THERAPEUTICS

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CHRONIC DEVELOPMENTAL LIVER DISEASE
– THE ROLE OF EXOSOMES IN LIVER PATHOPHYSIOLOGY AND THEIR THERAPEUTIC POTENTIAL

SUMMARY

Chronic hepatopathies [Chronic viral hepatitis, NAFLD (Non-Alcoholic Fatty Liver Disease), and Haemochromatosis] are characterised by altered protein and coagulation factor synthesis, insufficient detoxification of metabolic products, and biliary excretion.

In Western countries their incidence in the general population is gradually increasing. Recent studies have documented that hepatocyte-derived exosomes, important means of intercellular communication, promote both hepatocyte proliferation in vitro and regeneration in vivo.
– Special mention must be made of the lyophilized swine liver Neorland® (Epatoguna), which has been shown to contain biologically active exosomes; these are internalised by human hepatocytes, stimulate mitochondrial metabolism, and protect hepatocytes from acetaminophen-induced damage.
This article also provides a review of the most significant papers on Epatoguna.

KEY WORDS **CHRONIC HEPATOPATHIES, EXOSOMES, EPATOGUNA**

INTRODUCTION – EPIDEMIOLOGY

Chronic Liver Disease is a major public health problem and a frequent cause of death worldwide.
Its incidence is increasing.

– In the Western world, the most frequent causes of Chronic Liver Disease are alcohol-related liver diseases, Chronic Viral Hepatitis (HBV; HCV; HBV and HDV), **Non-Alcoholic Fatty Liver Disease (NAFLD)** and Haemochromatosis (1-4).

The incidence of NAFLD is 2-44% amongst the European population (including the paediatric population suffering from Obesity) and 43-69% in patients with type 2 Diabetes Mellitus.
NAFLD correlates with the incidence of Obesity in the general population (2).

– **Hepatocarcinoma (HCC)**, which constitutes 70-90% of primary liver tumours, develops with symptoms of Cirrhosis in more than 80% of cases.
HCC-related mortality is approximately 47,000 deaths/year (2;5-7).

Therefore, it is necessary to act on the modifiable factors of Chronic Liver Disease and its prevention, as well as on the early diagnosis of the disease, in order to treat it or at least slow down its evolution.
The most common aetiologies of Chronic Liver Disease are set out in TAB. 1.

Liver transplantation is the therapy of choice for terminal liver failure and HCC.

CHRONIC LIVER DISEASE

- Alcoholic Liver Disease
- Non-Alcoholic Fatty Liver Disease (NAFLD)
- Chronic Viral Hepatitis (HBV; HCV; HBV and HDV)
- Hepatopathy from genetic causes
  - Alpha 1 antitrypsin deficiency
  - Hereditary haemochromatosis
  - Wilson's disease
  - Primary Biliary Cirrhosis (PBC)
  - Primary Sclerosing Cholangitis (PSC)
  - Autoimmune Hepatitis (AIH)
- Drug-related hepatopathies (e.g. amiodarone, isoniazid, methotrexate)
- Vascular disorders (e.g. Budd-Chiari Syndrome)
- Idiopathic/Cryptogenic Hepatopathy

Revised from: Sharma A. & Nagalli S. – Chronic Liver Disease. Chronic Liver Disease. 2022 Jul 4. In: StatPearls [Internet]. Treasure island (FL): StatPearls Publishing; **2022** Jan.

TAB. 1

Approximately 5500 liver transplants are performed each year in Europe; however, the number of patients on the waiting list is greater than the number of available and usable donors (8-11).

– To overcome this major problem and to increase the number of usable liver grafts for transplantation purposes, a number of alternative programmes to whole liver transplantation from brain-dead donors have been developed, such as the use of split liver (right and left liver on two different recipients), living donor liver transplantation and the use of donors who have been confirmed with cardiac death criteria (DCD) (10,11).

The survival of liver graft and transplant patients has improved considerably over the years thanks to improved surgical techniques, better preoperative selection of patients and improved management of immunosuppressive therapy and post-transplant complications.

– Overall survival, including all indications for transplantation, is currently at about 83% one year after transplantation (8,9,11).

PATHOPHYSIOLOGY

Chronic Liver Disease is characterised by a progressive deterioration of physiological liver functions such as protein synthesis, the synthesis of coagulation factors, the detoxification of metabolic products and the excretion of bile.

– From a pathophysiological point of view, chronic liver disease is characterised by a chronic evolutionary process showing a continuum of inflammation, destruction and regeneration of the hepatic parenchyma that leads to symptoms of Fibrosis and Cirrhosis in the terminal stages over the years.
The evolution of the fibrotic process and the duration of its progression are linked to multiple factors such as disease aetiology, environmental factors and host-related factors (e.g. genetic polymorphisms).

– Liver Fibrosis, especially in its early stages, is a potentially reversible process, whereas Cirrhosis represents the final stage of the disease, characterised by complete disruption of the liver architecture, formation of diffuse nodules (macro- or micro-nodules), neo-angiogenesis, vascular reorganisation and an abnormal deposition of **extracellular matrix (ECM)** (12).

Liver Fibrosis is related to ECM deposition in response to chronic liver damage, regardless of aetiology.
– In this process, a key role is played by the hepatic stellate cells (HSCs), located in the space between the sinuosuites and the hepatocytes, which under normal conditions are quiescent and dedicated to the storage of vitamin A.

In response to chronic liver damage, hepatic stellate cells are activated on fibrogenic myofibroblasts that increase the expression of inflammation receptors (e.g. ICAM-1 chemokine receptors) and other inflammation mediators through the release of chemokines.

This pro-inflammatory phase or initiation phase also leads to genetic and phenotypic changes in liver cells, making them more responsive to inflammatory cytokines and leading to the perpetuation of hepatic stellate cell activation leading to ECM accumulation and progressive Fibrosis (13).

EXOSOMES

Exosomes are extracellular microvesicles with a round or oval shape (30-100 nm) that originate from macrovesicles and fuse with the plasma membrane, releasing intraluminal vesicles, which are subsequently excreted from the cell through a process of exocytosis (14-16).

Exosomes are secreted by all cell types and can be found in many body fluids, including **the blood**, **urine** and **saliva** (17-20).

– Exosomes were initially regarded as

waste products of cell production; however, scientific research in recent years has shown that they are involved in numerous biological processes, especially in relation to their role in both short- and long-range **intercellular communication** (21,22).

Among the many functions of exosomes, the most important is the exchange of information and transfer of material between cells.

Exosomes communicate with cells via three main mechanisms (23):

1. binding through receptors in the target cell
2. direct fusion with the plasma membrane of the target cell
3. entry into the target cell through a mechanism of endocytosis.

Cells produce exosomes under both physiological and pathological conditions.

The composition of exosomes is characterised by a part common to all exosomes (proteins associated with their formation and secretion) and a variable part consisting of proteins, growth factors, nucleic acids [mRNA, microRNA (miRNA) and other non-coding RNAs] specific to the cell of origin (24,25).

The components that make up exosomes reflect the status in which the cell from which they originate is at the time of their formation (25).
– The material contained within the exosomes, known as 'cargo', is transferred to the recipient or 'target' cell and is able to modulate intracellular regulatory pathways, affecting the behaviour of the recipient cell (15,26,27).

Due to their ability to act as intercellular carriers, exosomes are the subject of great interest from scientific research in relation to their potential clinical implications, both as biological markers, which can be used as a diagnostic and monitoring tool, and as transporters of drugs or genetic material.

– For an in-depth discussion of the characteristics of exosomes and their functions, see the review by
Ferroni O. – *Exosomes, the shuttles of intercellular communication. Their emerging role and diagnostic and therapeutic possibilities*, published in PRM 2022 (FIG. 1).

LIVER-DERIVED EXOSOMES

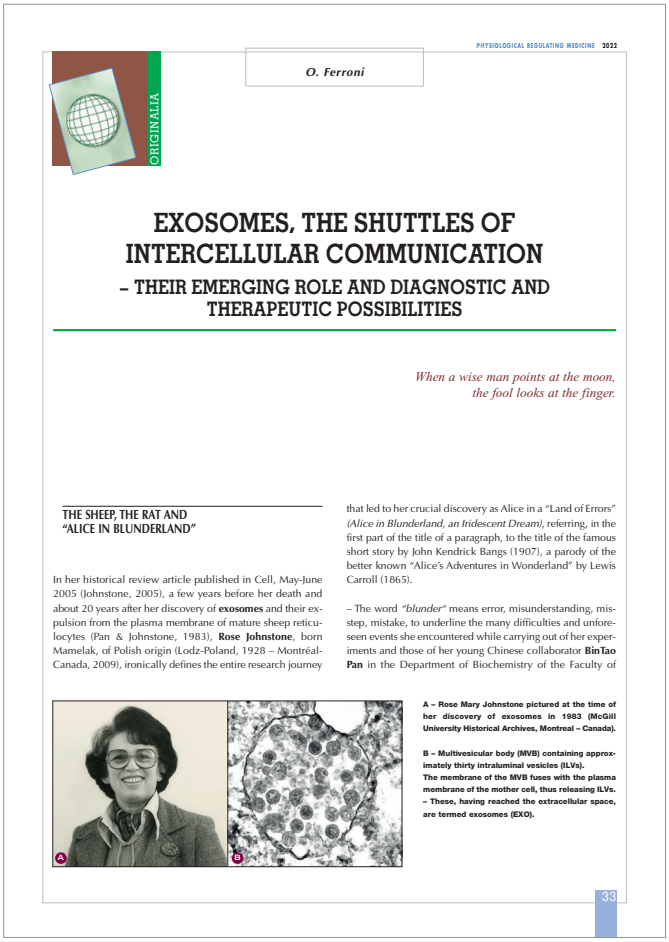
The liver performs a wide range of physiological functions; its homeostasis is maintained by a complex exchange of information between all cellular components, both parenchymal (hepatocytes, which constitute 80% of the total volume of the liver) and non-parenchymal (hepatic stellate cells, sinusoidal endothelial cells, cholangiocytes, Kupffer cells).
All parenchymal and non-parenchymal liver cells are capable of secreting exosomes.

– Hepatocytes release exosomes that act on surrounding hepatocytes and non-parenchymal cells to regulate liver regeneration.
Non-parenchymal cells are capable of secreting exosomes that are useful in regulating hepatic remodelling in response to damage (24).
Recent scientific evidence has shown that exosomes are crucial in the pathogenesis of multiple liver diseases, including viral hepatitis, alcoholic liver disease, hepatocarcinoma, and NAFLD (25,26) (FIG. 2).

HEPATOCYTE-DERIVED EXOSOMES

Hepatocytes are cells with peculiar characteristics in that, while remaining in a dormant state under physiological conditions, under conditions of liver damage they are able to enter the cell cycle and proliferate in order to repair

FIG. 1



PHYSIOLOGICAL REGULATING MEDICINE 2022

O. Ferroni

ORIGINALIA

EXOSOMES, THE SHUTTLES OF INTERCELLULAR COMMUNICATION
– THEIR EMERGING ROLE AND DIAGNOSTIC AND THERAPEUTIC POSSIBILITIES

When a wise man points at the moon, the fool looks at the finger.

THE SHEEP, THE RAT AND "ALICE IN BLUNDERLAND"

In her historical review article published in Cell, May-June 2005 (Johnstone, 2005), a few years before her death and about 20 years after her discovery of **exosomes** and their expulsion from the plasma membrane of mature sheep reticulocytes (Pan & Johnstone, 1983), **Rose Johnstone**, born Mamelak, of Polish origin (Lodz-Poland, 1928 – Montréal-Canada, 2009), ironically defines the entire research journey that led to her crucial discovery as Alice in a "Land of Errors" *(Alice in Blunderland, an Iridescent Dream)*, referring, in the first part of the title of a paragraph, to the title of the famous short story by John Kendrick Bangs (1907), a parody of the better known "Alice's Adventures in Wonderland" by Lewis Carroll (1865).

– The word *"blunder"* means error, misunderstanding, misstep, mistake, to underline the many difficulties and unforeseen events she encountered while carrying out of her experiments and those of her young Chinese collaborator **BinTao Pan** in the Department of Biochemistry of the Faculty of

A – Rose Mary Johnstone pictured at the time of her discovery of exosomes in 1983 (McGill University Historical Archives, Montreal – Canada).

B – Multivesicular body (MVB) containing approximately thirty intraluminal vesicles (ILVs). The membrane of the MVB fuses with the plasma membrane of the mother cell, thus releasing ILVs. – These, having reached the extracellular space, are termed exosomes (EXO).

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the liver damage itself.

This regenerative capacity, however, is lost when liver damage is severe (e.g. massive Liver Necrosis) or when the functional liver reserve is progressively depleted (e.g. Cirrhosis) (27).

– Studies have documented that hepatocyte-derived exosomes are able to promote both cell proliferation *in vitro* and liver regeneration *in vivo*, by mediating the transfer of **Sphingosine kinase-2 (SK2)** to target cells and inducing the up-regulation of intracellular **Sphingosine-1-phosphate (S1P)** (23,28).

– In the study by Nojima *et* Al. (28), when exosomes isolated from murine hepatocytes were administered to mice subjected to ischaemia/reperfusion injury or a partial hepatectomy, they were able to induce intracellular SIP synthesis within hepatocytes in order to promote cell proliferation.

It should be highlighted that these findings were not observed when hepatocytes were treated with exosomes derived from non-parenchymal liver cells such as Kupffer cells or sinus ductal cells.

– This observation also suggests that SK2 may be a specific (cargo) instruction of hepatocyte-derived exosomes.
Exosomes are also involved in the spread of infection during viral hepatitis.

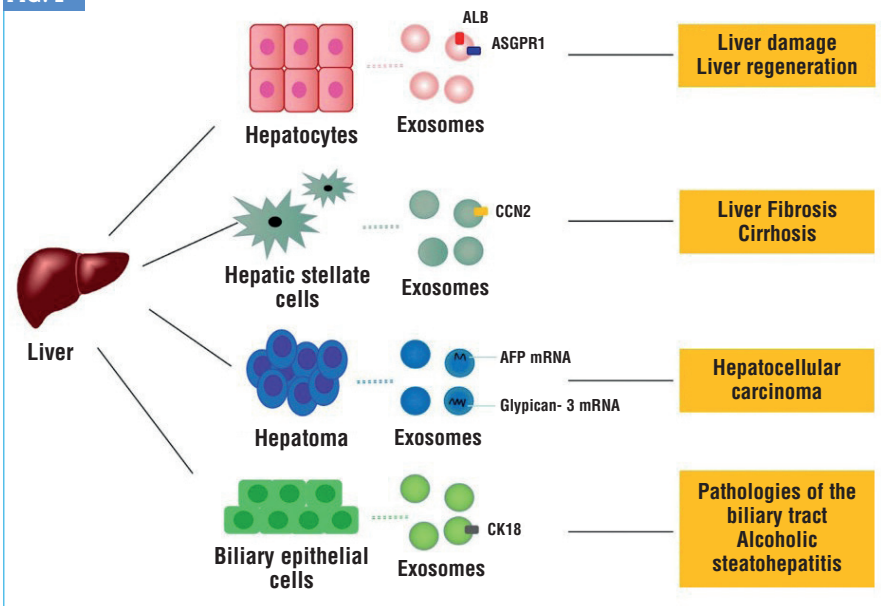
Studies in cell cultures have shown that HCV-infected hepatocytes release HCV-RNA-containing exosomes that infect healthy hepatocytes.
– This finding indicates that exosome-mediated hepatocyte-hepatocyte communication contributes to the spread of HCV viral infection (29).

Other studies have shown that, in response to liver damage, damaged hepatocytes release exosomes that are internalised by hepatic stellate cells that switch from a state of quiescence to a state of activation in myofibroblastic form, exacerbating the process of hepatic fibrogenesis (30).

EXOSOMES – POTENTIAL APPLICATIONS

The main applications of exosomes (31,32) lie in their characteristics of:
1. biohumoral markers
2. carriers of therapeutic agents.

FIG. 2



Revised from: Jiao Y., Xu P., Shi H., Chen D., Shi H. – Advances on liver cell-derived exosomes in liver diseases. J Cell Mol Med. **2021** Jan; 25(1):15-26.

The particularity of exosomes to reflect the conditions of the cell of origin in their content (cargo) both under physiological and pathological conditions suggests that they can be used as biological markers under physiological and pathological conditions (31,32).

For instance, the Connective Tissue Growth Factor (CTGF), an exomal fibrosis-related cargo, has a potential application in the non-invasive monitoring of liver Fibrosis (33).

– It should be emphasised, however, that levels of markers of exosomal origin may not exclusively reflect hepatic exosomal production, but also that of other cells and organs.

In order to use exosomes as organ-specific biomarkers for the diagnosis and monitoring of pathological conditions, it is desirable to identify organ-specific exosomal biomarkers.

– In this regard, in a study conducted by Lee *et* Al. (34), miR-192 lysosomal derived from damaged hepatocytes appears to be a potentially useful marker for assessing liver damage in the progression of Fibrosis from NAFLD to Non-Alcoholic Steatohepatitis (NASH). These results, although very promising, require further studies in order to confirm them.

So far, most dedicated studies have focused on evaluating the therapeutic effects of **mesenchymal stem cells (MSCs)** in relation to their immunosuppressive and immunostimulating properties (24).

– While extensive research is currently underway to evaluate the use of MSC-derived exosomes, relatively few studies are available on the use of liver cell-derived exosomes.

– In this context, special mention should be made about the Neorland® lyophilized swine liver contained in **Epatoguna**.

In the study conducted by the research

group coordinated by Ventura (35) that we refer to, the conclusions state that:

1. Neorland® lyophilized swine liver contains exosomes
2. they are internalised by human hepatocytes and are biologically active.

In research conducted by Berni *et* Al. (36) the *in vitro* effects of lyophilized swine liver in terms of cell cytotoxicity (MTT assay) and proliferation assays (bromodeoxydeoxyuridine incorporation and direct cell counting) were studied in 2 different cell types:

1) immortalised human-derived liver HepG2 cell line.

– The use of primary cultures of liver cells is rather complex since they lose most of their characteristic functions when expanded *in vitro*.

The HepG2 cell line maintains many differentiated liver functions and is a widely used model in the study of *in vitro* hepatocyte biology.

2) Primary culture of mesenchymal stromal cells (MSCs) derived from adipose tissue (At-MSCs).

– Mesenchymal stromal cells (MSCs) are cells distributed throughout mesodermal Tissues involved in maintaining Tissue homeostasis, controlling inflammatory processes and regenerating damaged Tissues in both physiological and pathological conditions.

– At concentration levels between 100 and 500 µg/mL, lyophilized swine liver stimulates mitochondrial metabolism as assessed by the MTT test ($p \leq 0.001$ for HepG2 and for At-MSCs) and induces an increase in bromine-deoxyuridine incorporation in both cell types ($p \leq 0.01$ for HepG2 and $p < 0.001$ for At-MSCs).

Furthermore, the cell count showed a statistically significant increased prolific activity in the At-MSC treated cell line ($p < 0.001$).

These results demonstrate that lyophilized swine liver is not cytotoxic; on the contrary, it induces a positive metabolic stimulus on the cell lines under consideration.

A study by Tassinari *et* Al. (37) evaluated the action of extracellular microvesicles isolated from lyophilized swine liver on human-derived HepG2 cells.

– In this study, it was shown that swine-derived extracellular vesicles were internalised by human HepG2 cells and had no toxic effect on them.

Furthermore, the study assessed whether extracellular vesicles played a role in protection in a model of acetaminophen-induced acute liver damage, showing that pretreatment of HepG2 cells with extracellular vesicles isolated from lyophilized swine liver were able to attenuate cell death, allowing cell growth in the 24 hours following damage.

CONCLUSIONS

Chronic Liver Disease and its clinical consequences have a major impact on the general population in terms of morbidity and mortality, representing a serious public health problem.

Given their evolutionary course, it is desirable to reach a diagnosis in the early stages where intervention aimed at improving the clinical picture and/or slowing down chronic liver damage may still be possible.

In recent years, scientific research has paid particular attention to the study of exosomes, important intercellular communication tools with diagnostic (biohumoral markers) and therapeutic (transporters of drugs and/or biological agents) potential.

– In this perspective, special mention should be made of the lyophilized swine liver contained in **Epatoguna**.

Several studies have documented that the lyophilized swine liver contained in Epatoguna contains exosomes capable of being internalised by human liver cells and being biologically active.

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