-gal activities. We then confirmed the presence of the blue-labeled cells under inverted microscopy (BX-FLA, Olympus, Tokyo, Japan).

Labeled MSCs were cultured with complete medium in 75 cm^2 tissue culture flasks. On the day of transplantation, the cells in each flask were washed with 6 ml of Hank's Basic Salt Solution (HBSS), and then trypsinized with 1 ml of trypsin-EDTA solution. The detached cell suspension was gathered in 2 ml of complete medium and the cell density was measured by a hematocytometer. Afterwards, the cells were collected by centrifugation at 1500 rpm for 5 min and re-suspended in a complete medium without serum at a concentration of 1-$6x10^6$cells/ml. The cell suspensions prepared in tuberculin syringes were placed on ice until injection into rats [38].

Intravenous injection of MSCs into adult rats:

Labeled MSCs were injected through the portal vein, tail vein or directly to the liver of adult SD and Lewis rats. After inducing the trauma under general anesthesia, a total of $1x10^6$ cells were injected into each rat. Subsequent developments during the course of the project changed the injection dose from $1x10^6$ cells per rat to $3x10^6$ cells to eventually $6x10^6$ cells.

Liver collection after euthanasia

The left lateral lobe was collected and fixed in 2% paraformaldehyde in PBS. The staining for b-galactosidase activity was performed as described above, with adding 0.02% Nonidet P-40 and 0.01% deoxycholate to the staining

solution. The staining was performed for 24 hours. After X-gal staining, liver specimens were cut into three – four sections, embedded in paraffin and coronal sections 5 mm thick were mounted on a set of gelatin coated glass slides. The slides were stained using Congo Red stain. Figure 5 shows a section of traumatized liver without stem cells injection.

Results:

Weight drop model:

The weight drop model was applied to 17 rats. With the aim of achieving a gross trauma score of 3-4 (Table 1), the force used ranged from 0.44 to 1.44 J.

Only 5/17 (29.4%) rats survived the experiment. Among the rats that died, five died on the table while doing the surgery. The cause of death was uncontrolled hemorrhage in four rats and high dose anesthesia in one rat. The other seven rats died within 24 hours of the surgery. The most common reason for death was hemorrhagic shock. Table 3 illustrates the criteria of the rats and different causes of mortality.

MSCs were injected in only one of the surviving rats through the tail vein. Liver histology was performed and did not show stem cells.

Kelly clamp crash model:

Due to the high mortality faced with the weight drop model, it was decided to apply a less traumatic model.

-Pilot test of the model:

Initially, we tested the survival potential of the model by applying it to three SD rats: one received trauma only, another received trauma and stem cells and the

last received stem cells only. All of the rats survived the procedure. The trauma Plus stem cells as well as the stem cells rats received $3x10_6$ stem cells each. The trauma rats received 1 ml of normal saline as control using the tail vein. All of the rats were euthanized 48 hours after the trauma. Histological studies failed to find the stem cells in the livers of the rats that received the stem cells.

In the next step, we changed the strain of rats to Lewis to avoid potential cross immunity between the strains of the donor and recipient rats. Thus, three Lewis rats received trauma and stem cells. The first and second rats received 6 million stem cells, and the third rat received 12 million stem cells. The route of injection was the tail vein. All the rats survived the procedure. Euthanasia was done 48 hours after the surgery for the first and third rats, and after one week for the second rat.

Lungs and liver were collected from the rats. Stem cells were identified in the lungs (Figure 6) of the rats euthanized after 48 hours but not in their livers. No stem cells were found in the rat euthanized after seven days.

To avoid potential sequestration of the stem cells in the lungs, it was decided to inject the stem cells into the portal circulation rather than the systemic circulation. Therefore, two rats underwent trauma and stem cell injection through the portal vein. They were both euthanized 48 hours after the trauma. Histological studies successfully located the stem cells in the liver of both rats. The stem cells were found to exit the blood vessels to migrate to the trauma site (Figure 7). Furthermore, MSCs were found in the trauma site and started to

trans-differentiate to liver like cells by taking the hexagonal shape of liver cells (Figure 8).

Applying the model:

After success with the pilot step of the experiment, the following groups were proposed:

	Euthanasia after 48 hours	Euthanasia after one week
1-Trauma+ stem cells	5 rats (TS48)	6 rats (TS7)
2- Stem cells only	5 rats (S48)	6 rats (S7)
3-Trauma only	3 rats (T48)	6 rats (T7)

All of the rats survived the procedure except for one from the TS7 group and one from the S7 group.

A scant amount of disintegrating stem cells/debris were found in the livers of 2/5 of the TS48 group, 3/5 of the TS7 group, 2/5 of the S48 group and 3/5 of the S7 group.

Failure to demonstrate MSCs in the liver in this step led us to delay the injection of MSCs within 4-24 hours of the trauma. In this last step, the following three routes of injection were attempted: Portal vein, tail vein, and direct injection to the liver.

Table 4 details the groups along with the times of euthanasia. Table 5 details the results of the histological studies from each group. MSCs were found only

in the portal vein group. In 1 of 3 rats euthanized 48 hours after portal vein injection, stem cells were found in multiple sites adjacent to the trauma area. Some cells differentiated into liver-like cells (Figure 9). Regarding the rats that were euthanized 7 days after portal vein injection, Stem cells were found around the trauma area in the first rat (Figure 10). Some differentiated into liver-like cells. Multiple degenerated stem cells with traces of blue staining were found in the second rat. Cellular debris was found in the third rat.

Discussion:

Despite the advances in managing liver trauma, these traumas still pose a major health problem. High-grade liver injuries can lead to high mortality rates and full healing can take a long time for these injuries as detailed previously. Findings in this study indicate the ability of MSCs to locate to and engraft traumatized areas in mechanically injured livers.

The success in using the stem cells to treat different forms of non-traumatic liver injuries in animal models provided incentive to carry out human trials. Terai et al injected autologous bone marrow cells into nine cirrhotic patients. They examined the patients' liver function tests (LFTS) and Child-Pugh scores and found improvement in the LFTS and Child-Pugh scores over the course of 24 weeks[39]. Although the study was limited by the small number and a lack of a control group, it still showed the potential benefit of recruiting stem cells in the multimodality treatment of those challenging patients. At the time of writing this paper, there were 262 registered clinical trials at

www.clinicaltrail.gov that involve the use of stem cells to treat different forms of liver injuries (both acute and chronic). Nevertheless, studies utilizing stem cells to treat liver injury in the setting of trauma are still lacking, hence the impetus for our study. We hypothesized that injected MSCs can localize and transdifferentiate to liver-like cells after mechanical liver trauma in a rat model.

In order to examine our hypothesis, we sought to design a reproducible trauma model that leads to grade 3 or 4 liver trauma (as per Cox et al modified score) without resulting in high mortality rates. The concept of the weight-drop model developed by Cox et al and Karamercan et al was adopted initially for a number of advantages. The force of impact is measurable, it is reproducible, and the impact element mimics the trauma seen in real life such as in motor vehicle collisions. We attempted to grade the trauma immediately after inciting the injury rather than waiting for few days as in cox model. This was in order to avoid the conceivable harm to the rats from the stresses of undergoing two surgeries.

The learning curve related to learning the weight-drop model technique led to a number of deaths of our study animals. However, the main cause of mortality was hemorrhagic shock resulting from high-grade liver injuries. Our attempts to calibrate the force of trauma to achieve a high grade of injury that would not lead to mortality failed despite the use of fluid resuscitation. We therefore switched to the Kelly clamp crush trauma model.

The Kelly clamp crush trauma model offered a more controlled, repeatable trauma that is designed to induce a 3cm laceration that corresponds to a grade 4 trauma in the Cox gross trauma grading system.

After successfully establishing the trauma model, we began experimenting with MSCs injections. The first step was to establish an optimal route of MSC delivery. We tested three routes of injection: the tail vein, the portal vein and directly into the liver. Each of these routes has its own advantages and disadvantages. The tail vein is an easily accessible and minimally invasive route of injection. It does not require anesthesia or laparotomy, avoiding the need for a second laparotomy for invasive injection. However, it is a systemic injection that has the potential of sequestering the MSCs in the lungs. Studies on this issue show conflicting results. Several non-traumatic liver injury reports mention successful engraftment of the stem cells to the liver using peripheral veins, such as the tail vein[11, 31, 40]. However, other reports describe a "pulmonary first pass effect", in which the systemic route of stem cell injection (tail vein for example) leads to cell entrapment in the lungs and hence the passage of only a minority of the injected cells into the target tissue. A study by Fischer et al investigated this phenomenon by injecting four types of stem cells through the left internal jugular vein. A few minutes after injection, they found that the majority of the stem cells injected were entrapped in the lungs with only minimal number of cells passing through the lungs and reaching the

systemic circulation [41]. Another report by Li et al tracked labelled MSCs after induction of CCL4 injury using three different routes: Inferior vena cava (IVC), intrahepatic injection and inferior mesenteric vein injections. The MSCs injected through the IVC were entrapped in the lungs and did not distribute to other organs throughout seven days of follow up [42].

The histological studies from the rats that had tail vein injections in our group failed to show localization of the MSCs to the liver. They were rather found in the lungs, which led to the conclusion of them being sequestered. This prompted us to change the injection site to the portal vein.

Both the portal vein and direct injection to the liver have the advantage of bypassing the lungs and delivering the cells directly to the liver. However, both of these techniques are invasive as they require laparotomy. Furthermore, portal vein injection is a technique that requires training and can lead to bleeding that can be difficult to control. However, portal vein injection has the advantage of distributing the cells throughout the liver while directly injected cells tend to clump at the site of injection [42].

Several studies reported the successful engraftment of MSCs to the liver after portal vein injection [33, 43]. Xiang Guo-an et al studied the homing ability of the MSCs to the liver after inducing CCL4 chemical injury. They compared the number of stem cells engrafted to the liver by injection through the tail or portal veins and did not find significant difference between the two groups[44].

In our study, the portal vein was the only route of injection that led to successful homing and engraftment of the MSCs into the liver.

We also took the timing of euthanasia into consideration, i.e., the time required for the MSCs to locate to the liver after systemic injection. Qiang le et al tracked MSCs and hematopoietic stem cells (HSCs) after injection through the tail vein in a CCL4 mouse liver fibrosis model. The MSCs as well as HSCs were found to locate in the lungs first within 2 hours of injection. They then started to appear in the liver within 24 hours[45]. This led us to divide the rats into two groups with respect to the time of euthanasia: The first group was euthanized within 48 hours of injection of the MSCs, as the extra day from Qiang's study gave more time for the MSCs to locate to the liver. The second group was euthanized within seven days of injection of the MSCS. The goal of the second group was to have an estimate of the longevity of the potentially localized stem cells to the liver.

The initial attempt of injecting the stem cells through the portal vein resulted in successful localization of the MSCs to the liver in three rats. Some MSCs were found to exit the hepatic portal vein branches and migrate to the trauma area. Furthermore, a group of MSCs was found to locate to the trauma area and to start to take the shape of liver hexagonal cells rather than the fusiform shape

known for the MSCs, showing evidence of trans-differentiation to liver-like cells.

Unfortunately, this result was not always reproducible on subsequent attempts. On the second attempt, a scant amount of disintegrating stem cells/debris was found in the livers of 2/5 of the TS48 group, 3/5 of the TS7 group, 2/5 of the S48 group and 3/5 of the S7 group.

One possible explanation for this finding was that the stem cells were injected too soon after the trauma. Hence, unlike the first successful attempt, injection was performed immediately after the trauma without ensuring achieving full hemostasis of the injury to the liver. Therefore, we theorized that the MSCs may have leaked outside the liver through the bleeding wound. Subsequently, postponing the time of injection to 4-24 hours after the trauma led to improved results. MSCs localized to the liver in the portal vein group in 1/3 of the rats euthanized within 48 hours of stem cells injection and 1/3 of the rats euthanized 7 days after the injection of the stem cells, with evidence of disintegrating stem cells in another rat from the 7 days group.

By using the portal vein route of injection, we managed to localize the stem cells to the liver and showed evidence of trans-differentiation. However, two important points that need to be addressed.

First, localization of the MSCs to the liver was not successful in every attempt. Second, the concentration of the MSCs appears to be decreased in the livers of the rats euthanized seven days of MSCs injection compared with the ones euthanized 48 hours after the injection.

The rate of liver engraftment with MSCs is reported to be low to begin with. Reports indicate low MSCs engraftment rates to target tissues[9]. Qiang Li et al reported 5% engraftment rates of MSCs to the liver after systemic injection. Tom Kuo et al studied a model of CCL4-induced fulminant hepatic failure in a murine model. They reported engraftment frequencies of 4.2% (± 0.88%) for transplanted donor cells in recipient liver at 4 weeks post-transplantation [46].

Another factor is the age of the stem cells used for the experiment. The MSCs were prepared in our lab using Caplan's method as detailed earlier in the methods section and stored in a refrigerator three years ago. At the time of the experiment, a colony was expanded for the use in implantation. Only 5-6 passages were performed each time to avoid sensitizing the cells. Additionally, these cells were tested based on morphology with no identifying cell markers. Using old MSCs could have affected their quality and ability to exert their function as stem cells. However, these cells localized successfully to the liver in some experiments representing an indication of their viability.
Another possibility is the loss of the LacZ signal with time due to either weakness of the signal or integration of the MSCs to the liver parenchyma or disintegration of the MSCs due to immune reaction.

It is not well known how long the MSCs can last in the liver after injection. One report mentioned the presence of viable stem cells with intact LacZ expression three months after injection of the MSCs, indicating the potential long term survivability of the stain using this technique [11]. With our current available data, we cannot confirm whether finding fewer stem cells in the seven-day euthanasia specimens or having negative results for different specimens is due to any of the aforementioned reasons. Future studies should incorporate more dependable and diverse markers and methods to find MSCs. To the best of our knowledge, this is one of the first studies that examined the localization and trans-differentiation of MSCs into bluntly injured livers. Our study designed a simple, reproducible blunt liver trauma model for rats that can be applied to different trauma scenarios. Future developments of this model should examine the biological markers of liver trauma that can be used to monitor the responses to different possible interventions.

We established the portal vein as the optimal route to deliver the MSCs to the liver. We were able to localize the MSCs to the liver. Further studies should focus on further optimizing the delivery of the MSCs to the liver and should examine the potential effect on healing.

In conclusion, MSCs can locate to the liver and differentiate to liver-like cells in a rat liver trauma model.

Tables:

Score	Lesion	Description
1	Hematoma	Smaller than 1 cm^2 in surface area
	Laceration	Less than 1 cm in length; affects only one lobe
2	Hematoma	1 to 2 cm^2 in area
	Laceration[a]	1 to 2 cm in length; may affect multiple lobes
	Discoloration[b]	1 to 2 cm^2 surface area; may affect multiple lobes
3	Hematoma	Larger than 2 cm^2 in area
	Laceration[a]	2 to 3.5 cm in length, may affect multiple lobes
	Discoloration[b]	2 to 3.5 cm^2 in area; may affect multiple lobes
	Hemorrhage[c]	Less than 4 mL
4	Hematoma	Larger than 3.5 cm^2 in area
	Laceration	More than 3.5 cm in length; may affect multiple lobes
	Discoloration	Larger than 3.5 cm^2 in area; may affect multiple lobes
	Hemorrhage[c]	4 mL or more

Table 1: Rat liver gross injury scale*.

[a]When accompanied by hematoma or discoloration of the same or a higher score, the overall injury score in- creases by 1. [b]Varied from white to light pink. Possibly an indication of hypoxia. [c]Must be present in injuries receiving scores of 3 or 4.

- Reproduced with permission from Cox et al[36]

Histological Grading of Liver Vascular Injuries	
Grade 0	No dilatation, stasis, or hemorrhage
Grade 1	Central venous and sinusoidal dilatation
Grade 2	Central venous stasis
Grade 3	Central venous and sinusoidal stasis
Histological Grading of Liver Parenchyma Injuries	
Grade 0	No cellular damage
Grade 1	Localized mild cellular damage
Grade 2	Localized severe cellular damage (cellular separation)
Grade 3	Diffuse mild cellular damage
Grade 4	Diffuse severe cellular damage (cellular separation)

Table 2: Rat liver histological injury scale*

*Reproduced with permission from Karamercan et al[37]

Rat number	Weight dropped (Kilograms)	Height from the impact point (Meters)	Impact force applied (Jouls)	Grade of trauma	Time of mortality	Cause of mortality	Stem cells injected	Results of histology
1	0.24	0.5	1.18	1	During surgery	Failure of hemorrhage control from PV* injection site	No	Not done
2	0.24	0.6	1.4	3-4	Within 24 hours of surgery	Hemorrhagic shock	No	Not done
3	0.24	0.5	1.18	3-4	The rat survived the surgery. **	Hemorrhagic shock?	No	Not done
4	0.24	0.7	1.646	4	During surgery	Immediate hemorrhage	No	Not done
5	0.24	0.6	1.18	3-4	Within 24 hours of surgery	?hemorrhagic shock	No	Not done
6	0.24	0.5	1.17	4	Within 24 hours of surgery	Hemorrhagic shock	No	Not done
7	0.19	0.4	0.74	-	While doing surgery	High dose of anesthetic	No	Not done
8	0.19	0.4	0.74	3-4	Within 24 hours of surgery	Hemorrhagic shock	No	Not done
9	0.167	0.4	0.65	1	While doing surgery	Hemorrhagic shock	No	Not done
10	0.179	0.4	0.70	1	Within 24 hours of surgery	Hemorrhagic shock	No	Not done
11	0.19	0.4	0.74	1	Survived the surgery	Euthanasia	No	Not done
12	0.214	0.4	0.83	4	Survived the surgery	Euthanasia	Yes ($1x\ 10_6$ cells)	No stem cells were found in the liver
13	0.214	0.4	0.83	3-4	Died within 24 hours of surgery	Hemorrhagic shock	No	Not done
14			0.77	-	Died on table	Injury to IVC*	No	Not done
15					Died within 24 hours of surgery	Hemorrhagic shock	No	Not done
16			0.77	1-2	Survived the surgery	Euthanasia	No	Not done
17	0.112		0.43	1-2	Died during the surgery	Failure of hemorrhage control from PV injection site	No	Not done

Table 3: Summary of the rats used for the weight drop model.

*PV: Portal vein, IL: Iliolumbar vein, IVC: inferior vena cava

** A re-laparotomy 3 days later showed grade 3-4 trauma as evidenced by discoloration of more than the left lobe.

After closure, that rat died within the next day.

	Euthanasia after 48 hours of trauma	Euthanasia after one week of trauma	Euthanasia after two weeks of trauma
Portal vein (PV)	3 rats (PV 48)	3 rats (PV7)	
Tail vein (TV)	3 rats (TV 48)	3 rats (TV 7)	
Directly to the liver (DI)	3 rats (DI 48)	1 rat (DI 7)	2 rats (D 14)

Table 4: Last experimental group. Stem cells were injected into either the portal vein, the tail vein or directly into the liver. There was a total of 6 rats in each group.

	Number of rats per group	Time of Euthanasia Days (# of rats)	Findings
Portal vein group (PV)	6	2 (3)	In 1 out of 3 rats, stem cells were found in multiple sites adjacent to the trauma area. Some differentiated into liver-like cells
		7 (3)	Stem cells were found around the trauma area in the first rat. Some differentiated into liver-like cells. Multiple degenerated stem cells with traces of blue staining were found in the second rat. Cellular debris was found in the third rat.
Tail vein group (TV)	6	2 (3)	Cellular debris was found in all rats
		7 (3)	No stem cells were identified
Direct injection group (DI)	6	2 (3)	Cellular debris was found in 3 rats
		7 (1)	Cellular debris was found in this rat
		14 (2)	Cellular debris was found in one rat

Table 5: Results for the last group, MSCs were found only in the portal vein group. In 1 of 3 rats euthanized 48 hours after portal vein injection, stem cells were found in multiple sites adjacent to the trauma area. Stem cells were found also in 1 of 3 rats of the rats euthanized after 7 days

Reference:

1. David Richardson, J., et al., *Evolution in the management of hepatic trauma: a 25-year perspective.* Ann Surg, 2000. **232**(3): p. 324-30.
2. Croce, M.A., et al., *Nonoperative management of blunt hepatic trauma is the treatment of choice for hemodynamically stable patients. Results of a prospective trial.* Ann Surg, 1995. **221**(6): p. 744-53; discussion 753-5.
3. Malhotra, A.K., et al., *Blunt hepatic injury: a paradigm shift from operative to nonoperative management in the 1990s.* Ann Surg, 2000. **231**(6): p. 804-13.
4. Mattox, K.L., *Trauma.* 2013(seventh edition): p. Chapter 29. Liver and biliary tract.
5. Kozar, R.A., et al., *Risk factors for hepatic morbidity following nonoperative management: multicenter study.* Arch Surg, 2006. **141**(5): p. 451-8; discussion 458-9.
6. Asensio, J.A., et al., *Operative management and outcomes in 103 AAST-OIS grades IV and V complex hepatic injuries: trauma surgeons still need to operate, but angioembolization helps.* J Trauma, 2003. **54**(4): p. 647-53; discussion 653-4.
7. Padalino, P., et al., *Healing of Blunt Liver Injury After Non-Operative Management: Role of Ultrasonography Follow-Up.* European Journal of Trauma and Emergency Surgery, 2009. **35**(4): p. 364-370.
8. Tinkoff, G., et al., *American Association for the Surgery of Trauma Organ Injury Scale I: spleen, liver, and kidney, validation based on the National Trauma Data Bank.* J Am Coll Surg, 2008. **207**(5): p. 646-55.
9. Duncan, A.W., C. Dorrell, and M. Grompe, *Stem cells and liver regeneration.* Gastroenterology, 2009. **137**(2): p. 466-81.
10. Courtney M. Townsend, R.D.B., B. Mark Evers and Kenneth L. Mattox, *SABISTON TEXTBOOK OF SURGERY.* 2012. **19th edition**.
11. Bellayr, I.H., et al., *Skeletal muscle-derived stem cells differentiate into hepatocyte-like cells and aid in liver regeneration.* Int J Clin Exp Pathol, 2010. **3**(7): p. 681-90.
12. Yoon, W., et al., *CT in blunt liver trauma.* Radiographics, 2005. **25**(1): p. 87-104.
13. Moore, E.E., et al., *Organ injury scaling: spleen and liver (1994 revision).* J Trauma, 1995. **38**(3): p. 323-4.

14. Kozar, R.A., et al., *Complications of nonoperative management of high-grade blunt hepatic injuries.* J Trauma, 2005. **59**(5): p. 1066-71.
15. Goldman, R., et al., *Delayed celiotomy for the treatment of bile leak, compartment syndrome, and other hazards of nonoperative management of blunt liver injury.* Am J Surg, 2003. **185**(5): p. 492-7.
16. Stassen, N.A., et al., *Nonoperative management of blunt hepatic injury: an Eastern Association for the Surgery of Trauma practice management guideline.* J Trauma Acute Care Surg, 2012. **73**(5 Suppl 4): p. S288-93.
17. Velmahos, G.C., et al., *High success with nonoperative management of blunt hepatic trauma: the liver is a sturdy organ.* Arch Surg, 2003. **138**(5): p. 475-80; discussion 480-1.
18. Helling, T.S., et al., *Treatment of liver injuries at level I and level II centers in a multi-institutional metropolitan trauma system. The Midwest Trauma Society Liver Trauma Study Group.* J Trauma, 1997. **42**(6): p. 1091-6.
19. Coimbra, R., et al., *Nonoperative management reduces the overall mortality of grades 3 and 4 blunt liver injuries.* Int Surg, 2006. **91**(5): p. 251-7.
20. Richardson, J.D., *Changes in the management of injuries to the liver and spleen.* J Am Coll Surg, 2005. **200**(5): p. 648-69.
21. Tiberio, G.A., et al., *Evaluation of the healing time of non-operatively managed liver injuries.* Hepatogastroenterology, 2008. **55**(84): p. 1010-2.
22. Kenneth L. Mattox, E.E.M., David V. Feliciano, in *Trauma*. 2013, The McGraw-Hill Companies.
23. Hall, P.A. and F.M. Watt, *Stem cells: the generation and maintenance of cellular diversity.* Development, 1989. **106**(4): p. 619-33.
24. DeLeve, L.D., *Liver sinusoidal endothelial cells and liver regeneration.* J Clin Invest, 2013. **123**(5): p. 1861-6.
25. Prockop, D.J., *Marrow stromal cells as stem cells for nonhematopoietic tissues.* Science, 1997. **276**(5309): p. 71-4.
26. Zhang, D., M. Jiang, and D. Miao, *Transplanted human amniotic membrane-derived mesenchymal stem cells ameliorate carbon tetrachloride-induced liver cirrhosis in mouse.* PLoS One, 2011. **6**(2): p. e16789.
27. Woodbury, D., et al., *Adult rat and human bone marrow stromal cells differentiate into neurons.* J Neurosci Res, 2000. **61**(4): p. 364-70.
28. Oyagi, S., et al., *Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl4-injured rats.* J Hepatol, 2006. **44**(4): p. 742-8.
29. Bruder, S.P., N. Jaiswal, and S.E. Haynesworth, *Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation.* J Cell Biochem, 1997. **64**(2): p. 278-94.

30. Wakitani, S., T. Saito, and A.I. Caplan, *Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine.* Muscle Nerve, 1995. **18**(12): p. 1417-26.
31. Abdel Aziz, M.T., et al., *Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver fibrosis.* Clin Biochem, 2007. **40**(12): p. 893-9.
32. Zhao, W., et al., *Intravenous injection of mesenchymal stem cells is effective in treating liver fibrosis.* World J Gastroenterol, 2012. **18**(10): p. 1048-58.
33. Yu, Y., et al., *Mesenchymal stem cells over-expressing hepatocyte growth factor improve small-for-size liver grafts regeneration.* Mol Ther, 2007. **15**(7): p. 1382-9.
34. Liu, Z.C. and T.M. Chang, *Transdifferentiation of bioencapsulated bone marrow cells into hepatocyte-like cells in the 90% hepatectomized rat model.* Liver Transpl, 2006. **12**(4): p. 566-72.
35. Hannoush, E.J., et al., *Role of bone marrow and mesenchymal stem cells in healing after traumatic injury.* Surgery, 2013. **153**(1): p. 44-51.
36. Cox, J.M. and J.E. Kalns, *Development and characterization of a rat model of nonpenetrating liver trauma.* Comp Med, 2010. **60**(3): p. 218-24.
37. Karamercan, M.A., et al., *Microscopic hematuria as a marker of blunt abdominal trauma in rats: description of an experimental model.* J Trauma, 2011. **71**(3): p. 687-93.
38. Jun, L., et al., *The immature heart: the roles of bone marrow stromal stem cells in growth and myocardial repair.* Open Cardiovasc Med J, 2007. **1**: p. 27-33.
39. Terai, S., et al., *Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy.* Stem Cells, 2006. **24**(10): p. 2292-8.
40. Liu, H., et al., *Human amniotic fluid-derived stem cells can differentiate into hepatocyte-like cells in vitro and in vivo.* In Vitro Cell Dev Biol Anim, 2011. **47**(9): p. 601-8.
41. Fischer, U.M., et al., *Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect.* Stem Cells Dev, 2009. **18**(5): p. 683-92.
42. Li, Z., et al., *Optimization of mesenchymal stem cells (MSCs) delivery dose and route in mice with acute liver injury by bioluminescence imaging.* Mol Imaging Biol, 2015. **17**(2): p. 185-94.
43. Sgodda, M., et al., *Hepatocyte differentiation of mesenchymal stem cells from rat peritoneal adipose tissue in vitro and in vivo.* Exp Cell Res, 2007. **313**(13): p. 2875-86.
44. Xiang, G.A., et al., *[A preliminary study of the homing capacity of allograft mesenchymal stem cells to rat liver].* Di Yi Jun Yi Da Xue Xue Bao, 2005. **25**(8): p. 994-7.
45. Li, Q., et al., *In vivo tracking and comparison of the therapeutic effects of MSCs and HSCs for liver injury.* PLoS One, 2013. **8**(4): p. e62363.

46. Kuo, T.K., et al., *Stem cell therapy for liver disease: parameters governing the success of using bone marrow mesenchymal stem cells.* Gastroenterology, 2008. **134**(7): p. 2111-21, 2121 e1-3.

Figures:

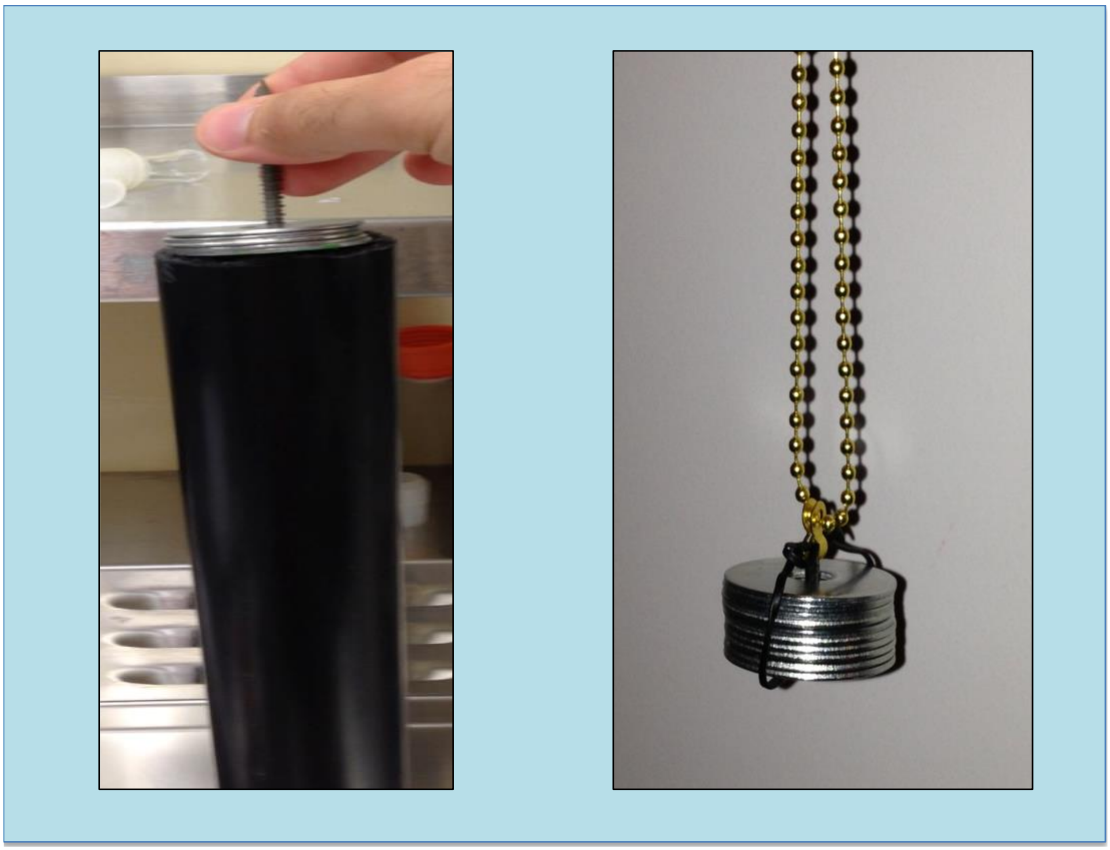

Figure 1:

weight drop trauma model. On the left; the PVC tube with the disks being ready to be dropped. On the right, a demonstration of the disks.

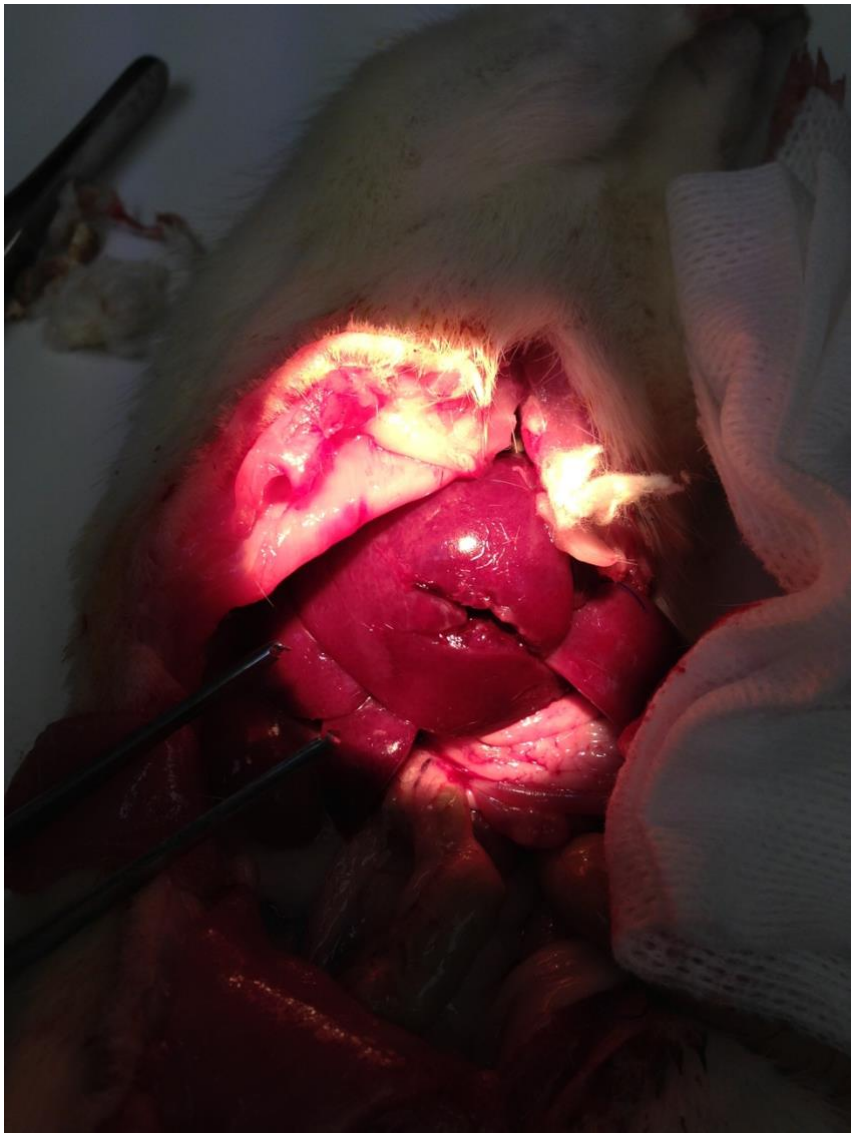

Figure 2: Grade 4 trauma, weight drop model. Force applied 1.17 J. free blood was found in the abdomen, almost 3 ml. The rat survived the procedure but was found dead the next day.

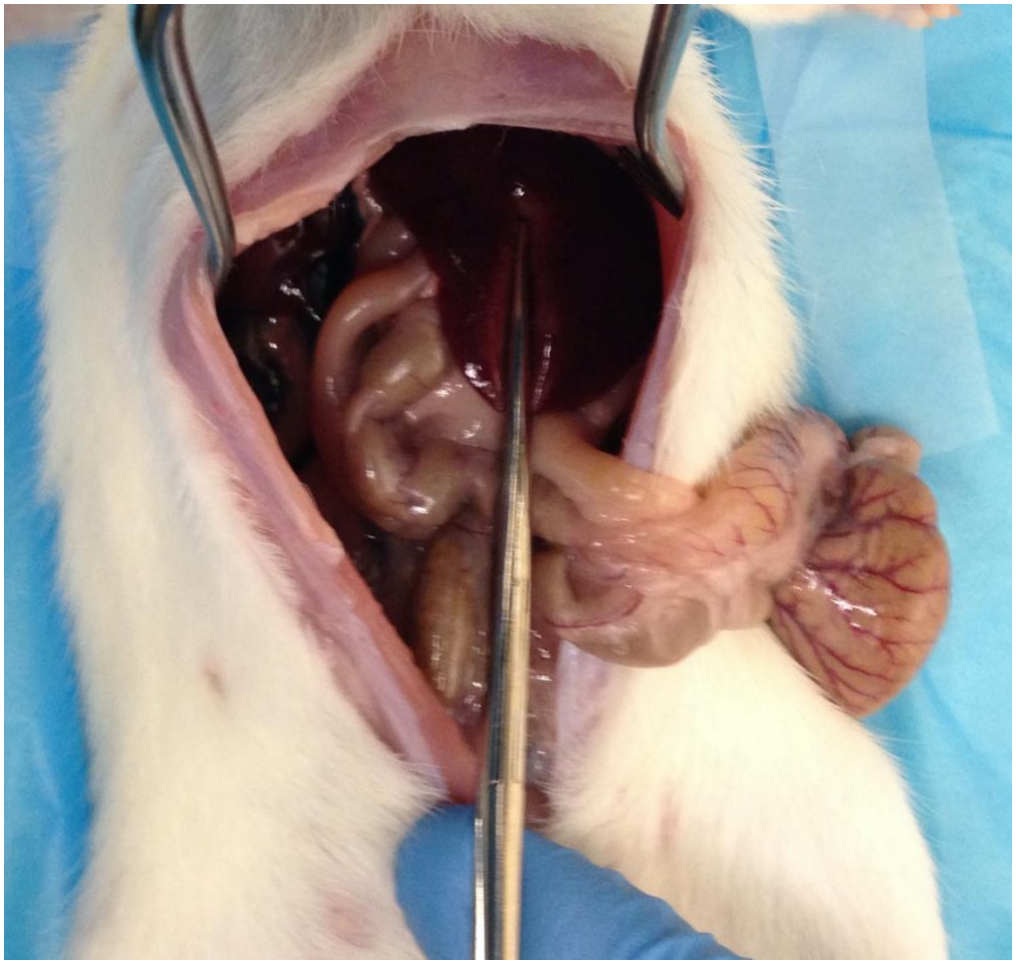

Figure 3: Kelly clamp crash model

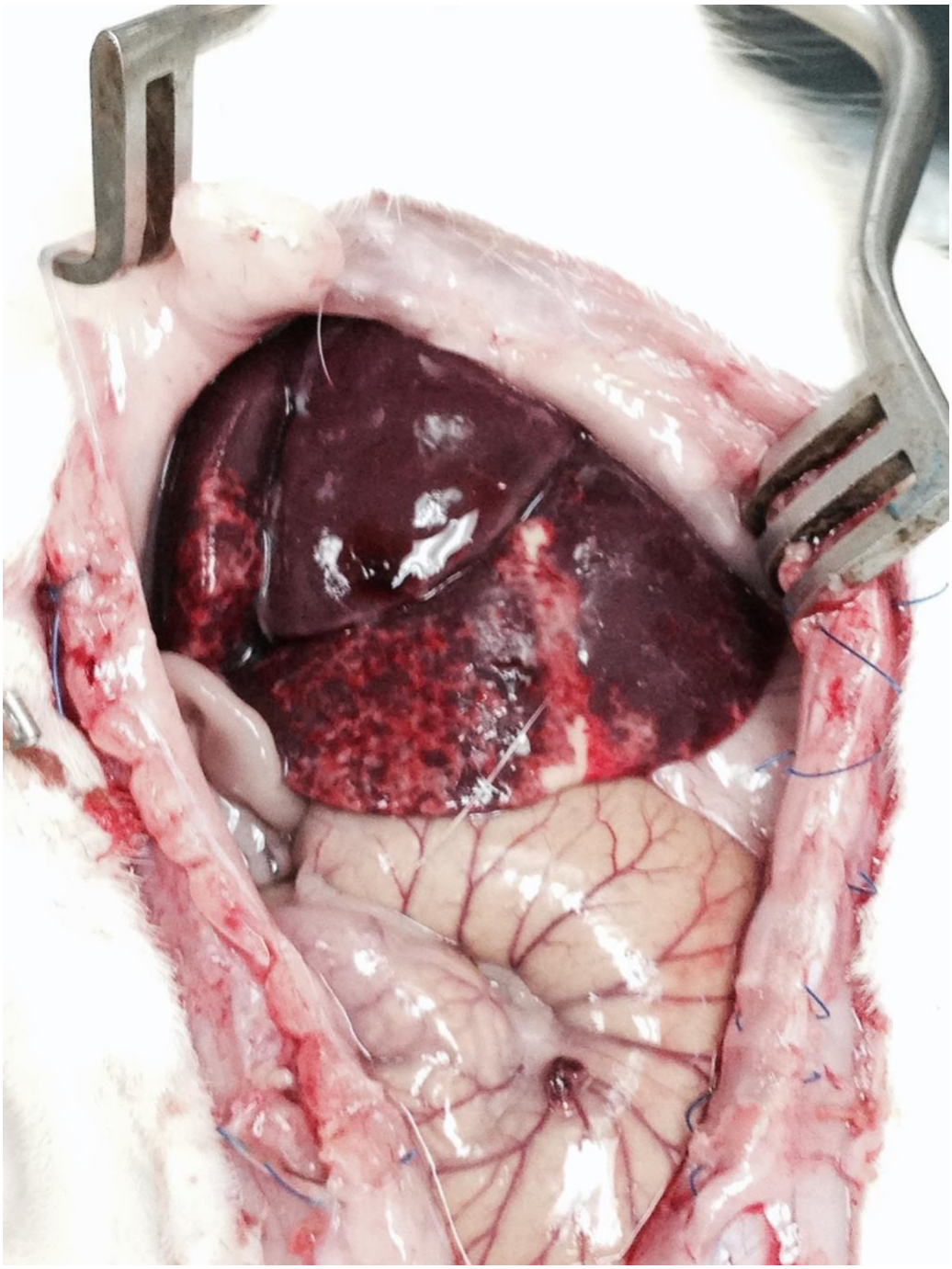

Figure 4: The liver of a rat from the Kelly clamp model 48 hours after receiving the stem cells. Stem cells were found under light microscopy.

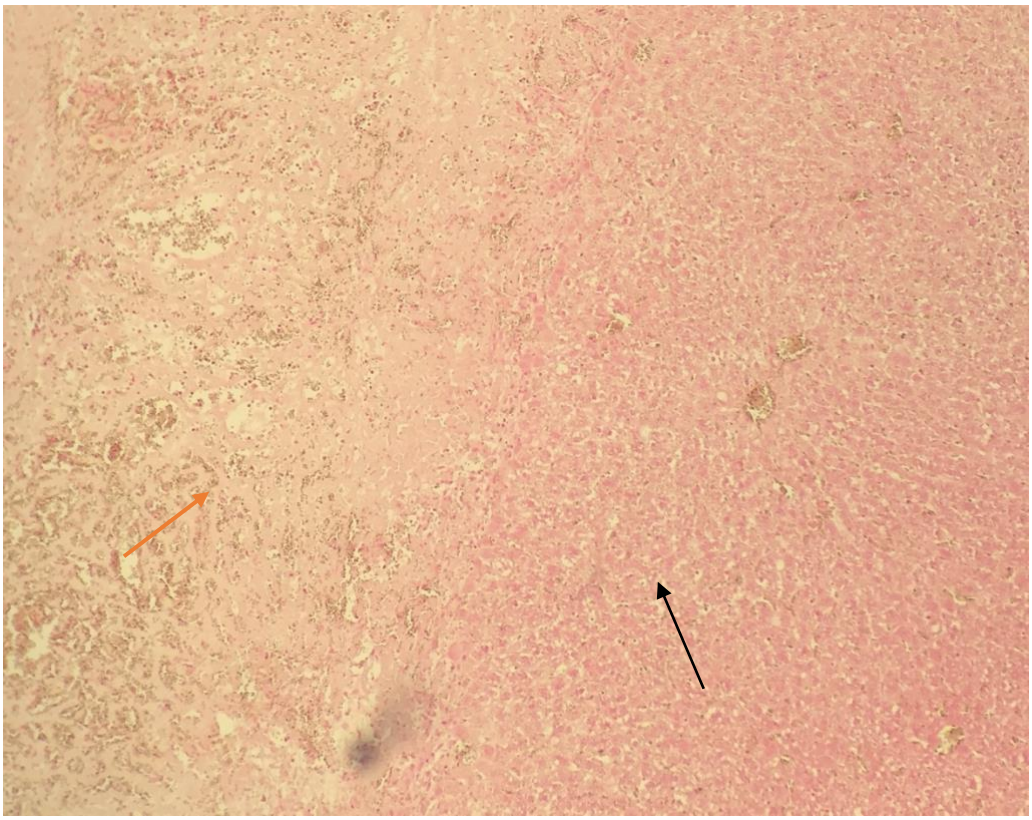

Figure 5: Liver under light microscopy after the trauma. The normal liver is on the right (black arrow) while to traumatized area in on the left (red arrow). (48 hours)

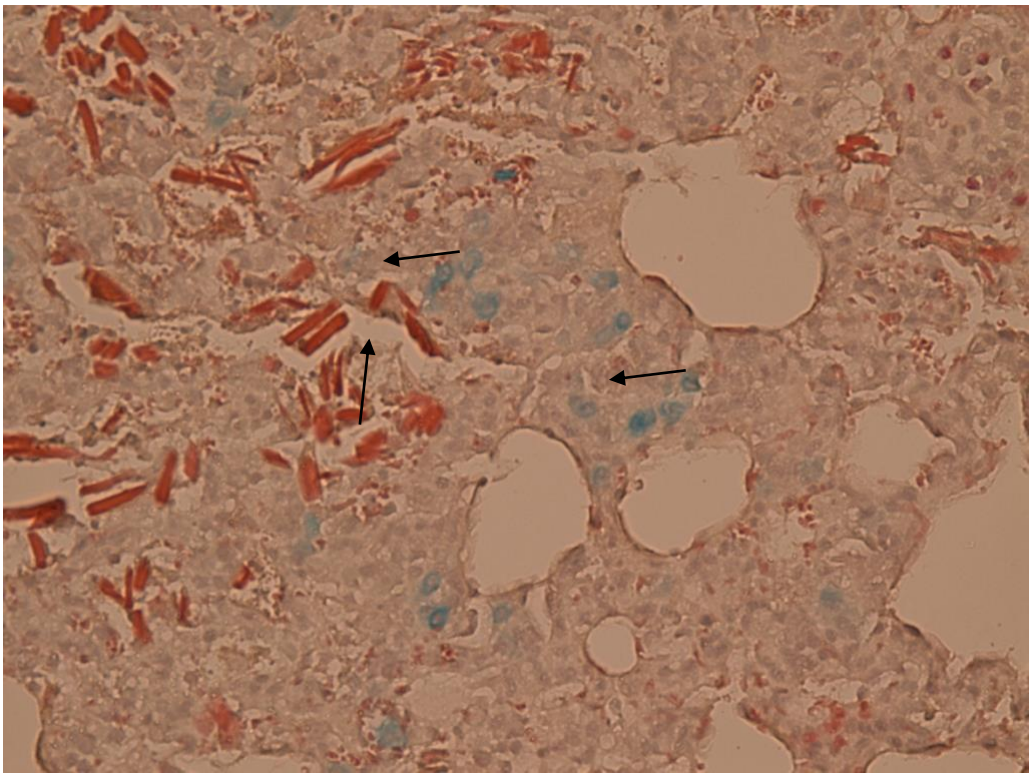

Figure 6: Stem cells (arrows) trapped in lungs

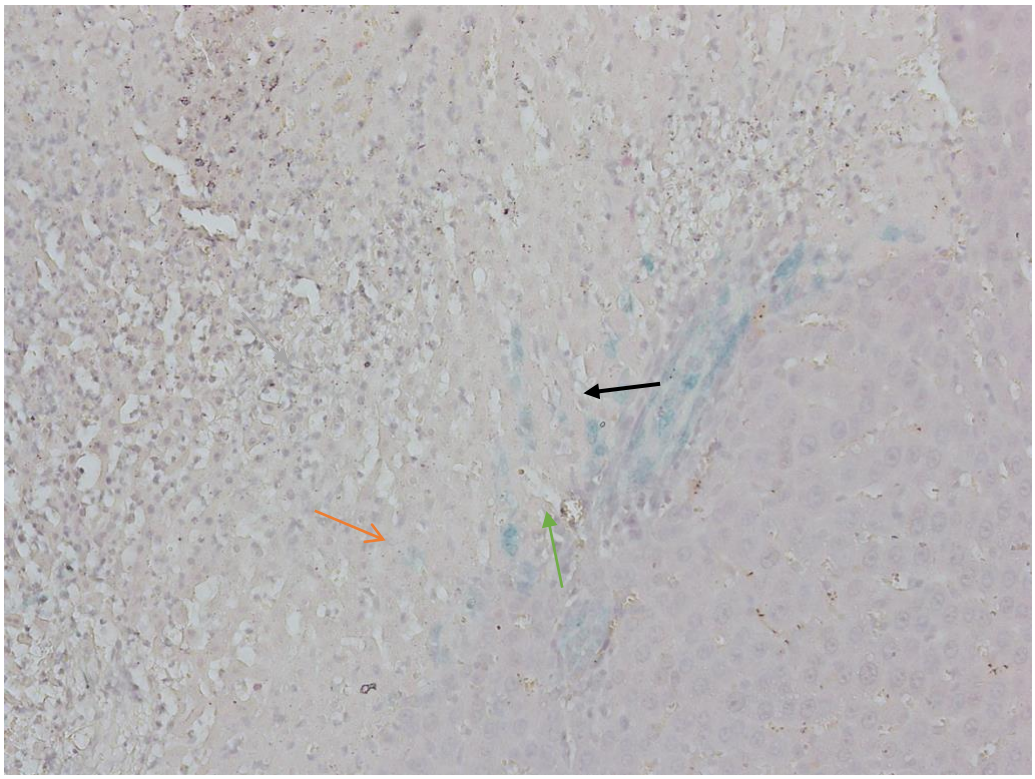

Figure 7: MSCs locate successfully to the liver after portal vein injection (black arrow) (Euthanasia after 48 hours of trauma). The MSCs can be seen exiting to portal sinusoids (orange arrow) and starting to move (red arrow) towards the trauma area (green arrow).

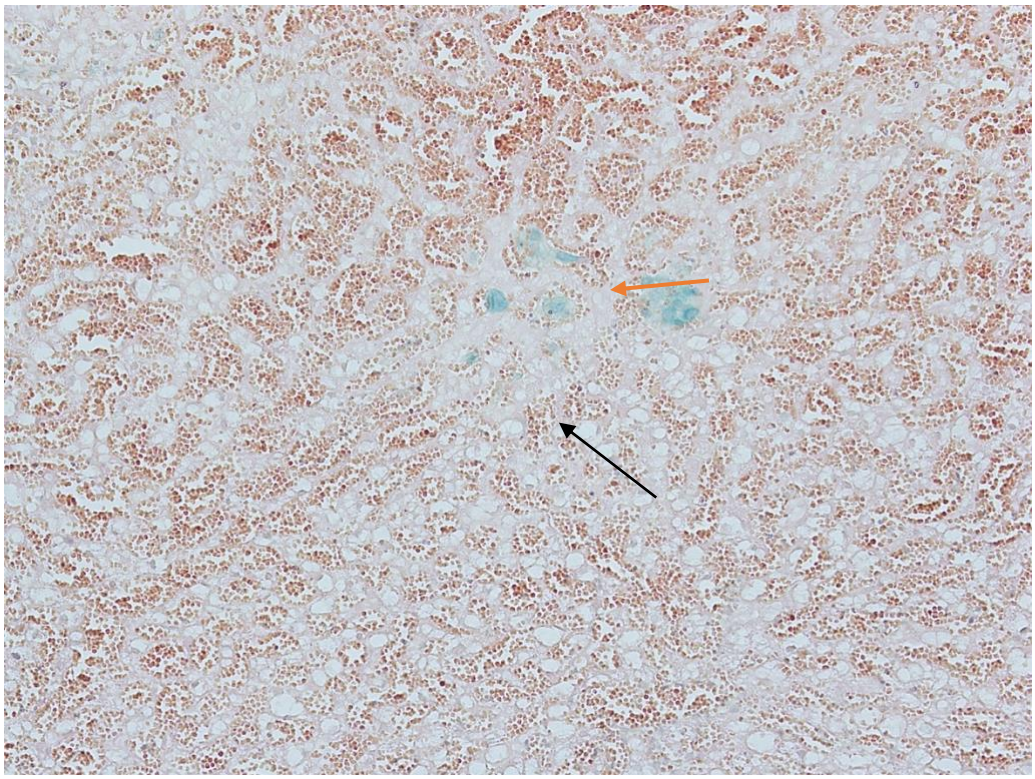

Figure 8: MSCs in the traumatized area of the liver (black arrow) starting to take the hexagonal shape of the liver cells (red arrow), showing evidence of trans-differentiation to liver-like cells.

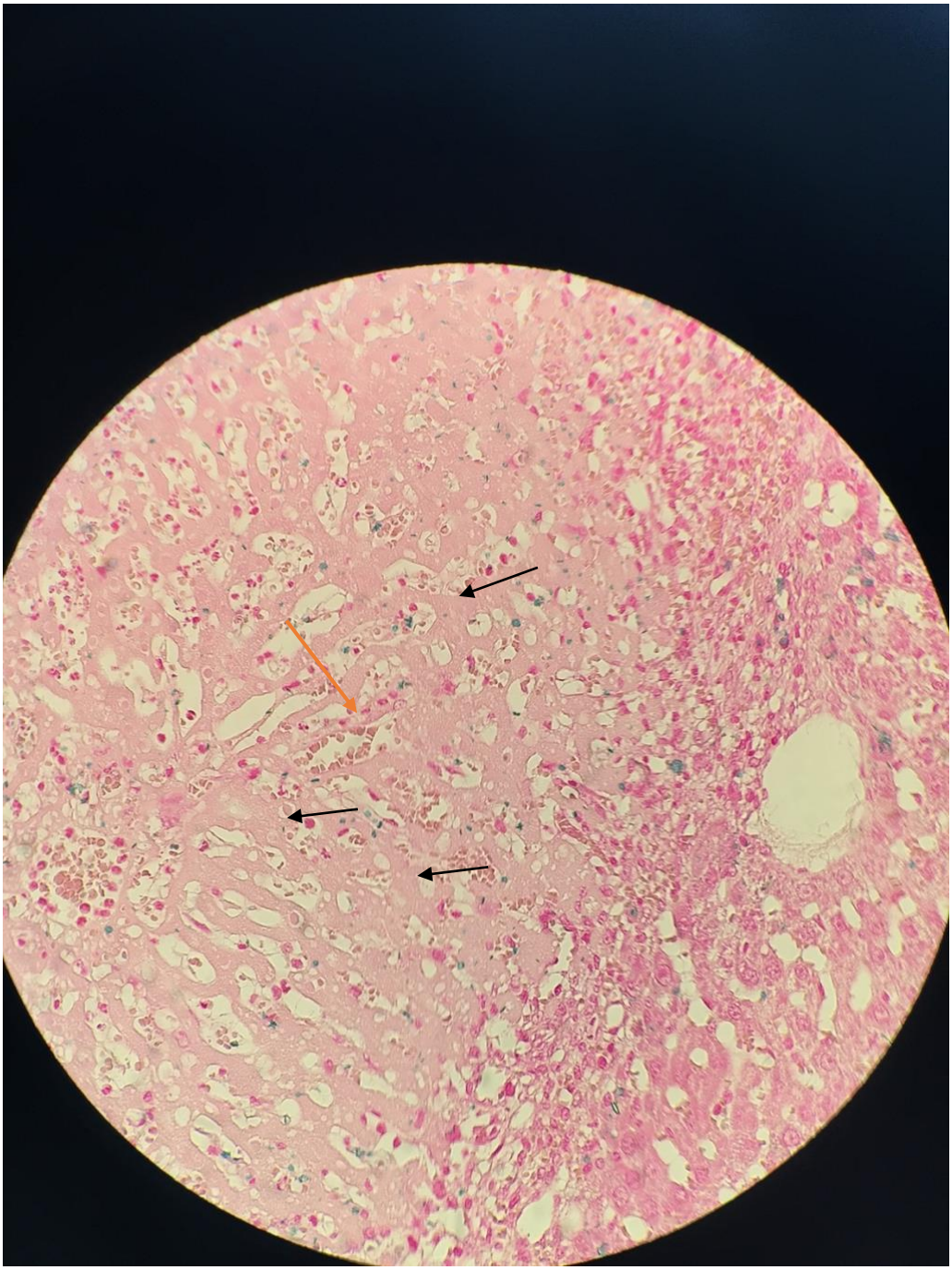

Figure 9: A slice of liver in a rat euthanized 48 hours post MSCs injection (portal vein). There is an abundance of stem cells in the trauma area (red arrow). Many of which started to take the form of liver-like cells (black arrows).

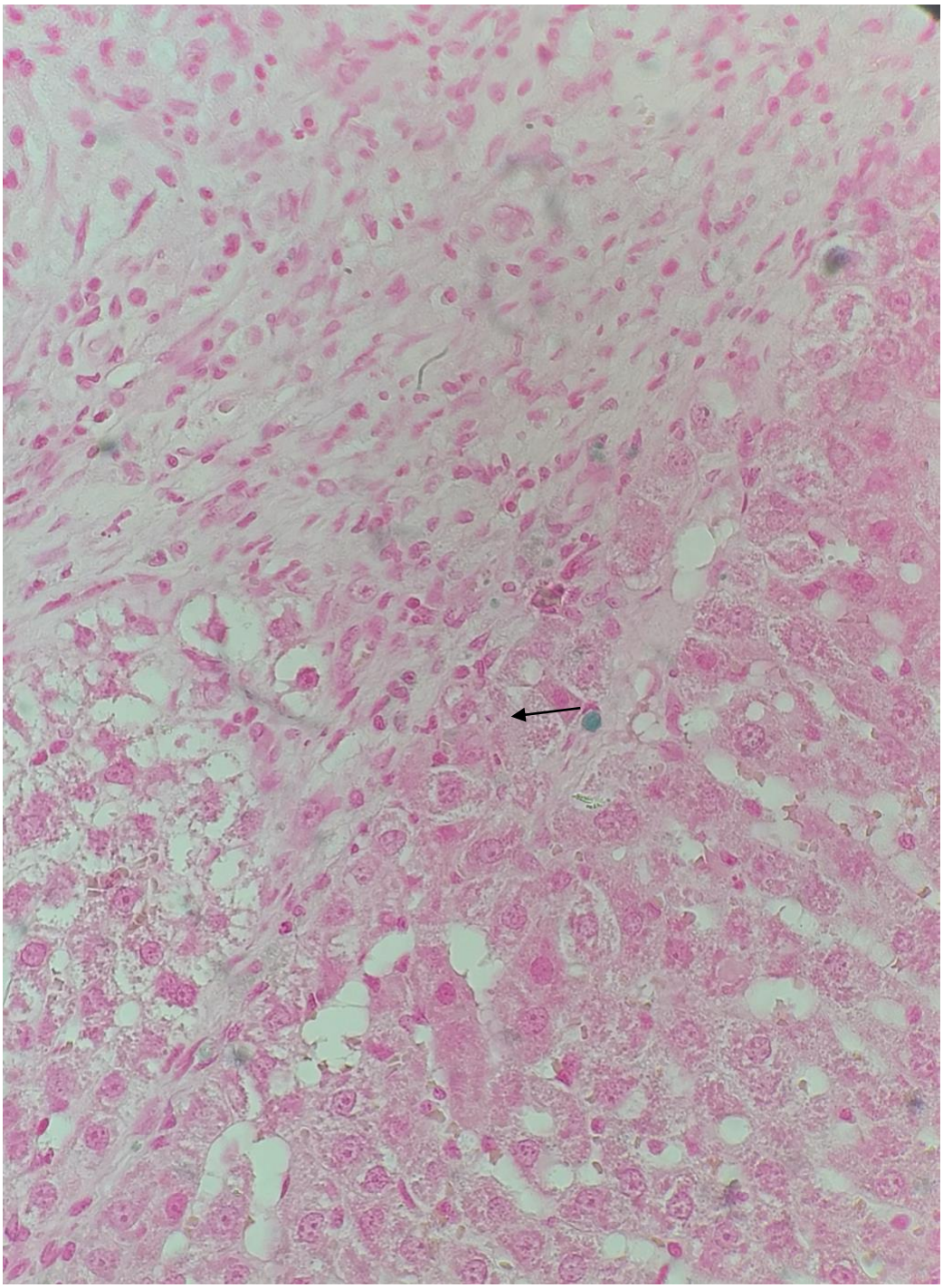

Figure 10: A single MSC (black arrow) cell is seen in a rat one week of euthanasia after the MSCs injection. It was noticed that the amount of MSCs found decreased with time when comparing the 2 and 7 day groups.