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Role of Liver Stem Cell-derived Extracellular Vesicles on ischemia-reperfusion injury in a murine liver experimental model

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1. ISCHEMIA-REPERFUSION INJURY (IRI)

1.1 Definition and risk factors

Ischemia-reperfusion injury (IRI) is defined as pathological process occurring after reperfusion of an ischemic organ. There are mainly two fundamental moments, the first corresponding to the interruption of blood supply to the organ and the second, that is represented by tissue reperfusion [1]. The consequent organ damage depends on ischemia extension and length. Furthermore, there are well known IRI risk factors. In occidental countries, ischemic events occur especially because of thrombo-embolic or arterio-embolic disorders. Not modifiable factors are age, genetic, male gender; modifiable ones comprehend smoking habit, diabetes, hypertension, obesity, alcohol consumption. Finally, IRI is also related to surgical procedures, such as organ resections and transplantation.

1.2 Ischemic phase

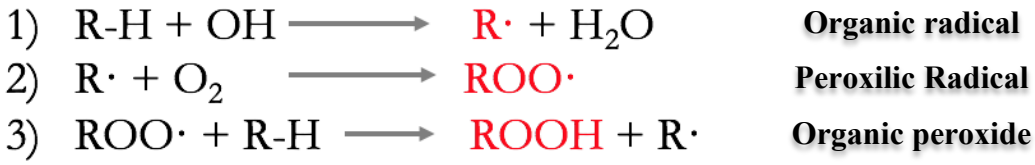
The first phase is the ischemic period, corresponding to interruption of blood supply and consequently damaging of metabolically active tissues. Without oxygen, in fact, aerobic metabolic processes are rapidly reduced. In particular mitochondrial activity is the most affected by oxygen deficiency. Mitochondrial ATP phosphorylation, an oxygen-dependent process, is the main source of cell energy. Lacking oxygen supply, ATP levels fall down, causing loss of ATP-dependent ion channel regulation, with passive ion shift trans membranes. In particular there is an augmented Na and Ca levels inside cell cytoplasm. Anaerobic metabolism is, moreover, associated to accumulation of metabolites such as lactic acid and lowering of intracellular pH [2]

1.3 Reperfusion phase

The second phase is the reperfusion period, when there is a reoxygenation and rewarming of previous ischemic tissue. Although oxygen reuptake triggers aerobic metabolism restart with ATP production and normalizes pH levels, it is responsible of harmful consequences. This concept was firstly evidenced in studies on hearts, where coronary reperfusion was related to accelerating necrosis after ischemic event[3]. Mechanisms at the basis of tissue damages after reperfusion are numerous and comprehend:

- *Oxygen reactive species (ROS) creation*

  Aerobic metabolism restarts but oxygen supply represents also a substrate for production of ROS, the main responsible of tissue injury. They are highly reactive components, able to oxidize every intracellular molecule, causing cellular disfunction. ROS are normally produced by cells at low concentrations and removed by cellular pathways. However, after rewarming of an ischemic tissue, ROS concentrations become so high to be converted in inert molecules. ROS are produced both by mitochondria and by enzymatic complex of plasmatic membrane. In the table below are reported the main ROS species.



ROS cellular injury is realized by following mechanisms of action: damage towards proteins, DNA, lipidic membranes; reduction of nitric oxide (NO) concentration, known for its vasodilatation and antiaggregant effects and anti-leucocyte adhesion action; alteration on intracellular signaling pathways.

Cellular response to oxidative stress is related to ROS levels: in case of low concentration, there is an activation of anti-oxidative elements; if ROS levels are intermediate, anti-oxidative response is associated to inflammatory activation and anti-apoptotic pathway; in case of high ROS concentration, injuries are so important to cause cellular death. Fig.1 shows the principal anti-oxidative defenses, divided in anti-oxidative enzymes (such as SOD, glutathione-peroxidase), natural molecules (for instance ascorbate, GSH) and metal-transporter (for example transferrin, ceruloplasmin). They work to delay or inhibit oxidative process in one specific substrate[4] .

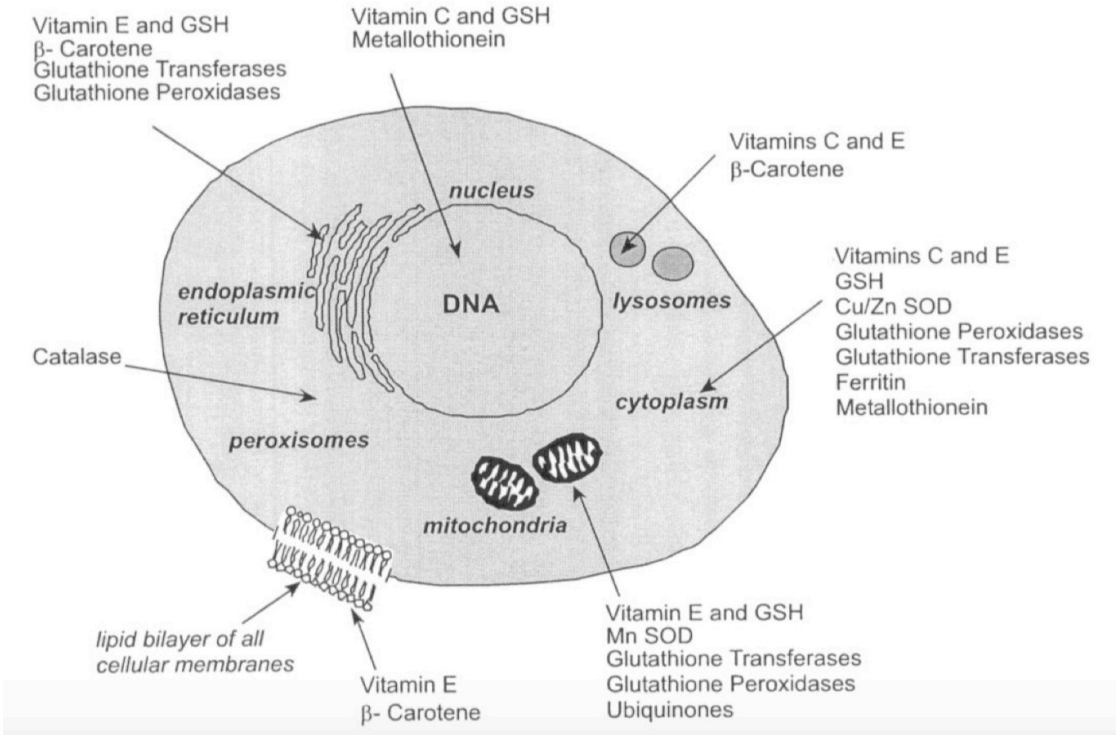


Fig 1. Anti-oxidant molecules. Picture by Halliwel and Gutteridge, Baillieres Clin Haematol, 1989

- *Ca accumulation*

  Normally intracellular Ca concentration is maintained within defined ranges by regulation systems. These are 3 different transporter type and work to remove Ca from cytosol. All are ATP-dependent: reticulum Ca- ATPase, mitochondrial Ca- ATPase and membrane Ca-ATPase. After ischemia, for ATP depletion, the above regulation mechanisms reduce their function, with increase in intracellular Ca levels. Furthermore, Ca is exchanged with Na (Na/Ca pump) in order to decrease intracellular sodium. The final step of elevated intracellular calcium concentration is an irreversible damage, with plasmatic membrane rupture and cell death[4] .

- *Mitochondrial disfunction*

  MPT-pore (Mitochondrial Permeability Transition pore) plays a crucial role in IRI and in the progression to cellular death. MPT-pore, first described by Hawort and Hunter [5] is a protein transmembrane channel located in mitochondrial membrane. During ischemic phase it remains closed for acidosis. After reperfusion, MPT-pore opens because of elevated intracellular Ca levels, oxidative stress and acidosis correction. As a consequence, it occurs a shift of H+ and H2O inside mitochondria for deregulation of

transmembrane gradient. Water causes mitochondrial ballooning with following rupture and depletion of ATP reserve and consequently death for necrosis [6]

- *Microvascular disfunction*

  IRI is associated to vascular alteration including: permeability increase; unbalance between vasodilatation and vasoconstriction agents; complement, endothelial cells and platelets activation. Microvascular alteration occurs especially in transplanted organs. As demonstrated by Ogawa et al. [7], cultures of endothelial cells exposed to a hypoxic setting increase their permeability. In analogous way, mice exposed to hypoxic environment (8% oxygen for 4-8 hours) show pulmonary edema, albumin reduction and inflammatory cytokines. A reduction of vasodilatation causes the so called "no reflow phenomenon", characterized by increased resistance to blood flow after reperfusion. It seems due to neutrophils activation and adhesion to endothelium. Neutrophils induce enhancement in extravascular edema with consequent microcirculation collapse.

- *Inflammatory response*

  It is normally activated in an injured tissue, independently from the cause, to confine the pathogen noxa and create substrate for repair process. Inflammatory response due to IRI, except for intestinal ischemia, is defined sterile inflammation since there are no microorganisms. It consists in massive neutrophils recruitment and elevated production of cytokines, chemokines and other pro-active molecules[4]. Inflammatory response comprehends innate (for instance Toll-like receptors, INF) and adaptative (T-lymphocytes for the main part) immune response. Furthermore, sterile inflammation is characterized by inflammatory cells accumulation, that contribute to increase tissue injury. For example, endothelial cells play an important role in microvascular alteration, while neutrophils release ROS and different cytokines (IL-1, IL-6, TNF-α); moreover platelets, activated by tissue damage, tend to aggregate and adhere to endothelium, causing potential thrombogenic events; finally mastocytes are stimulated in degranulation with release of pro-inflammatory mediators; macrophage and Kupfer cell role will be describes in next paragraph.

1.4 Cellular death

For many years scientific community considered cell death due to IRI occurred only for necrosis, related to different factors such as ATP depletion, mechanical damage to cellular structures and inflammation action. Nowadays other mechanisms of cellular death are known. They are: apoptosis, necroptosis and autophagia.

Necrosis

It is defined as death of cells in tissue or organ inside living organism. Necrotic cells share some characteristics: increased eosinophilia, nuclear alteration (ballooning, disappearance), intracellular organ alterations and loss of cellular borders [4]. Necrosis is generally associated to inflammatory reaction. Different aspects of necrosis are described: coagulative, colliquative, caseous, steatonecrosis.

Apoptosis

Also called planned death. It is the death of single cells in a tissue, with the purpose of eliminating damaged or not necessary cells. Apoptosis needs different pathways activation and, consequently, it is a energy-consuming process. It is present in physiological processes, like planned death of cells during embryogenesis, tissue involution in adult age, disruption of cells after their function (neutrophils after acute inflammatory phase). Furthermore, apoptosis can occur in pathological settings; for instance after cellular DNA alteration or oncogenic mutations. Apoptotic cell morphology is characterized by initial reduction of cellular volume and subsequent cytoplasmic external offshoots until apoptotic bodies creation[4] (Fig. 2). Apoptotic bodies will be destroyed by macrophages within 12-16 hours, without any cellular release. There is, consequently, no inflammatory response. Two specific pathways are described in apoptosis mechanism: intrinsic and extrinsic way.

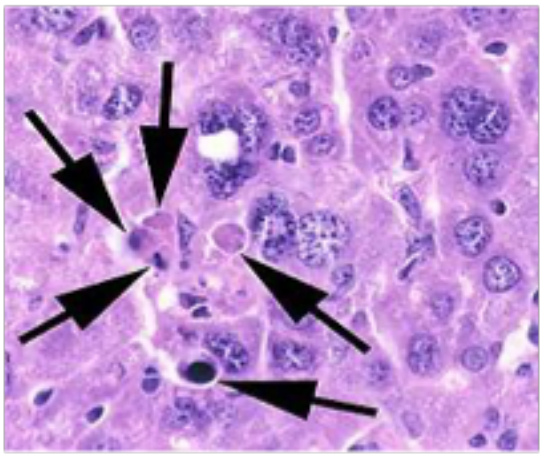


Fig 2. Apoptotic bodies indicated by arrows

Necroptosis

Also called planned necrosis. It shares peculiarities both of apoptosis and necrosis. Similarly to apoptosis, it depends by planned cellular pathways. As necrotic cells, in this type of death, cells show ballooning, intracellular organs alteration and finally cellular membrane disruption [4].

Autophagia

It is a process where the cell digests its own content, through transport and degradation of cytoplasmic materials in the lysosome. Autophagia occurs also in physiologic settings, to maintain cellular integrity in stress conditions [4].

1.5 Therapeutic strategies towards IRI

It is already known that hypothermia represents an important strategy to reduce tissue injury, since it reduces cellular metabolism and, so, also oxygen and energy demand.

In the same way, rapid reperfusion is the best option to decrease post-ischemic and post-reperfusion damages.

Therapeutic methods to minimize IRI effect comprehend: ischemic preconditioning, anti-oxidant agents, nitric oxide (NO) administration and anti-leucocyte factors.

Ischemic preconditioning consists in short ischemic periods with rapid reperfusion, to prevent subsequent tissue damages after prolonged ischemic phase[8-9]. Its advantages were demonstrated both in vitro (inhibition on macrophages accumulation, prevention on intracellular phosphate depletion) and in vivo (mortality and morbidity reduction after myocardial infarction). Preconditioning causes two protective phase: early and late. Early protection period occurs immediately after preconditioning and lasts 2 hours. During this period tissue is in latent condition. After 24 hours starts the second protective phase, the late one, that prevent organ injury for the following 72 hours. In cardiac tissue, it was demonstrated that preconditioning was able to reduce ischemic area of 40-75% in early phase and of 25% in the late one. Mechanisms of action of ischemic preconditioning are complex and not completely identified. Key role seems to be made by protein kinase C (PKC).

As described previously, ROS represent one of the main factors responsible of tissue injury. Pharmacological research focused on individuation of anti-oxidant agents. Some examples are:

iron- transporters, acid arachidonic metabolites, ACE-inhibitors, xanthine-oxidase inhibitors, mannitol, allopurinol, vitamin C, vitamin E. Despite encouraging experimental results, their efficacy was not confirmed in clinical setting.

NO is mainly employed in lung transplant and ARDS setting, by inhalation form. Its advantages are due to vasodilatation action that enhances ventilation/perfusion rate in most injured areas.

Also as regards anti-leucocytes agents, pharmacological experiments were not converted in clinical conditions.

2. ISCHEMIA-REPERFUSION INJURY IN LIVER

2.1 Physiopathological details

IRI hepatic setting was firstly studied by Toledo-Pereyra [10] in 1975 in experimental studies on liver transplant. Despite double circulation (arterial and portal) and glycogen reserve to use in case of ATP depletion, hepatic tissue can be involved by ischemia-reperfusion injury. Mild damage occurs mainly in central area of hepatic lobules, while severe damage involves all areas in the lobule and causes ischemic hepatis. The most susceptible cells to IRI are the hepatocytes and endothelial-sinusoidal cells. The firsts are sensible to warm ischemia damage (flow interruption in normothermic condition), while the seconds suffer in cold ischemia phase (flow interruption in hypothermic condition).

Numerous factors influencing hepatic IRI were recognized.

The principle one is represented by the ischemia length. In fact, short periods (less than 60 minutes) are well tolerated and cause reversible damages; on the other hand, reperfusion after a long ischemic period (120-180 minutes) induces an irreversible injury. That is the reason to identify 90 minutes as the cut off value that guarantees hepatocytes survival [11].

Another relevant factor that influences IRI consequences is liver steatosis. Many papers evidenced that steatotic livers are more susceptible to ischemia-reperfusion injury if compared to normal livers, because of their own microvascular disfunction and major sensibility to ROS[12-13]. In particular, steatotic organs develop massive necrosis as consequence to reperfusion, differently to apoptosis seen in normal livers [14].

Patient age is related to increased IRI entity, as demonstrated by experimental studies where it was observed pronounced neutrophils activity and increased intracellular ROS levels [15].

Finally, clinical patient status was recognized as important factor conditioning IRI. For instance, a good nutritional aspect seems to protect the liver, since means high glycogen supplies to employ in case of ATP depletion [16].

Studies conducted by Jaeshke and collegues [17] show that liver damage due to IRI can be divided in two phases. The initial period, within 2 hours since reperfusion, is characterized by oxidative stress due to Kupfer cells; the late phase, 6 hours after reperfusion, has in neutrophils recruitment its main feature.

The exact death mechanism of hepatic cells after IRI is still unclear. Some scientists described apoptosis as the main form of death, while others focused on necrosis. As a consequence, nowadays it is used the term necroapoptosis, that comprehend peculiar characteristics of both necrosis (high inflammatory cytokines) and apoptosis (severe ATP depletion)[18].

Hepatic IRI can cause systemic consequences on other organs, such as acute renal insufficiency, that represents the most common associated complication, ARDS as regards lung involvement, intestinal alterations in motility and absorption, rarely acute pancreatitis.

In the following paragraphs will be described the two principal settings in hepatic surgery where IRI could develop.

2.2 Hepatic resection

Liver resection is a common surgical procedure, employed mainly in oncologic setting but also for benign hepatic and biliary diseases. The central part of the surgery is represented by parenchyma transection, realized through different coagulator tools. In order to reduce hematic loss, during parenchymal transection, surgeon employs vascular clamping for the necessary period. At the end of procedure, vascular pedicle is declamped and blood flow restored. Ischemia-reperfusion injury can occur, particularly in case of difficult or multiple hepatic resection, that mean prolonged vascular clamping. In post-operative period an increase in transaminase level, in particular if more than 20 times normal value, reflects suffering of residual liver parenchyma. It is important to notice that biochemical parameters like bilirubin and INR are the main signals of hepatic insufficiency.

2.3 Liver transplant

Independently by the cause, liver transplantation procedure comprehends a series of determined phases: hepatectomy, after vascular clamping and section of vein cave, portal vein and hepatic artery; implant of graft with performing of vascular sutures; deplamping with organ reperfusion; biliary anastomosis. In transplant setting, ischemia-reperfusion injury involves the

transplanted graft. Organ is exposed to ischemic period that starts since procurement until reperfusion, after vascular declamping in the recipient.

The principal factors conditioning IRI in liver transplant are divided in recipient and donor characteristics. Among the recipient one there are: general patient condition, as described above for hepatic resection, and cause of liver disfunction. Cirrhotic patients have generally coagulation disorders, with tendency to pro-thrombotic pattern. Pro-thrombotic profile, particularly elevated in steatohepatitis, increases risk of IRI. Donor risk features comprehend: hepatic steatosis, age, long ischemic period, non-heart beating donors.

Liver from old donors, as steatotic livers and non-heart beating grafts, are called "marginal donors" since are related to increased primary non function or delayed non function after transplant.

As regards steatosis, it seems to be the most relevant factor influencing graft function. In particular macrosteatosis (>30%) is related to increased transaminase levels and inferior hepatic function[19] . Talking about age, livers from donors aged more than 70 years are more susceptible to IRI, if compared to younger grafts [20-21]. Furthermore, non-heart beating livers, in comparison with graft preserved in cold storage, need to replace blood flow interruption during warm ischemia with severe ATP storage depletion[22.] These types of livers are, consequently, at increased risk of developing IRI.

2.4 Therapeutic strategies towards hepatic IRI

Hepatic IRI can be minimized through classical approaches and innovative methods.

Among classical strategies there are: ischemic preconditioning (IP) and pharmacologic agents. As described in previous paragraphs, IP consists in exposing the organ, in this case the liver, to short ischemic periods followed by rapid reperfusion. The aim is to prevent tissue injury after a longer ischemic period. The effect of preconditioning seems to be a sort of increased resistance towards oxidative stress in treated organs; possible mediators of this response are nitric oxide and adenosine. IP efficacy was evaluated in different experimental models of hepatic ischemia, until identification of the protocol of liver protection. It consists in ischemic periods of 3-5 minutes followed by reperfusion phase lasting 15 minutes. Two hepatoprotective frames are described: the classical one (acute or early), that starts few minutes after reperfusion and lasts 4 hours; the delayed frame develops for 48-72 hours, but is less represented in liver[23].

Some studies show that preconditioning could manifest protective effect not only on the treated organ, but also in distant tissues. For instance, experimental model of IP in hemi-liver demonstrated protection towards IRI in the liver itself and, moreover, in the kidney (remote

preconditioning)[24] . A recent paper evidenced the clinical benefits of liver preconditioning after transplantation, in terms of decrease of transaminase levels and infectious complications [25] .

Pharmacological agents described in literature to prevent liver IRI are the following:

- Serine-protease inhibitors: limit warm ischemia damage in experimental model of non-heart beating organs [26]
- Streptokinase: enhances hepatic microcirculation [27]
- N- acetilistidine: in liver grafts with micro vesicular steatosis, inhibits inflammatory response and increases resistance to oxidative stress [28]
- Epidermal Growth Factor (EGF) and Insulin Growth Factor (IGF): enhance hepatic values of liver damage in experimental models of liver transplant [29]
- RAAS system modulators: angiotensin inhibitors (AT-1R and AT-2R) display a protective effect mainly in steatotic livers, in terms of reduction in cellular necrosis and enhancement of global survival [30]
- Modulators of cytokines and oxidative stress: monoclonal antibodies anti-LPS are associated to better outcomes if compared to controls in murine model of hepatic ischemia; methylprednisolone therapy reduces necrosis and inflammatory cytokines, but not in obese rats [31]
- Lipidic metabolism regulators: manipulation of lipidic supplies entails benefits in macro steatotic livers [32]
- Adenosine modulators: in experimental model of liver transplant in pigs, adenosine or selective agonists of A2 receptor reduce transaminase levels and increase biliary production [33]
- Complement modulators: C1 inhibitors decrease hepatocyte damage e promote tissue regeneration [34]
- EPO: erythropoietin, an hypoxia-induced cytokine, plays protective role in experimental model of liver graft [35]
- Melatonin: anti-inflammatory activity through regulation of TNF-α and iNOS in a rat model of liver IRI; increases neutrophils apoptosis in clinical study of hepatic resection[36]

Innovative approaches to contrast hepatic IRI are represented by machine perfusion systems and staminal cells.

Machine perfusion (MP) is a recent revolutionary system, studied about 10 years ago as graft preservation method alternative to static cold storage (SCS). It is employed in standard clinical practice in numerous transplant centers. Many organs can be treated with MP It consists in a perfusion circuit where the liver is placed after the procurement, through portal and arterial connections. Depending on the type of machine, the graft is exposed to normothermia or hypothermia. Moreover, it is supplied by oxygen, erythrocytes and nutrients elements (such as biliary acids, glucose), in order to maintain active its metabolism. During machine perfusion, clinicians can assess liver function by evaluation of biliary production and study of biochemical and pH parameters.

Hypothermic machine perfusion (HMP) employs hypothermia principle to reduce cellular metabolism, in order to prevent ATP depletion. Numerous experimental studies demonstrated HMP efficacy both in preclinical and clinical settings, in particular in DCD (donation after circulatory death) organs. Lee and colleagues show an increased survival, associated to marked histological and biochemical enhancement, in DCD grafts treated with HMP for 5 hours [37].

Normothermic machine perfusion (NMP) exploits normothermia (37°C) to maintain metabolically active hepatic cells. The advantage of this device is to avoid ischemia-reperfusion phase, since the graft is placed in the machine immediately after procurement and removed to be implanted in the recipient. NMP is the principal example of pre-transplant liver assessment. In fact, during the perfusion, the quality of the graft can be evaluated in a condition very similar to the recipient setting. Oxford group compared NMP and SCS in porcine grafts including DCD and non-DCD donors: groups treated with NMP showed reduced IRI and transaminase levels after transplant, in comparison to the control group (SCS)[38]. Efficacy of NMP was already demonstrated in clinical trials, in terms of cellular damage reduction, transaminase and cholestasis decrease and biliary production enhancement [39].

Stem cells represent an attractive strategy to prevent ischemia-reperfusion injury. In past years it was already demonstrated the protective role towards injured tissues of mesenchymal stem cells (MSC) and their derived molecules [40]. In the following paragraphs we will focus our on the effect of Human Liver Stem Cells (HLSC).

3. HEPATIC STEM CELLS AND DERIVED PRODUCTS

3.1 Stem cells

Stem cell is defined as a cell with two essential characteristics: immortality in replication and self-renewing. In fact, stem cells are able to divide themselves in unlimited way over time; every division creates two son cells with differential pathway: one become identical to mother cell, in order to maintain the staminal pool; the other one proceeds towards following differential steps and will become a mature differentiated cell.

According to their capacity to create different mature cell lines (potency), stem cell can be classified in [41]:

- Totipotent stem cells: able to differentiate in every type of cell line, embryonal and extra-embryonal; so, they are able to generate a complete organism. Zygote and embryonal cells within first two-three cellular divisions are considered totipotent stem cells.
- Pluripotent stem cells: able to differentiate in each type of germinal sheets; as consequence they can generate every kind of cells of adult but not extra-embryonal tissues. For instance, embryonal cells in blastocysts phase are considered pluripotent.
- Multipotent stem cells: able to differentiate in a limited type of cellular lines, belonging to the same differentiating line. An example is represented by hematopoietic stem cells, that can generate all blood cells, but not other cell variety.
- Oligopotent stem cells: able to differentiate in restricted kind of cells. Lymphoid and myeloid staminal cells can be considered oligopotent.
- Unipotent stem cells: able to differentiate in a single cell line, that correspond to the original tissue. They differ from non-staminal cells for their ability of self-renewing. For instance, muscular stem cells are unipotent.

Adult stem cells (ASC) are staminal cells that can be detected in the tissues of the organism, after complete embryonal development. They are also called Somatic Stem Cells or Tissue-Specific Stem Cells. Their role is to guarantee the renewal of that tissue, both in physiological and pathological conditions. They are generally multipotent, oligopotent or unipotent cells. Adult pluripotent stem cells are, in fact, very rare and limited in number.

Some examples of ASC are represented by Mesenchymal Stem Cells (MSC), hematopoietic stem cells, adipose tissue-derived stem cells and endothelial stem cells. Nevertheless, groups of ACS were discovered in other organs, such as brain, skeletal

muscle, skin, spleen, pancreas and liver. That is ad indirect proof of their key role in tissue regeneration[42] .

It is generally thought that ASC are limited to their original tissue as regards differential capacity; nevertheless, different studies suggest they are able to differentiate also in other cell lines [43] .

Nowadays MSC represent an excellent interest point as regards regenerative medicine, both for their own characteristics and because, for their production, it is not necessary an embryonal tissue. In fact, they could be employed as mean to restore injured tissues in order to maintain physiological function [44] .

3.2 HLSC

The liver has unique regenerative ability, described for the first time in 1931 by Higgins and Anderson, that observed a complete restore in hepatic mass after subtotal hepatectomy in rats.

Liver embryonal development is well known and consists in differentiation of hepatoblasts, progenitor cells, into hepatocytes and cholangiocytes. On the contrary, hepatic renewal mechanisms in adult liver are still unclear and under debate. It is important to make a distinction between the physiological turnover and the proliferative response after harmful event. The first process occurs slowly and is realized by mature hepatocytes; in the second case, it is possible that multipotent stem cells resident in liver parenchyma play an active role. In particular, when the damage occurred is relevant or chronic, the normal turnover is not sufficient to restore tissue injury; is then necessary ASC activation [45]. Hepatic ASC nature, location and mechanism of action are not completely clear. Many studies on rats demonstrated the presence of intrahepatic niches, containing stem cells able to promote tissue regeneration when physiological turn-over is insufficient [46-47] . This type of cells is also called “oval cell”; their molecular profile is well characterized in murine models [48] , but their counterpart in human liver was never isolated and described comprehensively [42, 49] . In humans, important studies demonstrated the presence of small cells with staminal characteristics within Hering canals[50]; these cells are able to generate, when necessary, hepatocytes and cholangiocytes through a switch on/off mechanism, mediated by paracrine molecules, secreted by cells in response to inflammatory reaction [51, 52] .

In 2006, Camussi and colleagues isolated a population of cell with ASC features, from adult liver biopsies [53] . These cells, defined Human Liver Stem Cells (HLSC), express

typical marker of MSC (such as CD29, CD73; CD44 and CD90), but in the same time express liver-specific markers (for example albumin, αFP). We can affirm that show a partial hepatic commitment. On the contrary of hematopoietic stem cells, HLSC do not express specific markers of hematopoietic compartment; furthermore, they cannot be identified with oval cells, for their differential ability and for absence of superficial markers, like CK19, CD117 and CD34. If cultured with growth factor as HGF and FGF4, HLSC can differentiate in mature hepatocytes. But they can also differentiate in other cell types, if cultured with specific means. For instance, they can generate structures similar to pancreatic insulae or differentiate towards osteogenic or endothelial lines.

Thanks to their demonstrated potency and their relatively easy detection, HLSC become in few times a promising instrument for regenerative medicine.

Numerous papers reported HLSC properties in experimental models. Herrera demonstrated HLSC ability to promote hepatic regeneration after acute liver insufficiency induced by N-acetil-p-aminophenole in SCID mice[52-53]. Another interesting study showed the protective role played by HLSC in a lethal model of fulminant liver insufficiency in SCID mice; HLSC administration by systemic way or by direct injection in liver parenchyma was able to guarantee animal survival, restore liver function and complete restitutio as integrum of liver tissue [54]. Finally, ex-vivo experiments regarding re-cellularization of hepatic rat bio-scaffolds, demonstrated regenerative capacity of HLSC and their organ-specific differentiating ability [55].

3.3 Extracellular vesicles

Extracellular vesicles (EV) are membrane vesicles, released by cells in order to maintain homeostasis and intercellular communication. Firstly, EV secretion was describes as a modality to eliminate discarded cellular components, but subsequently, their role was identified as transporters of important molecules, such as proteins, nucleic acids and lipids both in physiological and pathological settings [56] .

The term EV comprehends, actually, two different vesicles chategories:

- Exosomes: intraluminal vesicles with diameter range 50-100nm, released by endosomal membrane and, then secreted through endosome fusion with lysosomes or plasmatic membrane

- Microvesicles: vesicles with diameter range 100nm-1μm, originating by external budding from plasmatic membrane.

EV content can comprehend different bio elements: proteins, lipids, nucleic acids.

As regards proteins, they can derive from plasmatic membrane or cytosol. Examples of proteins are adhesion molecules (CD9, CD11, CD18, CD146, CD166) and heat shock proteins (Hsp70 and Hsp90).

Lipids contained inside EV are numerous; were identified cholesterol, phospholipids, sphingolipids, diglycerides and many others. It was demonstrated that can be present also bio active lipids, such as prostaglandins and leukotrienes [57].

Relatively to nucleic acids, different kind were characterized: RNA, mRNA and miRNA. miRNA is generally linked to Argonaute proteins (Ago) and, through them, can influence hundreds of mRNA [56, 58].

EV mechanism of action seems to be mediated by different modality: membrane receptors activation, internalization and plasmatic membrane fusion.

Extracellular vesicles play an important role both in physiological and pathological conditions. For instance, during embryonal development, they are implied in the correct tissues organization; furthermore, in nervous system, EV are essential for intercellular communication. As regards pathological contests, EV were studied in cancer. As shown in the Figure 3, cancer cells release particular vesicles, named oncosomes, that contribute to create environment for cell growth, vascular invasion and metastatization [59].

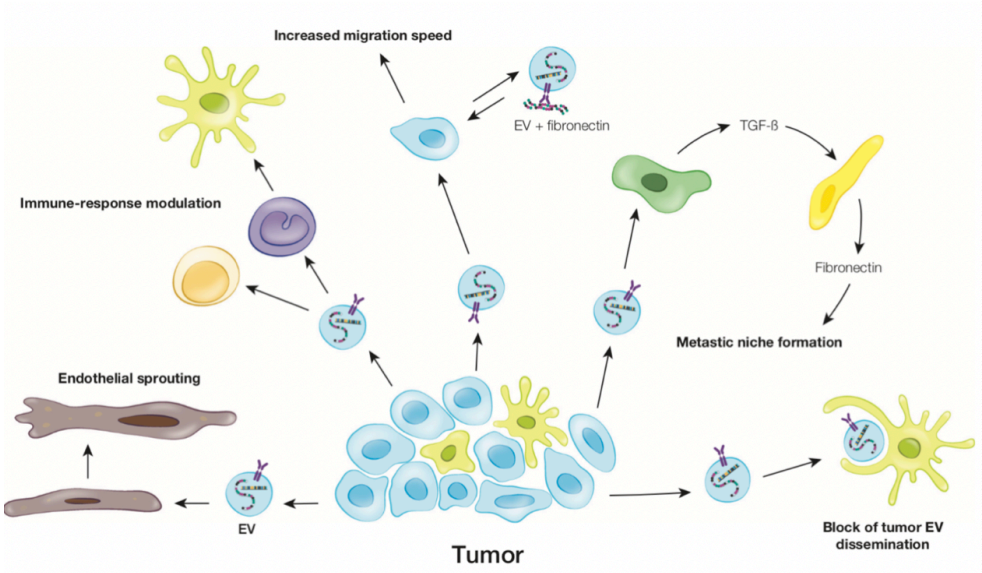


Fig 3. Role of EV released by cancer cells. Picture adapted by Trend in Cell Biology 2017

Moreover, EV can also be produced by stem cells. Several studies suggest that the regenerative effect of EV is comparable to their staminal cell of origin and is ascribed to a horizontal transfer of proteins, lipids and specific subsets of mRNA and miRNA[57, 60, 61].

EV isolation is a quite complex procedure, for their dimension and frailty. For this reason, in many studies EV are purified from cellular cultures, through centrifugation process that consents to separate vesicles from cellular debris. One possible problem could be the risk of fragmentation or fusion of vesicles during centrifugation procedure, in particular if employed high acceleration values.

Pharmacological employment of EV was studied since 10 years ago. In particular, exosome from MSC derived from bone marrow, umbilical cord and placenta. MSC-EV efficacy consists in immunomodulatory and anti-inflammatory actions, through paracrine mechanism. Some experimental examples where MSC-EV were exploited are represented by hepatic IRI, acute liver insufficiency, hepatic fibrosis, bowel inflammatory diseases, myocardial IRI, acute renal injury glicerole-induced, cerebral stroke, pulmonary hypertension and graft-versus host disease in skin transplant. In all the listed models it was evidenced organ function restoration, damaged cells regeneration, reduction in inflammatory cytokines, anti-apoptotic action and anti-oxidative effect, induced by EV [61].

3.4 HLSC-EV

Human liver stem cell-derived extracellular vesicles (HLSC-EV) are a category of EV with promising potentiality in regenerative medicine setting. They have diameter of 174±64 nm and express typical exosomal markers, such as CD81, CD63 and Hsp90, and MSC markers.

Several studies demonstrated regenerative and protective effects of HLSC-EV towards injured tissues. In nephrological setting, they promote functional restoration in a murine model of acute kidney insufficiency glicerole-induced [62]. Furthermore, Kholia described HLSC-EV effect in preventing kidney fibrosis in a murine model[63].

As regards liver experiments, HLSC-EV efficacy was evidenced in numerous models on small animals. HLSC-EV were able to reduce apoptosis and promote hepatocytes proliferation in a mouse model of partial hepatectomy; furthermore, the treatment with EV reduce significatively transaminase levels and produced better outcome in comparison to control groups[64.] In this paper was also identified α4-integrine as key molecule in internalization of HLSC-EV inside hepatocytes (Fig.4). More recently, Rigo and colleagues described the beneficial effect of

HLSC-EV on rat livers perfused *ex-vivo* in NMP under hypoxic conditions [65]. In detail they demonstrated HLSC-EV can integrate in hepatocytes after 4 hours of perfusion; hypoxic livers treated with EV during NMP showed decreased AST and LDH values and reduced apoptosis and necrosis, in comparison to control organs. Furthermore, Camussi and colleagues conduced an in vitro study in order to evaluate HLSC-EV efficacy in Citrullinemia type 1. It is an autosomic recessive pathology due to enzyme Arginine-Succinate Sintase 1 (ASS1) defect. HLSC-EV isolated from liver biopsy of affected patient, were able to transfer both mRNA coding for ASS1 and the own enzyme. Consequently, ASS1 activity was restored, as urea production [66]. Finally, a recent paper by Lopatina [67] demonstrated as HLSC-EV, on the contrary of MSC-EV, are able to inhibit tumoral angiogenesis, by suppressing gene expression of related molecules.

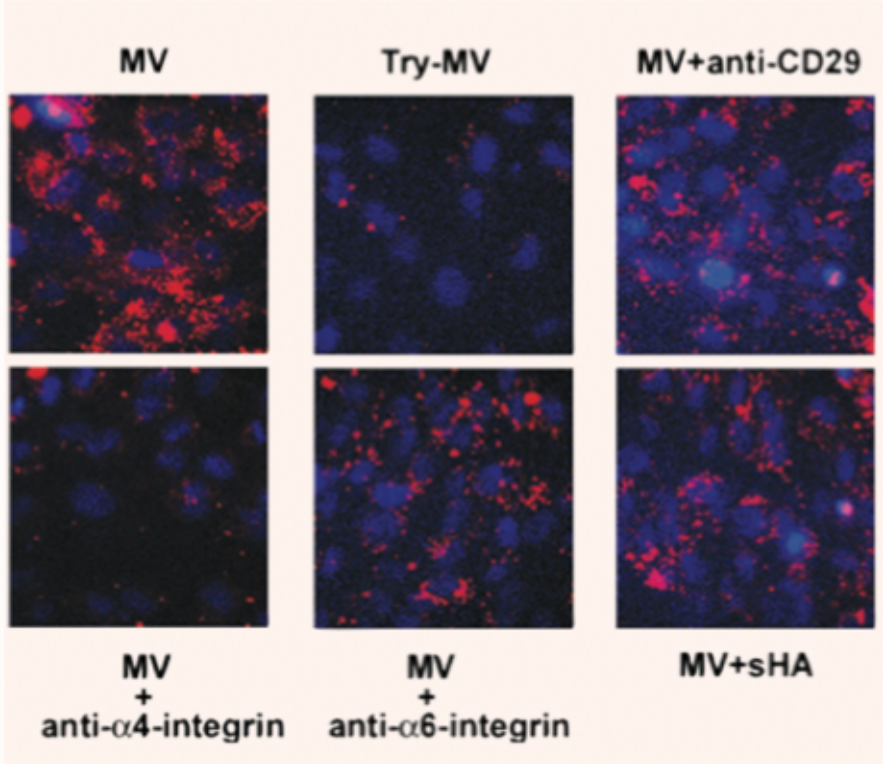


Fig. 4 HLSC-EV microscopies: pre-incubation of HLSC-EV with antibodies anti-α4-integrine inhibits their internalization in the hepatocyte. Adapted images from Human liver stem cell-derived microvesicles accelerate hepatic regeneration in hepatectomized rats, Herrera MB, 2010

Among advantages of HLSC-EV, there is the same protective action of cells of origin, without collateral effects of stem cells (immunity, rejection). Moreover HLSC-EV can be easily preserved at -80°C for 6 months, maintaining their properties.

4. EXPERIMENTAL STUDY

Aim of the study

Reflecting on the key role of ischemia-reperfusion injury in relevant liver settings, taking into account the previous brilliant results of MSC-EV in treating hepatic IRI and considering the possibility of HLSC-EV to integrate in damaged liver parenchyma, we propose an experimental study on a murine model of hepatic IRI.

The aim is to evaluate the effects of HLSC-EV in this model and thereby to assess their therapeutic potential in tissue repair.

5. MATERIALS AND METHODS

5.1 Mouse model

Animal studies were performed following the protocol approved by the Ethic Committee of the Italian Institute of Health (Istituto Superiore di Sanità, N.62/2016-PR), and conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

Experiments were conducted on 42 male C57BL/6 mice at 8-12 weeks of age.

Animals were located in cages at controlled temperature and humidity, with free water access until the experiment and free food access until the night before the experiment.

Murine liver is the major organ, located in abdominal cavity in right subcostal side. It is composed by 5 distinctive lobes, divided by deep fissures, as shown in Figure 5. Hepatic hilum is extrahepatic and formed by portal vein, hepatic artery and bile duct. Like in humans, there is the gallbladder.

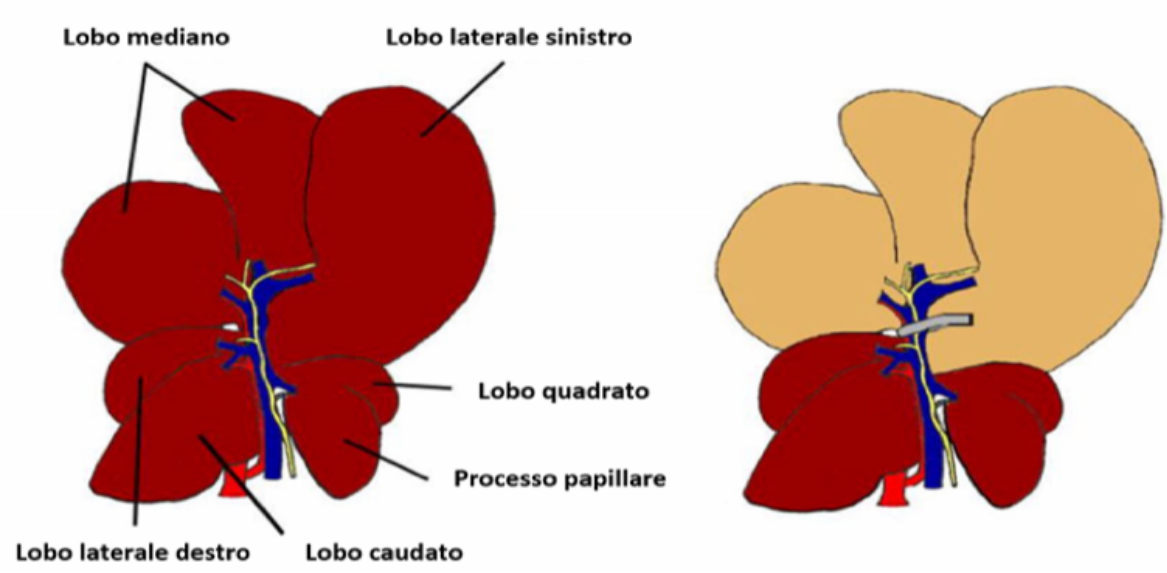


Fig. 5 On the left normal anatomy of murine liver. On the right, partial ischemic liver after selective hilum clamping. Abe et al Free Radical Biology & Medicine, 2009

5.2 Surgical operation

All animals underwent total anesthesia, through an intramuscular injection of Zolazepam+Tiletamina (Zoletil) (0.2 mg/Kg) and Xilazina (Rompun) (16 mg/Kg). Surgical procedure, was preceded by thoracic and abdominal trichotomy and alcoholic disinfection. Animals were placed on rigid support, with four arms fixed with atraumatic devices. Surgical procedure, illustrated in the pictures below, was conducted using stereoscopic microscope. First surgical time was a midline xifo-pubic laparotomy, with particular caution to avoid liver, bowel or bladder injury. The falciform ligament was cut, the liver mobilized and small bowel disposed on the left abdominal quadrants, allowing the exposure of the hepatic hilum. An atraumatic clamp (Micro Serrafine, length 17mm) was then placed on left vascular pedicle, in order to interrupt both arterial and portal blood supply to the left lateral and median lobes (approximately 70% of total liver parenchyma).

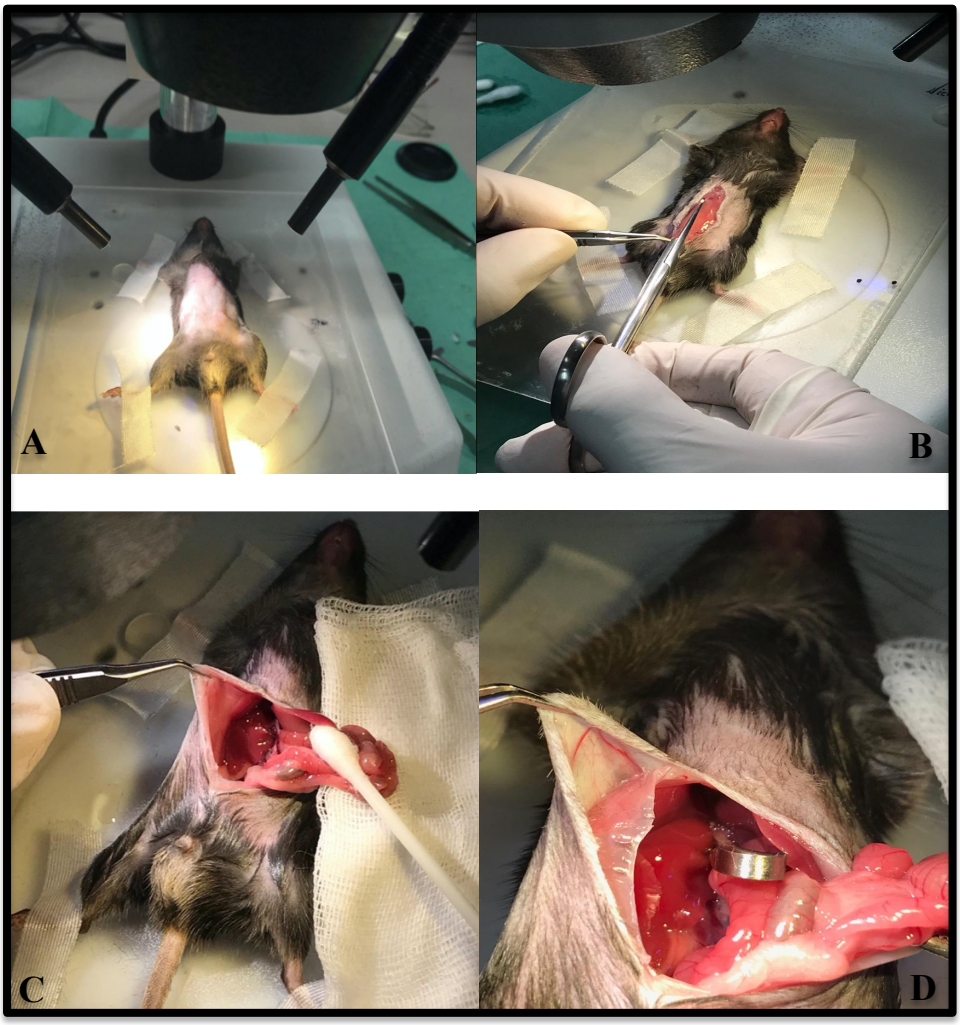


Fig. 6 Surgical procedure phases: A: animal positioning; B: abdominal opening; C: hepatic hilum exposure; D: selective clamping

If no macroscopic variation in liver color is observed, a repositioning of the clamp has to be considered. The non-ischemic lobes (right lateral, caudate and square lobes) guaranteed a portocaval shunt that avoided intestinal congestion during the ischemic period. The clamp was left in place for 90 minutes. Small bowel was replaced on physiological position. To prevent hypothermia, the abdomen was closed through a cutaneous running suture (silk 6/0) and the animal was kept warm under an infrared lamp. After 90 minutes of warm ischemia, the abdomen was re-opened and the clamp removed, allowing reperfusion of the total parenchyma. According to group division, immediately after reperfusion, HLSC-EV or saline solution was administered through tail vein injection. After 6 hours from reperfusion animals were sacrificed to collect blood and tissue samples.

5.3 HLSC culture and HLSC-EV isolation

The HLSC were isolated from human cryopreserved hepatocytes obtained from Lonza, Bioscience (Basel, Switzerland). The HLSC were cultured in a medium containing a 3:1 proportion of α-minimum essential medium and endothelial cell basal medium-1, supplemented with L-glutamine 2 mM, penicillin 100 U/mL, streptomycin 100 µg/mL and 10% fetal calf serum (α-MEM/EBM/FCS), and maintained in a humidified 5% CO_2 incubator at 37°C. HLSC at passages 5 to 8 were used in all the experiments.

The HLSC-EV were obtained as previously described[53]. Briefly, the HLSC were starved overnight in RPMI medium deprived of FCS at 37°C in a humidified incubator with 5% CO_2. Supernatants were collected and centrifuged at 3500 rpm for 15 minutes to remove cell debris. Supernatants were then collected and ultracentrifugated at 100,000 g for 2 hours at 4°C (Beckman Coulter Optima L-90 K, Fullerton, CA, USA). EVs were collected and labelled with 1 µM of Dil dye and 1 µM of Did dye (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate**;** Molecular Probes Life Technology, New York, NY, USA), then washed in phosphate buffered saline (PBS) and ultracentrifugated for 1 hour at 4°C. The collected Dil-Did stained EVs were used fresh or stored at −80°C after re-suspension in RPMI and 1% dimethyl sulfoxide (DMSO). No differences in biological activity were observed between fresh and stored EVs (data not shown). Quantification and size distribution of EVs were analyzed by using NanoSight LM10 (NanoSight Ltd, Minton Park, UK) with the NTA 1.4 Analytical Software as described previously [68].

5.4 Study groups

42 male mice C57, comparable in size and weight, were divided into five groups:

a) **EV1** group (n°10) was treated with $3x10^9$ HLSC-EV diluted in 120 µl of saline solution;
b) **EV2** group (n°9) received $7.5x10^9$ HLSC-EV diluted in 120 µl of saline solution;
c) **EV3** group (n°4) was treated with $10x10^9$ HLSC-EV diluted in 120 µl of saline solution;
d) control group (**CTRL**) (n°10) received 120 µl of saline solution;
e) **SHAM** group (n° 9) underwent the same surgical procedure of the other four groups, except for the clamping and the intravenous injection.

GROUPS	WEIGHT
SHAM	20,81 g ± 2,09
CTRL	22,06 g ± 2,71
EV1	23,29 g ± 2,38
EV2	22,29 g ± 3,47
EV3	23,63 g ± 2,17

Tab. 1 Animals weight in the study groups

5.5 Immunofluorescence analysis

HLSC-EV uptake was analyzed by immunofluorescence microscopy. After mouse death, tissue biopsies were taken from ischemic lobes and non-ischemic ones and criopreserved at -80°C. Through a cryotome, liver parenchyma slices of 3-5 mcm were obtained by cryopreserved tissue samples and then are fixed in acetone. After rinsing in PBS, slices were incubated for 5 minutes at 4°C with a permeabilization solution containing 20 mmol/l Hepes, 50 mmol/L NaCL, 300 mmol/L sucrose, 3 mmol/L MagCl2, 0,5 % Triton X- 100, pH 7.4. After washing with PBS, the slices were incubated for 1 hour at room temperature with a blocking solution of PBS added with 3% bovine albumin (both from Sigma-Aldrich) and incubated overnight at 4°C with an anti-mouse cytokeratin-8 primary antibody (1:200) (Abcam). At the end of the incubation, slices were washed with PBS and then incubated for 1 hour at room temperature with the Alexa Fluor 488-coniugated secondary antibody (1:200) (Invitrogen),

used as fluorescent marker. Thereby, the slices were washed with PBS and nuclei were stained with DAPI *(4', 6 diamidino-2-phenylindole)*. After a final washing in PBS, the slides were mounted with Fluoromount (Sigma-Aldrich). Microscopy analysis was performed using a Cell Observer SD-ApoTome laser scanning system (Carl Zeiss).

5.6 Biochemistry analysis

Blood samples (around 1 ml) were collected by cardiac puncture after mouse death. Serum was then separated by centrifugation (10 minutes at 3500 rpm) and stored at -80°C. Serum levels of aspartate amino-transferase (AST), alanine amino-transferase (ALT), lactate dehydrogenase (LDH), alcaline phophatase and phosphate were assessed by the Biochemistry Laboratory (Laboratorio Baldi e Riberi, Molinette - Città della Salute).

5.7 IVIS (In Vivo Imaging System) analysis

IVIS Spectrum CT (PerkinElmer, Waltham, MA, USA) is a platform of optic imaging, used to obtain small animal images in different preclinical settings. Its work modality are: bioluminescence and fluorescence.

In our experiments, we could perform biodistribution analysis of HLSC-EV, previously marked with fluorescent marker (Dil- Did), using epi-fluorescence modality.

The figure below shows schematically the mechanism of action of IVIS technology.

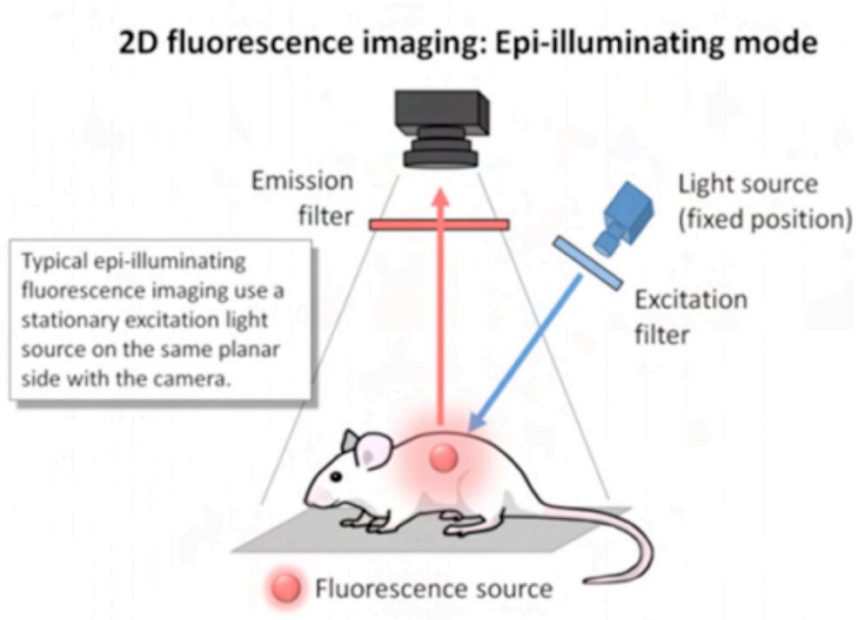


Fig.7 Functioning of Fluorescence in epi- fluorescence modality, at the basis of IVIS.
Picture adapted by "Perkinelmer.com"

Analysis was performed as the following timing:

T0: imaging obtained in vivo immediately after treatment administration;

T3: imaging obtained in vivo 3 hours after treatment administration;

T6: imaging obtained in vivo 6 hours after treatment administration;

ORGANS: imaging obtained ex-vivo after animal sacrifice

In vivo imaging is realized through anesthesia reinforce to avoid animal movements. Ex-vivo imaging is obtained by positioning the organs studied (liver, kidneys, heart, lungs, spleen, pancreas) in a non-fluorescent Petri dish (Fig.8). Images were acquired and analyzed using Living Image 4.0 software (PerkinElmer) through the designation of regions-of-interest (ROI). The fluorescence intensity was obtained and expressed as the Average Radiant Efficiency ([p/s/cm^2/sr] / [µW/cm^2])

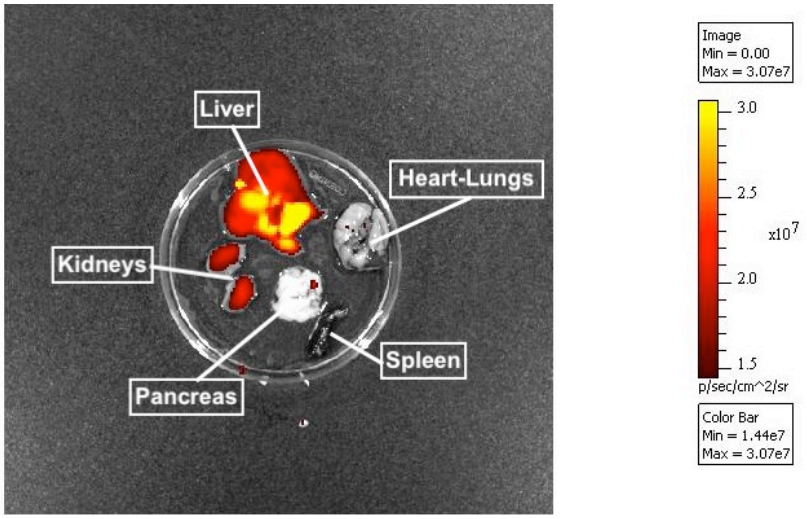


Fig.8 Fluorescence at IVIS of different organs, placed on Petri dish

5.8 Histological analysis

After mouse death, tissue biopsies were taken from ischemic lobes (IRI lobe) and non-ischemic lobes (NO IRI lobe) and then formalin fixed and paraffin embedded for hematoxylin-eosin staining (H&E). The severity of histological damage was quantified using the Suzuki's Score, that consider three components: sinusoidal congestion, hepatocyte necrosis and ballooning degeneration. Every criteria was graded from 0 to 4 (Tab 2)[21] Histological evaluation was blindly scored by an experienced liver pathologist (E.D.).

POINTS	CONGESTION	BALLOONING	IDROPIC DEGENERATION
0	Absent	Absent	Absent
1	Minimal	Minimal	Single cells
2	Slight	Slight	30%
3	Moderate	Moderate	60%
4	Severe	Severe	>60%

Tab.2 Suzuki score

In consideration of the short time to observe cells alteration (6 hours), the concept of necrosis was changed in "idropic degeneration". It corresponds to cells with picnotic nucleus with unclear margin with high probability to become necrotic over time.

Cellular proliferation was studied through PCNA (Proliferating Cell Nuclear Antigen) coloration. It is an immunohistochemical analysis that allow to evidence cellular nuclei in phase G1 and phase S of cellular cycle. For every slide, we obtained 10 representative pictures by optic microscope at 200x magnification. On micrographs it was performed blind manual count of positive and negative cells, with software ImageJ. Proliferation Index is obtained by rate of positive cells on total cell number. It is expressed in percentage.

TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) is a specific coloration used to identify apoptotic cells. It allows to detect fragmented DNA by caspase action. As or PCNA analysis, it was performed a blind manual count of positive and negative cells, with software ImageJ, after acquisition of representative micrographs at 200x magnification.

5.9 Quantitative reverse transcription polymerase chain reaction

In order to investigate HLSC-EV molecular mechanism of action, we analyzed, by RT-PCR, gene expression of possible molecules involved in cellular damage pathways.

The analysis is focused on genes coding for inflammatory citochines and chemochines (TNFα, CXCL-10 , CCL-2, IL-10, TLR-4 and IL-6); pro-apoptotic marker (BAX); anti-apoptotic marker (BCL-2); anti-oxidant and cytoprotective molecules (HO-1 and SIRT1); hypoxia-induced marker (HIF-1α (hypoxic-induced factor)); pro-fibrotic molecules.

In the table below are reported all the primers used for RT-PCR analysis.

Gene	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
BAX	TTTTGCTACAGGGTTTCATCCAG	GCCGGAGACACTCGCTCA
BCL-2	GAGGCTGGGATGCCTTTGT	CCAGGTATGCACCCAGAGTGA
CCL2	AAGCTGTAGTTTTTGTCACCAAGCT	TGGTTCCGATCCAGGTTTTTA
CXCL10	GTGTTGAGATCATTGCCACGAT	GGCTAAACGCTTTCATTAAATTCTTG
HIF1α	TTCTGGATGCCGGTGGTC	GTCGCCGTCATCTGTTAGCA
HO1	GTCGCCGTCATCTGTTAGCA	GTTGCCAACAGGAAGCTGAGA
IL-6	AAGAGTTGTGCAATGGCAATTCT	TGATTATATCCAGTTTGGTAGCATCC
IL-10	GACTTTAAGGGTTACTTGGGTTGC	TCCTGAGGGTCTTCAGCTTCTC
SIRT1	CTACCGAGACAACCTCCTGTTGA	CCAGTCACTAGAGCTGGCGTG7
TGFβ1	CAACAACGCCATCTATGAGAAAAC	CTCTGCACGGGACAGCAAT
TIMP1	GACCTGGTCATAAGGGCTAAATTC	TTAGTCATCTTGATCTTATAACGCTGGTA
TLR4	GAATCCCTGCATAGAGGTAGTTCC	GAATCCCTGCATAGAGGTAGTTCC
TNFα	AGGGATGAGAAGTTCCCAAATG	GCTTGTCACTCGAATTTTGAGAAG
ACTβ	GATTACTGCTCTGGCTCCTAGCA	GCCACCGATCCACACAGAGT

Tab.3 Genes and relative primers employed in RT-PCR. The selected mouse genes are: ACTβ (Actin β), BAX (BCL2-associated X protein); BCL-2 (B cell leukemia/lymphoma 2); CCL2 (Chemokine C-C motif ligand 2); CXCL10 (Chemokine C-X-C motif ligand 10); HIF1α (Hypoxia inducible factor 1, alpha subunit); HO1 (Heme oxygenase 1); Il-6 (Interleukin 6); IL-10 (Interleukin 10); SIRT1 (Sirtuin 1); TGFβ1 (Transforming growth factor β 1); TIMP1 (Tissue inhibitor of metalloproteinase 1); TLR4 (Toll-like receptor 4); TNFα (Tumor necrosis factor α).

In practice, mouse hepatic tissue was suspended in 1 ml of TRIzol™ solution (Ambion,Thermofisher) and homogenized in a Bullet blender (Next Advance Inc., New York, NY, USA) at a speed of 8 rpm for 3 minutes using 0.5 mm size zirconium beads. The homogenized tissue was collected and centrifuged at 12,000 g for 15 min at 4°C, and the supernatant from homogenized tissue was transferred to clean tubes and subjected to RNA isolation using TRIzol™ as per manufacturer's protocol. Isolated RNA was quantified spectrophotometrically using NanoDrop 2000 (ThermoFisher Scientific). High-Capacity cDNA Reverse Transcription (RT) Kit (Applied Biosystems™) was used to synthesize the cDNA from 200 ng of RNA. Then, a Real-Time Polymerase Chain Reaction (RT-PCR) (Applied Biosystems™) was performed on duplicate cDNA samples according to the chemistry of Power SYBR® Green PCR Master Mix (Applied Biosystems™), using the primers described in Table 1. Comparative ΔΔCt method was used to calculate the relative expression levels of the genes of interest normalized to the house-keeping gene expression Actinβ. Samples from the experimental sham group were used as reference for the quantitative analysis.

6. STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of the mean (SEM). Normality was tested through Shapiro-Wilk and Levene's tests. Statistical analyses were performed using one-way or two way ANOVA with Newman-Keuls multi comparison test where appropriate (GraphPad Prism, version 6.00, USA). A p value <0.05 was considered as statistically significant.

7. RESULTS

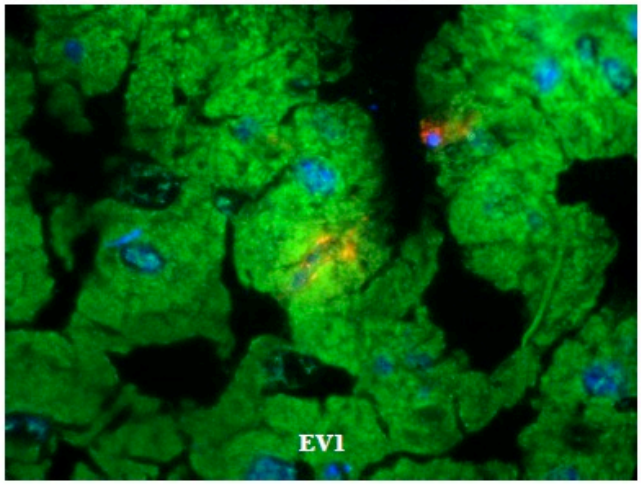
7.1 HLSC-EV treatment and animal survival

Survival of SHAM, CTRL, EV1 and EV2 groups was 100%. On the contrary, mice treated with maximal dose of HLSC-EV (EV3,$10x10^9$ particles) died within 3-5 minutes after trreatment administration. That is the reason of animal recruitment interruption (only 4 animals in the study group EV3). Consequently, we could consider the dose of $10x10^9$ particles the lethal dose.

7.2 HLSC-EV biodistribution

Immunofluorescence analysis revealed HLSC-EV uptake in liver parenchyma of treated animals at the end of the experiments. In particular, as shown in Fig. 9, vesicles were found both in the cytoplasm and in the nucleus of hepatocytes. This type of analysis is only qualitative and, consequently, it does not allow any quantitative comparison between EV1 and EV2 groups.

In vivo IVIS images obtained at t0, t3 and t6 were no analyzed since it was not possible to perform adequate measurements of organs fluorescence. Nevertheless, we noticed a tendency of major fluorescence in abdominal cavity corresponding to liver parenchyma (Fig.10).



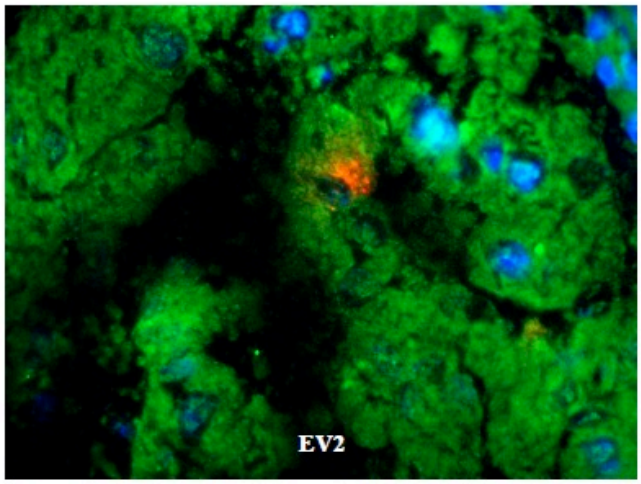


Fig.9 Representative micrographs showing DAPI-stained cell nuclei (blue), mouse anti-human cytokeratin-8 antibody immunofluorescence (green) and Dil/Did-stained HLSC-EV (red) (original magnification 630×).

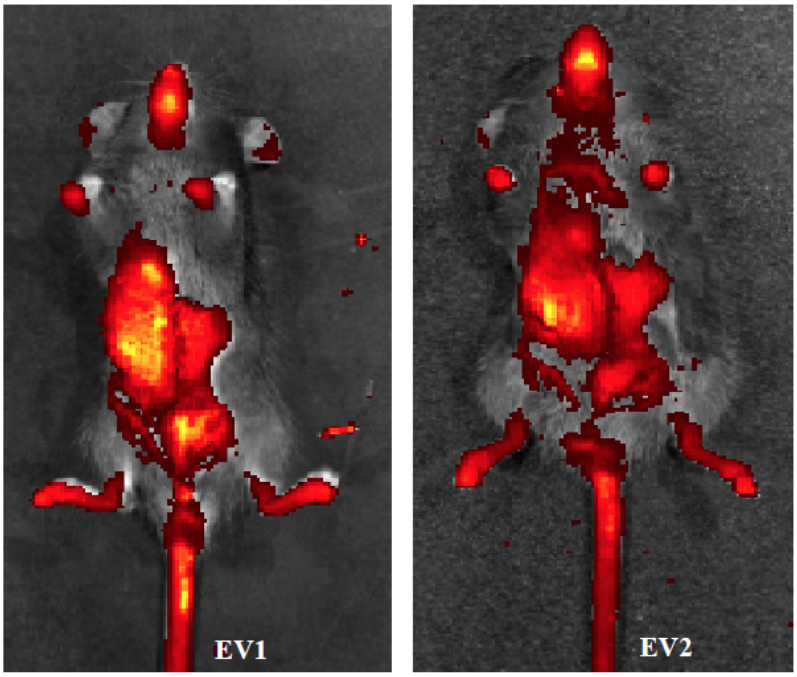


Fig. 10 IVIS images show major fluorescence in right abdominal cavity, corresponding to the liver.

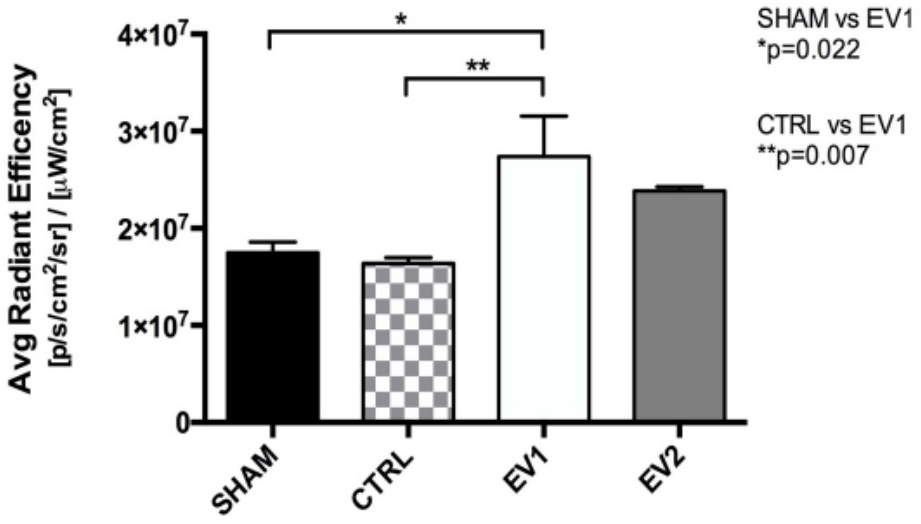
As regards IVIS analysis on dissected organs, we investigated two aspects: the first was the comparison between hepatic fluorescence in the different study groups; the second was the comparison of fluorescence in the different organs. The fluorescence intensity was expressed as the Average Radiant Efficiency ([p/s/cm^2/sr] / [µW/cm^2]).

Taking about hepatic fluorescence, in the table below are reported mean hepatic fluorescence values in the different groups.

N. animale	SHAM	CTRL	EV1 ($3x10^9$)	EV2 ($7.5x10^9$)
1	$1,68x10^7$	$1,76x10^7$	$1,33x10^7$	$2,36x10^7$
2	$1,94x10^7$	$1,55x10^7$	$1,65x10^7$	$2,43x10^7$
3	$1,98x10^7$	$2,00x10^7$	$1,61x10^7$	$2,36x10^7$
4	$9,08x10^6$	$1,56x10^7$	$4,85x10^7$	$2,53x10^7$
5	$1,90x10^7$	$1,59x10^7$	$2,11x10^7$	$2,14x10^7$
6	$1,79x10^7$	$1,44x10^7$	$4,10x10^7$	$2,51x10^7$
7	$1,75x10^7$	$1,39x10^7$	$4,67x10^7$	$2,46x10^7$
8	$1,80x10^7$	$1,53x10^7$	$1,96x10^7$	$2,20x10^7$
9	$1,99x10^7$	$1,82x10^7$	$2,55x10^7$	$2,47x10^7$
10		$1,74x10^7$	$2,56x10^7$	

Tab.4 Mean fluorescence values of animal livers.

From these data, through one way ANOVA with Newman-Keuls multi comparison test, we obtained the following results, shown in the graphic below.



Graph. 1 Statistical analysis of mean hepatic fluorescence obtained by IVIS

Firstly, hepatic fluorescence was almost the same in SHAM and CTRL group (p=0.99). On the contrary, EV1 group shows hepatic fluorescence significantly higher than control and sham groups (respectively p=0.007 and p=0.022). Furthermore, we did not evidenced relevant differences in hepatic fluorescence between EV2 and SHAM group (p=0.24) and between EV2 and CTRL group (p=0.18). Finally, the treated groups did not significantly differ between each other in liver fluorescence value (p=0.70).

It is important to underline that also control group animals own an intrinsic fluorescence, due to Iron atoms present in the blood and related to possible food interference.

As regards organs fluorescence analysis, our study focused on fluorescence comparison between liver, kidney, pancreas, spleen, heart and lungs. Fig.11 and graph. 2 show that in all groups hepatic fluorescence is significantly higher than in all the other organs (p<0.0001). Furthermore, it was observed a significantly higher fluorescence value in EV2 group compared to EV1 one in kidneys.

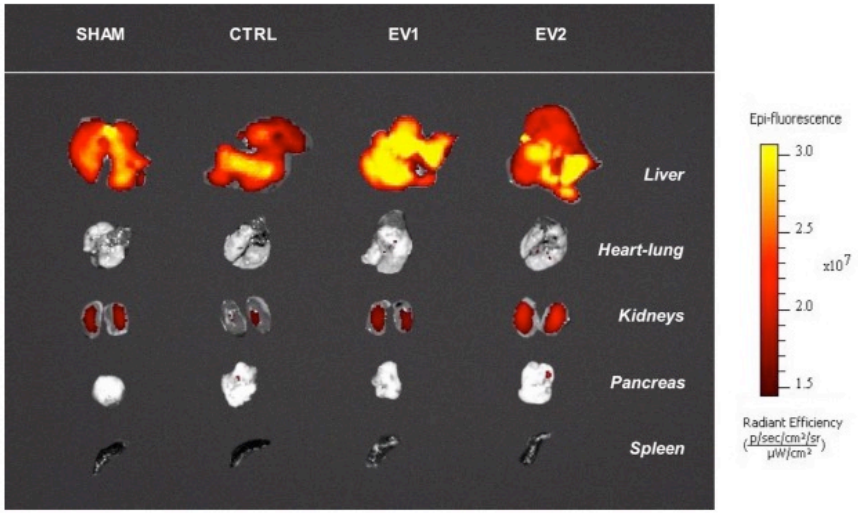
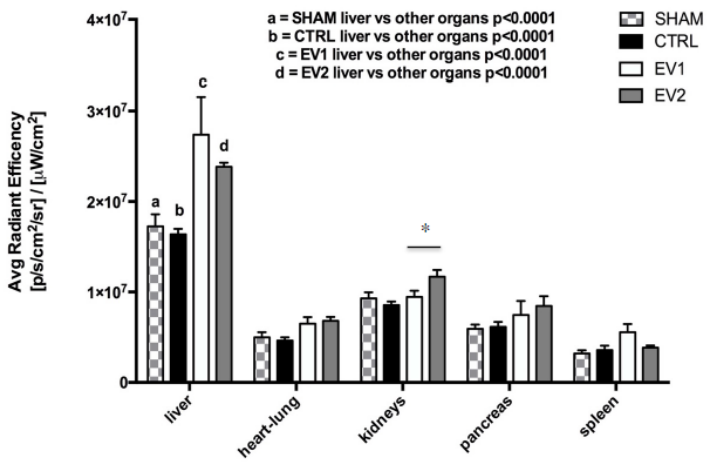


Fig.11 Liver, heart and lungs, kidneys, pancreas and spleen accumulation of Dil/Did-stained HLSC-EV



Graph. 2 Intensity of fluorescent signal detected *ex-vivo* after 6 hours. In all groups, liver vs other organs (p<0.0001), EV1 liver vs control liver (p<0.0001) and EV1 liver vs EV2 liver (p<0.01). Data are represented as mean ± SEM.

The biodistribution analysis illustrated above demonstrated firstly that livers treated with the EV1 dose were significantly more fluorescent than the livers treated with the EV2 dose and secondly that HLSC-EV were mostly localized in the liver, in comparison to other organs.

7.3 Biochemistry analysis

Aspartate transaminase (AST) is an enzyme present in the liver, but also in muscles, heart, pancreas and kidneys. In humans is used as parameter of liver function, since its increase is related to hepatocyte necrosis and, moreover, there is a linear correlation between injury entity and AST levels. After important hepatic damages, AST could rise within 4-6 hours and maintain elevated also for 4 days. Its value is generally measured together with ALT (alanine transaminase). ALT is more specific than AST because it is principally present in liver and only in small part in kidneys. LDH (lactate dehydrogenase) is an enzyme very little specific for hepatic injury because we can find elevated levels in different pathological settings (such as myocardial infarction, pulmonary infarction, hepatic cirrhosis, acute hepatitis, muscular dystrophy, renal insufficiency, shock status and neoplasia) but its sensibility to incoming injuries is strong.

In tables 5-6-7 are reported AST, ALT and LDH values at the end of 6 hours experiments.

N. animale	SHAM	CTRL	EV1 ($3x10^9$)	EV2 ($7.5x10^9$)
1	744	2.409	2.334	3951
2	525	399	216	1.605
3	114	840	537	1.638
4	294	2.619	765	4.584
5	213	1.056	849	816
6	222	2.343	987	1.635
7	486	1.416	333	435
8	255	1.860	981	2.301
9	234	2.142	*	4.329
10		2.073	1.302	
Media ± ES	343,0 ± 66,7	1.716 ± 236,8	922,7 ± 209,8	2.366 ± 514,6

Tab.5 AST values (UI/L)

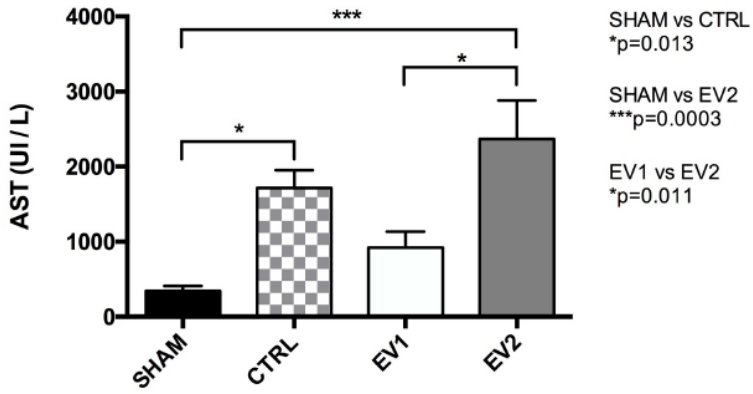
N. animale	SHAM	CTRL	EV1 ($3x10^9$)	EV2 ($7.5x10^9$)
1	108	2.553	39	4.668
2	129	657	189	1.527
3	30	1.101	108	1.551
4	108	1.170	1.716	3.600
5	54	2.694	1.002	684
6	57	2.649	381	1.863
7	87	1.044	423	420
8	60	2.025	447	5.172
9	81	1.755	57	2.763
10		1.680	979	
Media ± ES	79,33 ± 10,6	1733 ± 232,7	524,1 ± 168,3	2472 ± 565,1

Tab.6 ALT values (UI/L)

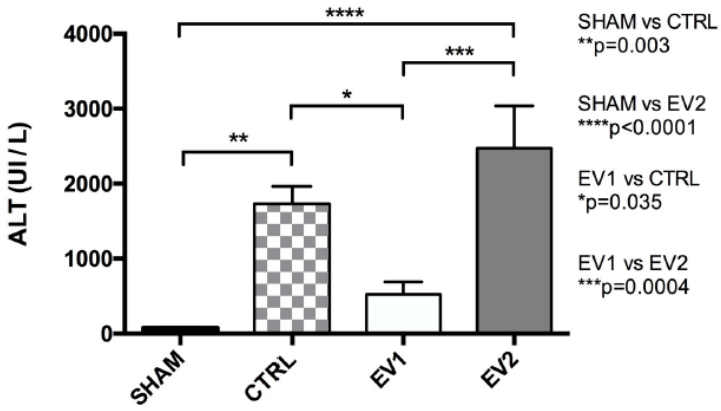
N. animale	SHAM	CTRL	EV1 ($3x10^9$)	EV2 ($7.5x10^9$)
1	5.325	19.038	921	25.272
2	4.479	8.946	3.408	10.092
3	2.154	12.375	5.235	10.299
4	1.893	17.025	14.835	20.697
5	2.364	21.447	9.612	7.176
6	2.613	21.237	13.098	11.607
7	4.392	11.853	4.713	7.023
8	2.235	20.487	8.235	25.863
9	1.776	10.848	9.966	15.858
10		10.884	9.030	
Media ± ES	3.026 ± 442,6	15.414 ± 1.552	7.9501 ± 1.374	14.876 ± 2.470

Tab.7 LDH values (UI/L)

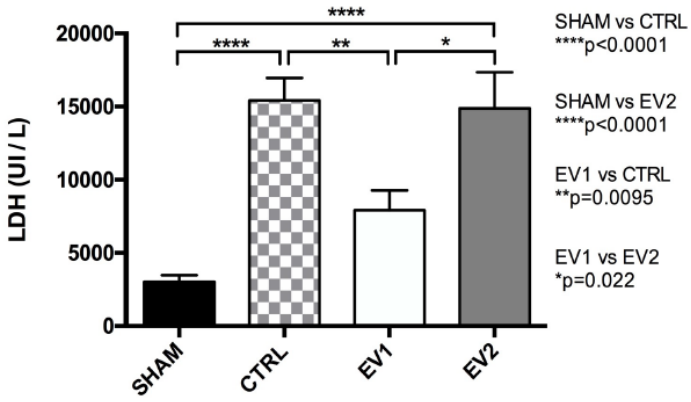
The graphs reported below (3-4-5) show the comparison of biochemical parameters between the study groups.



Graph. 3 Statistical analysis of AST values



Graph. 4 Statistical analysis of ALT values



Graph. 5 Statistical analysis of LDH values

After 6 hours from reperfusion, registered serum levels of AST, ALT and LDH were significantly increased in the control group if compared to the SHAM group ($p<0.01$).

The ALT, AST and LDH levels were reduced in EV1 group if compared to the control one. The difference is statistically significant as regards ALT ($p=0.035$) and LDH ($p=0.0095$), but not for AST ($p=0.25$).

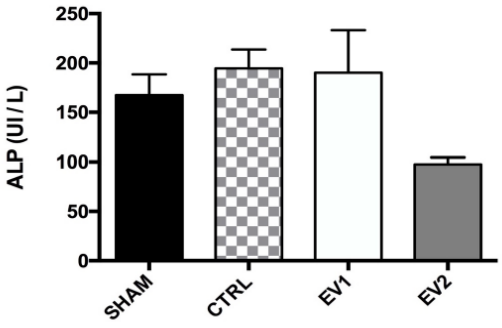
As regards comparison between SHAM group and EV1 group, we evidenced lower values of all three enzymes in the first group but this difference is not statistically relevant.

The treated groups significantly differ in ALT, AST and LDH levels, in terms of superior values in EV2 animals (respectively $p=0.0004$, $p=0.011$, $p=0.022$).

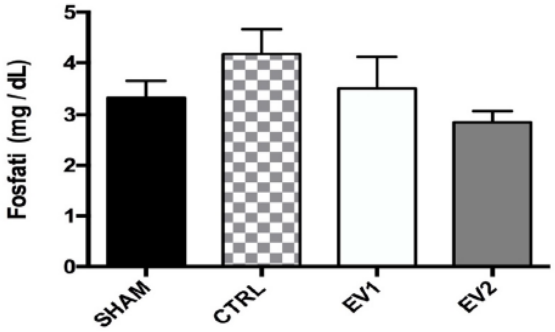
We did not registered statistically differences in cytolyses enzymes levels between control and EV2 groups.

Other biochemical parameters evaluated in study animals were ALP and phosphate.

ALP (alkaline phosphatase) levels are generally elevated in obstructive biliary diseases (such as primary or secondary biliary cirrhosis, sclerosant cholangitis, obstructive jaundice of different origins) and in osteo-metabolic diseases. As shown in graph. 6, we did not noticed statistically significant differences between groups as regards ALP levels.



Graph. 6 Statistical analysis of ALP values



Graph. 7 Statistical analysis of phosphate values

Also as regards phosphate, an indirect parameter of ATP availability, it was not registered any statistical relevant difference between the study groups (graph.7).

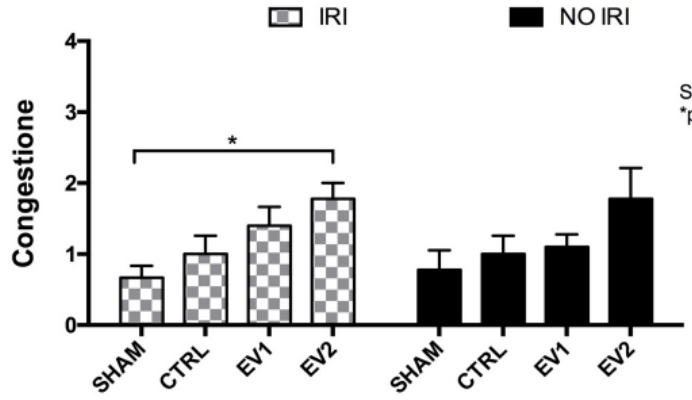
7.4 Histological analysis

Histological analyses focused on three main aspects: hepatic injury, apoptosis and proliferation evaluation.

HEPATIC INJURY

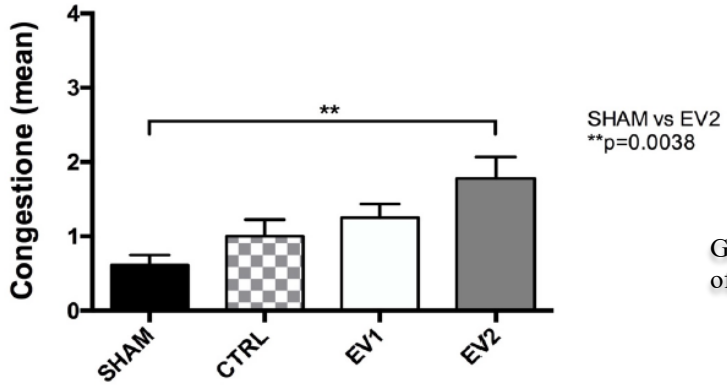
The evaluation was performed using the Suzuky score: congestion, ballooning and hydropic degeneration (considered a precursor of tissue necrosis). All the three parameters of the score were evaluated on IRI lobes and NO-IRI lobes separately, and on the means values of IRI and NO-IRI lobes together.

Graph.8 shows a statistically significant superior congestion in IRI lobes of EV2 group compared with SHAM one ($p=0.033$). No major differences are noticed as regards NO-IRI lobes.



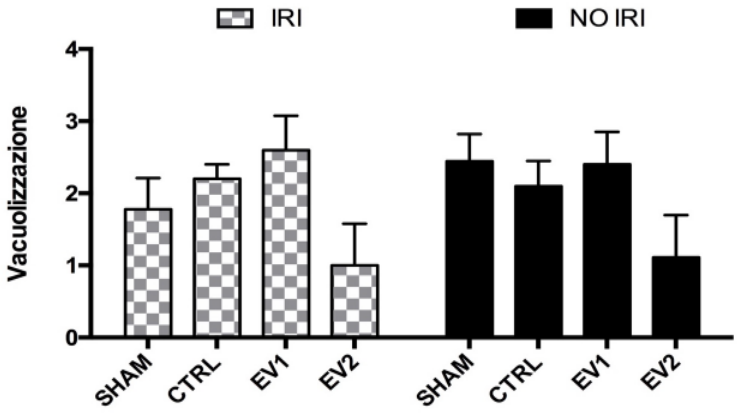
Graph. 8 Statistical analysis in IRI and NO-IRI lobes: congestion

The graph below (9), that combines the means of congestion in IRI and NO-IRI lobes, confirms the statistically relevant difference between EV2 and SHAM groups (p=0.0038).

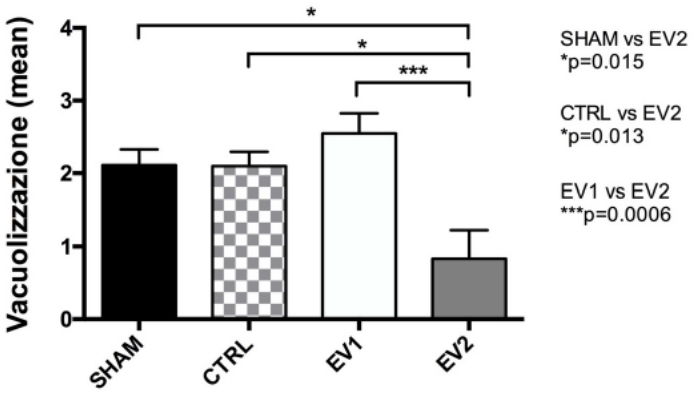


Graph. 9 Statistical analysis of means value of IRI and NO-IRI lobes: congestion

Talking about ballooning evaluation, our experiments revealed a tendency to inferior levels in EV2 group compared to the other groups with no statistically significant differences (graph.10). Furthermore, the analysis of mean values of IRI and NO-IRI lobes confirms lower values in EV2 group in comparison with SHAM, CTRL and EV1 group (p=0.015, p=0.013, p=0.0006 respectively) (graph.11).



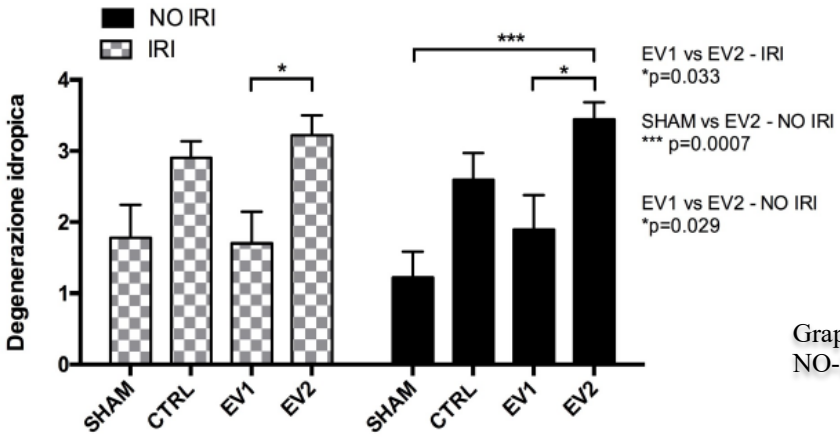
Graph. 10 Statistical analysis in IRI and NO-IRI lobes: balooning



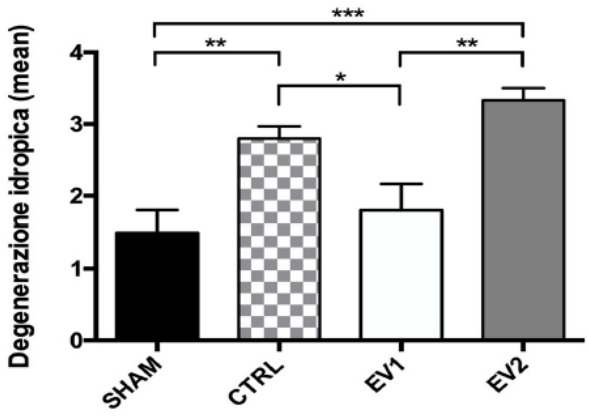
Graph. 11 Statistical analysis of means value of IRI and NO-IRI lobes: ballooning

Hydropic degeneration evaluation shows a trend to necrotic evolution particularly in animals treated with higher dose (EV2). In that group, in fact, the percentage of altered cells is often more than 50% of the total in a single slide. The analysis performed on the single IRI and NO-IRI lobes (graph.12) revealed higher levels on IRI lobes in EV2 group compared to EV1 one (p=0.033).

In the analysis of the mean values of IRI and NO-IRI lobes the differences are more evident (graph.13). Firstly, statistically superior levels in EV2 in comparison with SHAM (p=0.002) and EV1 (p=0.0016) group. Also the CTRL group has higher values compared to SHAM one (p=0.0084). Moreover, EV1 group shows inferior levels if compared to the CTRL group (p=0.049). Finally, EV1and SHAM groups do not differ between each other (p=0.86).



Graph. 12 Statistical analysis in IRI and NO-IRI lobes: idropic degeneration



Graph. 13 Statistical analysis of means value of IRI and NO-IRI lobes: idropic degeneration

APOPTOSIS AND CELL PROLIFERATION

The quantification of apoptosis and cell proliferation, performed through TUNEL and PCNA coloration respectively, did not revealed relevant results. In fact, we did not registered statistically significant differences between the four study groups both in apoptosis index and in proliferation index.

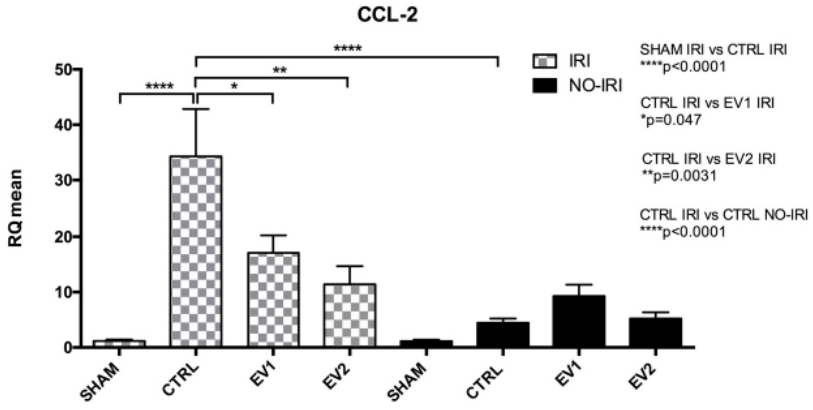
In the attached micrographs apoptotic and proliferating cells are colored in brown.

7.5 Molecular biology

RT-PCR analysis focused on different gene pathway evaluation: inflammation, apoptosis and hypoxia.

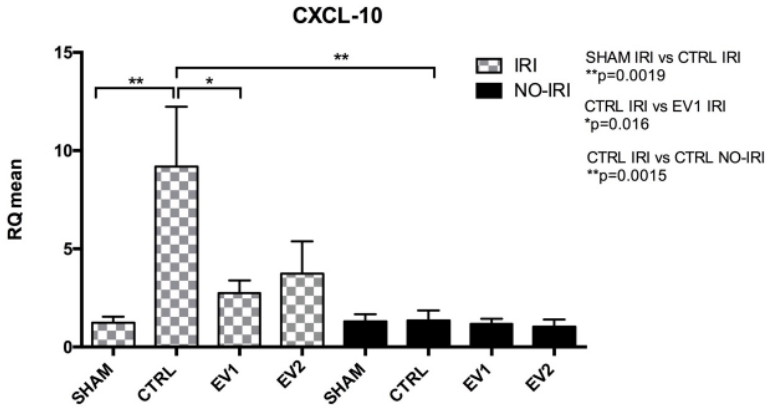
INFLAMMATION

Inflammatory pattern was accurately studied since IRI is, per definition, an inflammatory injury. As shown in graph 14, gene expression of CCL-2 is significantly increased in IRI lobes compared to NO-IRI lobes. In particular, the difference is evident between CTRL groups ($p<0.0001$). In IRI lobes it was registered higher cytokine level in CTRL group compared to SHAM ($p<0.0001$), EV1 ($p=0.047$) and EV2 ($p=0.0031$) group. Furthermore, the treated groups do not differ significantly from SHAM group.



Graph. 14 Gene expression CCL-2

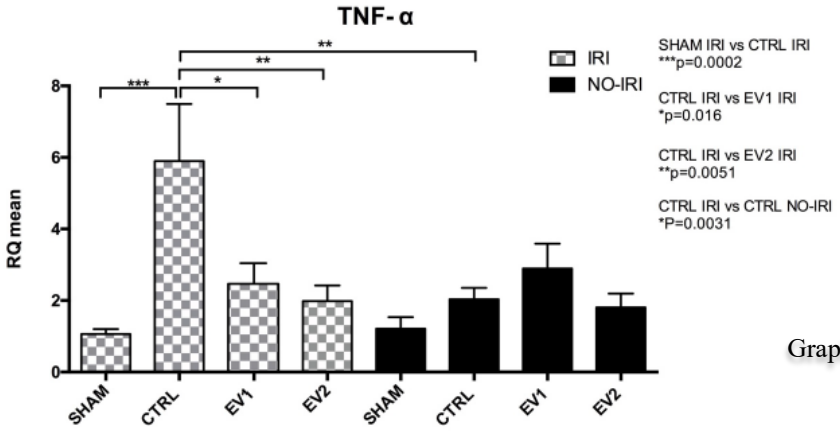
The graph below represents chemokine CXCL-10 levels in IRI and NO-IRI lobes. As for CCL-2, in IRI lobes higher values are registered in comparison to NO-IRI ones (CTRL IRI vs CTRL NO-IRI, p=0.0015). In addiction CXCL-10 levels are statistically significant inferior in EV1 group compared to CTRL and SHAM groups (p=0.016 and p=0.0019 respectively). No relevant differences are evidenced between EV1, EV2 and SHAM groups.



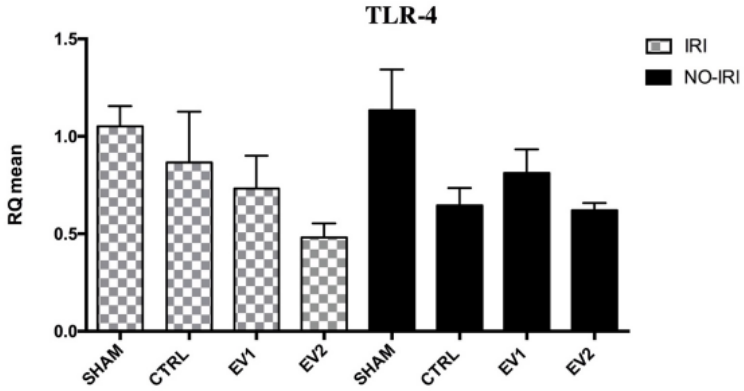
Graph. 15 Gene expression CXCL-10

As regards TNF-α expression (graph.16), in the control groups is registered a statistically difference between IRI and NO-IRI tissue, confirming the tendency to higher values in the IRI lobes (p=0.0031). Talking about IRI lobes, CTRL groups differ in relevant way from EV1 (p=0.024), EV2 (p=0.017) and SHAM (0.0009) ones.

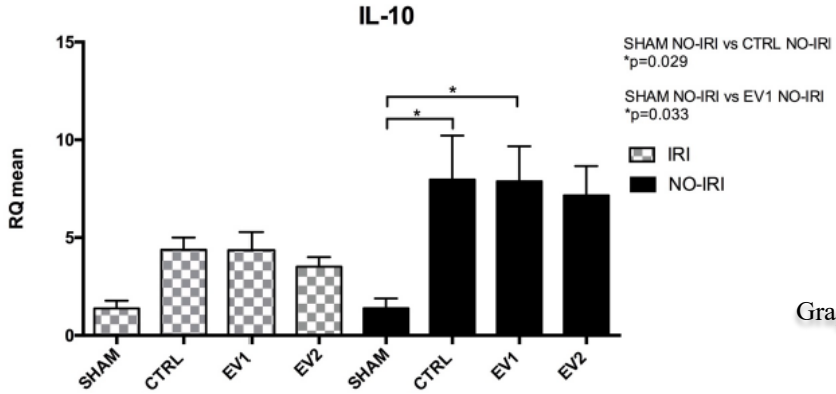
Graph 17 Represents TLR-4 gene expression, that does not differ between study groups.



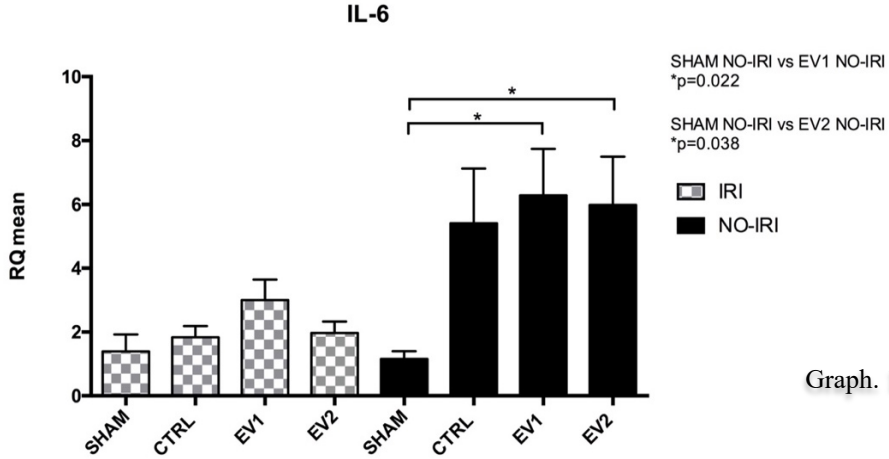
Graph. 16 Gene expression TNF-α



Graph. 17 Gene expression TLR-4

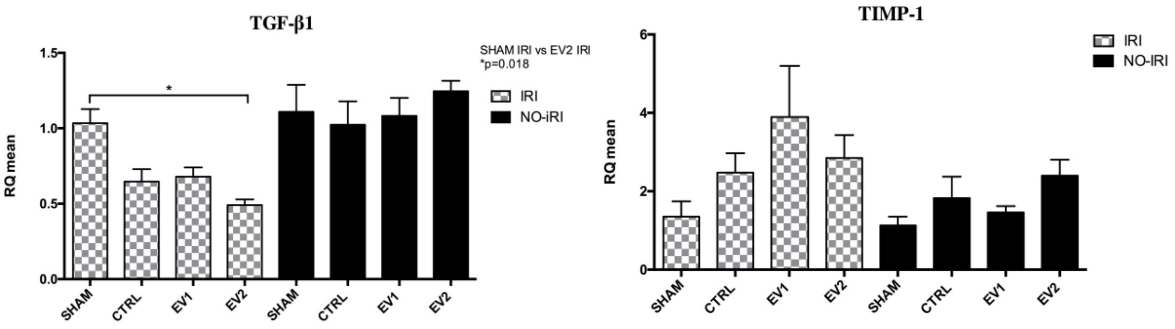


Graph. 18 Gene expression IL-10



Graph. 19 Gene expression IL-6

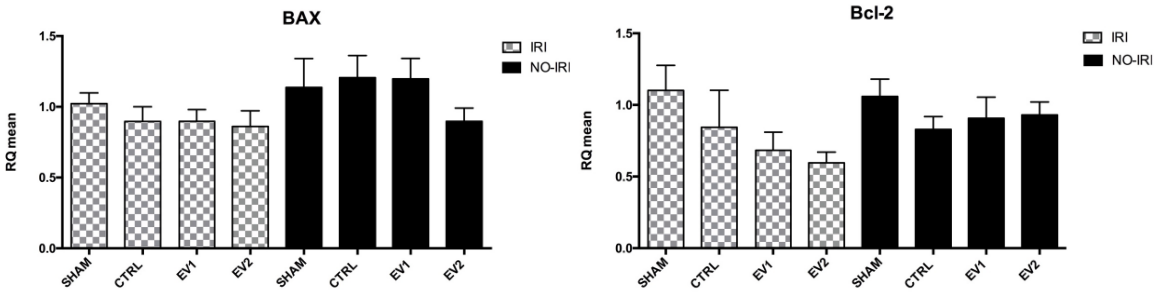
The graphs 18-19 show gene expression of IL-10, known anti-inflammatory molecule, and IL-6, mediator of both inflammatory and anti-inflammatory effects. Their levels do not differ significantly in IRI lobes. On the contrary, in NO-IRI lobes: IL-10 is more expressed in CTRL and EV1 groups if compared with SHAM group (p=0.029 and p=0.033); IL-6 has increased levels in EV1 and EV2 groups in comparison with SHAM one (p=0.022 and p=0.038).
Cytokines that regulate fibrosis process (TGF-β1 and TIMP-1) are expressed with no relevant differences in the study groups, except for lower expression of TGF-β1 in EV2 group rather than in SHAM group (p=0.0018) (graphs below).



Graph. 20 Gene expression of TGF-β1 and TIMP-1

APOPTOSIS

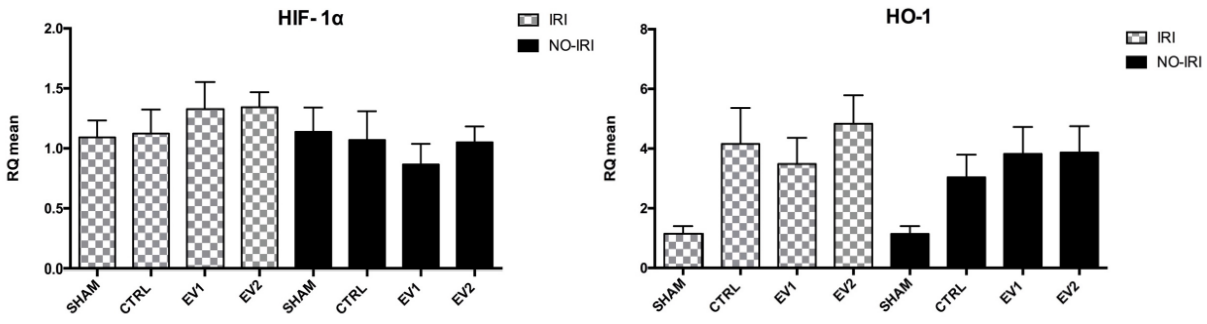
Gene expression of molecules regulating apoptosis (BAX and Bcl-2) does not differ in the study groups, both in IRI and NO-IRI lobes (graph 21.). This result is in line with TUNEL analysis, presented before.



Graph. 21 Gene expression of BAX and Bcl-2

HYPOXIA

Also as regards hypoxia-induced genes, HIF-1α and HO-1 represented in graph. 22, were not registered relevant differences in expression between the different groups analyzed.



Graph. 22 Gene expression of HIF-1α and HO-1

8. DISCUSSION

Despite new findings and continue scientific research, Ischemia-Reperfusion Injury still represents a challenging problem in different clinical conditions.

In this study we focused our attention on hepatic IRI. Both in transplantation and resection setting, post-operative organ function depends on multiple factors. First of all, it is important to consider comorbidities and age of the patient, liver parenchyma status (steatosis, chemotherapy damage) and graft characteristics (in case of transplant). These parameters are only partially modifiable.

But it is widely known that liver function recovery depends for a large part by the injury occurred after reperfusion. In last decade different procedures were studied to limit IRI. Ischemic preconditioning, for instance, is a method that consists in exposition of liver to short period of ischemia, followed by reperfusion, with the aim to protect hepatic tissue to the following prolonged ischemia [69]. Pharmacological research examined numerous molecules able to increase tissue tolerance to IRI, such as serine protease inhibitors [70], streptokinase [71], modulators of RAAS system, lipidic metabolism and adenosine [23, 24, 25]. Nevertheless it was not demonstrated real efficacy in vivo. One successful example of therapeutic tool towards IRI is represented by organ machine perfusion (MP), employed to optimize liver quality between procurement and transplant. Already applied in clinical practice, MP reached really important results in terms of reduction of primary non function or delayed non function.

An interesting research direction developed in last years is represented by staminal cells and their derived molecules. Firstly, MSC and MSC-EV, and then HLSC and HLSC-EV, were studied in different models of hepatic and renal injury, demonstrating their efficacy in terms of enhancement of organ function. In a murine model of acute renal injury, HSLC-EV were able to reduce creatinine and BUN plasma levels, to improve tubular injury and to promote proliferation[72]. More recently, HLSC-EV were effective when injected in animals with renal fibrosis and could down-regulate the expression of profibrotic genes[63]. In liver setting, HLSC-EV successfully promoted liver regeneration in a model of 70% hepatectomy in rats[64], showing similar biological effects to those of HLSC[55]. Moreover, it was recently demonstrated that HLSC-EV were able to reduce liver injury in a model of hypoxic normothermic machine perfusion.

From this encouraging basis, we developed our experimental study on a mouse model of IRI. Ischemia was induced by selective surgical clamping and involved more than half of liver parenchyma. The aim of the project was to analyze the potential protective effect of HLSC-EV administered after reperfusion and, if confirmed, the molecular pathways involved.

The first result obtained regards the dose-dependent effect. The higher dose employed (EV3=$10x10^9$ particles), was identified as lethal. In fact, it caused sudden death in all the animals. No problems were evidenced in the other two treated groups. It is known that, when injected at high concentrations, stem cell may cause pulmonary embolism due to aggregation phenomena.[73,74] Nevertheless, EV are nowadays considered an attractive alternative, as they usually present limited toxicity and immunogenicity. Of notice, in this work, we observed that HLSC-EV can be lethal when administered at high doses and concentrations (i.e. 10^{10} particles in 120 µl), suggesting the importance of dose-response analyses also in studies concerning the use of EV.

Secondly, this study demonstrated HLSC-EV engraftment inside the hepatocytes at the end of the experiments (almost 6 hours after administration). Differently from previous studies, our immunohistochemical analysis revealed presence of vesicles both at cytoplasmic and nuclear level.

Thanks to IVIS analysis on dissected organs, we investigated two aspects: the comparison of fluorescence in the different organs and the comparison between hepatic fluorescence in the different study groups. As regards the first one, it was evidenced an increased fluorescence in liver rather than in other organs, meaning a major concentration of HLSC-EV in hepatic parenchyma. This result is an indirect demonstration of hepato-specificity of vesicles. In fact, before reaching portal vein and then the liver, HLSC-EV are inoculated in systemic circulation. Therefore, they reach other organs (such as lungs and heart) before the liver where they could be engrafted. But HLSC-EV localized preferentially in hepatic tissue, probably addressed by mediators similar to HLSC deriving milieu.

The comparison of hepatic fluorescence in the study groups showed higher fluorescence in EV1 and EV2 groups compared to the control ones, even if only for EV1 the difference is statistically relevant. Consequently, EV1 dose could be considered effectively integrated in the hepatic parenchyma. This result confirms, moreover, HLSC-EV engraftment in treated animals. The unexpected evidence was the presence of fluorescence also in the control and sham groups, revealing that the liver had a strong background fluorescent signal.

The main focus of the study, that is hepatic damage evaluation, was performed through biochemical, histological and molecular parameters.

The release of cytolytic enzymes is widely considered an important marker of liver injury in mouse models of hepatic IRI[73, 75,76,77]. In our case series, EV1 dose significantly reduced serum ALT and LDH when compared to the control group. Furthermore, the EV1 group did not differ from the sham group in terms of ALT release, showing that HLSC-EV treatment strongly protected the hepatocytes from the ischemic insult. On the other hand, the EV2 group was comparable to controls as regards both ALT and LDH levels; consequently, we could consider that higher dose failed to fully protect the liver from IRI.

Histological analyses were consistent with the biochemistry results. The main parameter in Suzuky score correlating with IRI is hydropic degeneration, since represent a precursor of cellular necrosis. In our study, only the EV1 dose was able to reduce the amount of hydropic degeneration when compared to the control group, confirming the role of HLSC-EV on tissue integrity[65]. Hepatic parenchyma of EV1 group was properly preserved, similarly to those in the sham group. On the contrary, livers of EV2 group manifested relevant percentage of unpreserved cells.

Altogether, these results may suppose that, at higher concentrations of EV, the particles aggregate together, limiting their mechanisms of action.

Relatively to molecular analyses, our study revealed that HLSC-EV treatment resulted to mainly modulate the mRNA levels of TNF-α, CCL-2 and CXCL-10, key inflammatory molecules that participate in the post-reperfusion phase of IRI[78,790,80,81] without affecting the expression of TLR-4, IL-6 and IL-10. Furthermore, we noted that the mRNA levels of HO-1 and SIRT-1 in the IRI group were not different to that observed in the sham animals, suggesting the absence of oxidative damage at 6 hours of reperfusion. In addition, HLSC-treatment did not affect the apoptosis grade (as shown by TUNEL analysis) or the mRNA levels of BAX and BCL2, indicating the lack of modulating effects on apoptosis by HLSC-EV. Nevertheless, in mice treated with the EV1 dose, we observed a reduction in the hydropic degeneration, which is considered the precursor of necrosis stage. This evidence suggests that necrosis may be the main process involved in hepatocytes loss[82].

Finally, in our model the involvement of hypoxia or an early activation of fibrosis were discarded. This was supported by the fact that non evident increase of the mRNA levels of HIF1-α and TGFβ1 were observed in the ischemic groups when compared to the sham group. Moreover, we noticed that TIMP-1, which is activated downstream in the fibrotic process

initiated by TGF- β1, was not activated in our model, as expected, due to the lack of activation of TGF-β1.

Our data, analyzed together, suggest that the HLSC-EV are able reduce liver IRI by modulating, almost in part, the inflammatory status. These data allow us to hypothesize that HLSC-EV treatment could act at the beginning of the inflammatory cascade. In fact, it is known that TNF-α is involved in the activation of chemokines cascade and could be produced by activated macrophages, $CD4^+$ lymphocytes, neutrophils, mast cells and eosinophils, while CXCL-10 and CCL-2 are mainly secreted by Kupffer cells during hepatic inflammation, promoting neutrophils attraction[83,84]. Hence, the HLSC-EV could reduce the production of TNF-α and, as a consequence, the production of the two other chemokines CXCL-10 and CCL2, which are located downstream on the activation of inflammatory cascade, thus ameliorating the local inflammation induced by the ischemic reperfusion damage. Interestingly, also the EV2 dose was able to reduce the expression of TNF-α, CCL-2 and CXCL-10 genes, but this beneficial effect was observed only at molecular level and was not supported by biochemistry and histology results.

The current study presents some limitations. First of all, the selection bias of study animals, that are not representative of human population. In fact, experimental mice are healthy and young. The obtain results could not be completely transferred on clinical setting.

Secondly, this model of hepatic damage, through selective surgical clamping, could cause not homogeneous injury entity, related to possible patency of arterial supply. For that reason, Zhang and colleagues recently described a novel ischemia model, realized by occlusion of both inflow and outflow of lateral lobe (35% ischemia on total volume). The study showed worst results compared to the 70% ischemia model but more reproducible[85].

Furthermore, the small sample size of the study groups does not consent solid conclusions and represents a limit in interpretation of some results (for instance, there is a wide variation in the cytolysis in the same group). A larger number of cases in group EV1 and EV2 could reinforce the evidences obtained by the analysis.

Another limitation regards HLSC-EV treatment, since it is still unknown vesicles content and their signaling pathways. On the contrary of standard therapy, cellular treatments are referred to as number of particles and not mg or UI/ml. Then it is particularly important not only the quantity of vesicles but also their concentration, since could influence their final effect.

Finally, it is still unclear which is the correct timing of HLSC-EV administration. In our study we employed vesicles after reperfusion, while in other reports they are administered before ischemic phase.

A future study will be essential to confirm the demonstrated advantages of HLSC-EV in a larger number of cases and different hepatic IRI pattern, in order to obtain strong definitive conclusions.

The present study contributes to demonstrate the high potential translational role of extracellular vesicles in a wide range of clinical diseases. Thanks to their innate properties (sharing origin stem cell characteristics, transporters of genetic material, ability to cross biological barriers, expression of cell surface receptor, no immunogenicity) EVs represent an effective tool to applicate in regenerative medicine. In fact, it would be possible to engineer EV with a specific RNA or miRNA to be carried to a specific target cell. Some example of this application derived from neurologic field (Alzheimer disease [86]) and oncological setting (breast cancer [87] and leukaemia [88]). The current challenge for scientists is the so-called Good Manufacturing Practice (GMP), that means finding the right method to produce EV, that combines feasibility, reproducibility and ability to maintain EV properties. In this regard, HLSC could represent an important EV source, since, differently from MSC, they do not undergo senescence, maintain a stable karyotype up to 24th passage in expansion and the derived EVs share several properties both with HLSC and MSC.

9. CONCLUSION

This experimental study had a double objective: firstly, to evaluate HLSC-EV efficacy towards hepatic ischemia-reperfusion; secondly, to analyze a potential correlation dose-effect.

On the basis of the obtained results, it is possible to conclude that the $3x10^9$ dose HLSC-EV is able to protect the liver from IRI, in terms of biochemical, histological and molecular pattern; on the contrary, the intermediate dose $7.5x10^9$ HLSC-EV did not show this hepatoprotective effect except for an anti-inflammatory modulation observed with the RT-PCR analysis. Finally, the highest dose studied ($10x10^9$ HLSC-EV) was identified as the lethal dose.

Altogether, these data support the use of HLSC-EV as a therapeutic approach against hepatic IRI, warranting further investigations in different experimental settings. Future challenges could be represented by other models of liver damage (such as steatotic organs, DCD graft) and different treatment modalities (for instance administration route, timing, dose).

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