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EPATOGUNA – SWINE-LYOPHILIZED LIVER NEORLAND® IS THE SOURCE OF WELL-PRESERVED EXOSOMES, BIOLOGICALLY ACTIVE ON HUMAN STEM CELLS

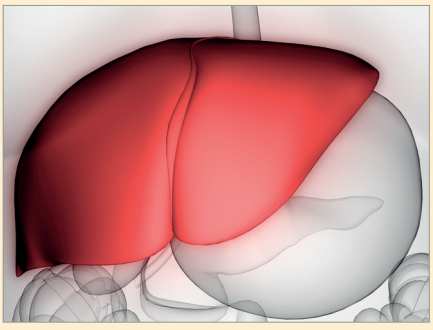
SUMMARY

Chronic Liver Diseases are a global health problem, accounting for 2 million deaths per year. In this context, many efforts have been made to identify new treatments able to restore, but in particular to preserve, liver function in the lifetime. Epatoguna is a food supplement, rich in choline and green tea extract, mainly composed of swine-lyophilized liver Neorland®. It's well known that liver contains a great amount of micro- and nano-vesicles, exosomes, arising from all the cell types contained in the organ.

– In this paper, we present our preliminary results regarding exosome isolation from lyophilized liver and their biological action on human stem cells. Exosomes from Neorland® lyophilized liver were immune-separated and quantified with ELISA. After that, human mesenchymal stem cells were treated with isolated exosomes. Human stem cells were able to internalize swine exosomes, as highlighted by BODIPY staining, and moreover exosome-treated cells displayed a cell cycle arrest, not later than 24 hours after exosome administration.
These preliminary data pave the way for new experiments, aimed at a comprehensive understanding of the role played by swine-lyophilized liver-derived exosomes in preserving a healthy liver in humans.

KEY WORDS

EPATOGUNA, LYOPHILIZED SWINE LIVER-DERIVED EXOSOMES, HUMAN MESENCHYMAL STEM CELLS, UPTAKE



INTRODUCTION

Chronic liver diseases (**CLDs**) constitute a global health problem.
– Despite considerable advances in their understanding and management, millions of people still suffer from chronic diseases associated with this vital organ.

It is currently estimated (and probably underestimated) that **844 million** people are carriers of CLDs, with a mortality of 2 million patients per year (1).
– These diseases present a worldwide clinical problem, comparable with that of other chronic diseases such as diabetes (422 million patients; 1.6 million deaths per year), lung diseases (650 million patients; 6.17 million deaths per year) and cardiovascular diseases (540 million patients; 17.7 million deaths per year) (2).

– The liver reflects an individual's state of health; the progression of a pathological state towards a chronic condition is determined by a combination of different factors [infections (HBV and HCV), alcohol abuse, genetic factors, etc.]. CLDs are not often recognised at an early stage, because the symptoms are initially scarce and generic; even abnormalities that can be detected by biological examinations are limited (2).

It is therefore essential to implement prevention programmes, and identify therapeutic strategies that prevent the progression of liver diseases.

– **Epatoguna** is a food supplement based on **lyophilized swine liver**, **choline** and **green tea**.
Neorland® lyophilized swine liver is obtained through a controlled lyophilization process specially designed to increase bioavailability and prevent the possible denaturation of the active ingredients, obtained from the fresh intact liver of young swine.
– This special procedure ensures the bioavailability of specific natural nutrients, for example the complete **vitamin pool** (especially group B vitamins), **minerals** (e.g. Iron) and essential **amino acids**, which help support and regulate hepatic metabolic processes.
It has also been hypothesised that the liver has a significant micro/nanovesicle content, notably **exosomes**, which are derived from all components of the animal's liver, including particularly numerous stem cells in young swine.

• Out of all the components of Epatoguna, we decided to study the microvesicle content, particularly exosomes, and their action in *in vitro* models.

– The cells that make up an organism are not entities in their own right; they must be able to both *perceive* and *relate* to the surrounding environment and pass "information" to the outside. There are many different mechanisms by which cells exchange information, and they vary according to the type of cell, but the secretion of signalling molecules, which make up the so-called cellular "secretome", is common to all cells.
The cellular "secretome" is made up of "naked" molecules (cytokines, chemokines, growth factors, etc.), and various types of extracellular vesicles (EVs), such as microvesicles, microparticles, nanovesicles, and exosomes.

Eukaryotic cells contain compartments surrounded by membranes, called endosomes, which have various functions.

One particular subclass of these, called the Multivesicular Bodies (MVBs), in turn contains vesicles with membranes. These can undergo various fates, including that of being released into the extracellular space, hence the name of **exosomes**.

Exosomes are a distinct population of extracellular vesicles with a diameter ranging between 30 nm and 150 nm. They have specific features (3) and specific endosomal proteins on the membrane, including CD9, CD63, CD81 and others, not found on other types of secreted vesicles which, by contrast, are formed by evagination from the plasma membrane (4).

Although the exosomes have been extensively studied, the exact mechanism by which they are secreted and the regulation of this process are still not well-defined (5).

Exosomes have been isolated *in vivo* from various biological fluids, e.g., blood, and *in vitro* from various types of cultured cells.

Exosomes mediate interaction between cells through nucleic acids (coding and non-coding RNA) and the proteins they contain, which can be transferred to neighbouring cells by endocytosis processes mediated by membrane antigens (6-9).

The mechanism by which exosomes are internalised by the recipient cells has also yet to be clarified.
This is because extracellular vesicles can be incorporated by various mechanisms, including phagocytosis, endocytosis, pinocytosis and fusion of the plasma membrane.

As mentioned, exosomes are secreted by cells specifically to mediate intercellular communication, since they are able to transfer various types of molecules to recipient cells, including proteins and different types of coding and non-coding RNA.

– This enables them to play a fundamental role in the **regulation** of both **physiological** and **pathological processes**, modifying the regulatory networks of the cell (10).

Exosomes are secreted by various types of cells and tissues. One part of the molecules they contain is always the same and is essential to the structure and sorting of the vesicles; however, they are partially differentiated in relation to cell-specific proteins and RNA, which probably reflect both their own biological function and that of the cell from which they originated (11).

The function of most of the proteins contained in exosomes is not well understood; however, many studies show that they clearly differ from those found in all other microvesicles secreted by cells (12).

Nucleic acids (mRNA and miRNA) are not active within the exosome, but become active when internalised by the recipient cell.
The study of exosomal miRNA has revealed a remarkable **specificity** according to the **cell from which it origins**, and has highlighted major differences, for example between different types of cancer cells (13,14).
– This pronounced variability of content suggests a selective process for the internalisation of miRNA, mediated by the cell that generates the exosome.

Many studies have investigated the mechanisms by which exosomes, within the same species, enter the recipient cells, release their contents (cargo) and mediate physiological and pathological processes.
On the other hand, the way in which they facilitate communication between cells of different species has not been well-researched (15), although it is known, for example, that approximately **80%** of the proteins found in the exosomes of mice and humans are conserved (homologous) between the two species (16).

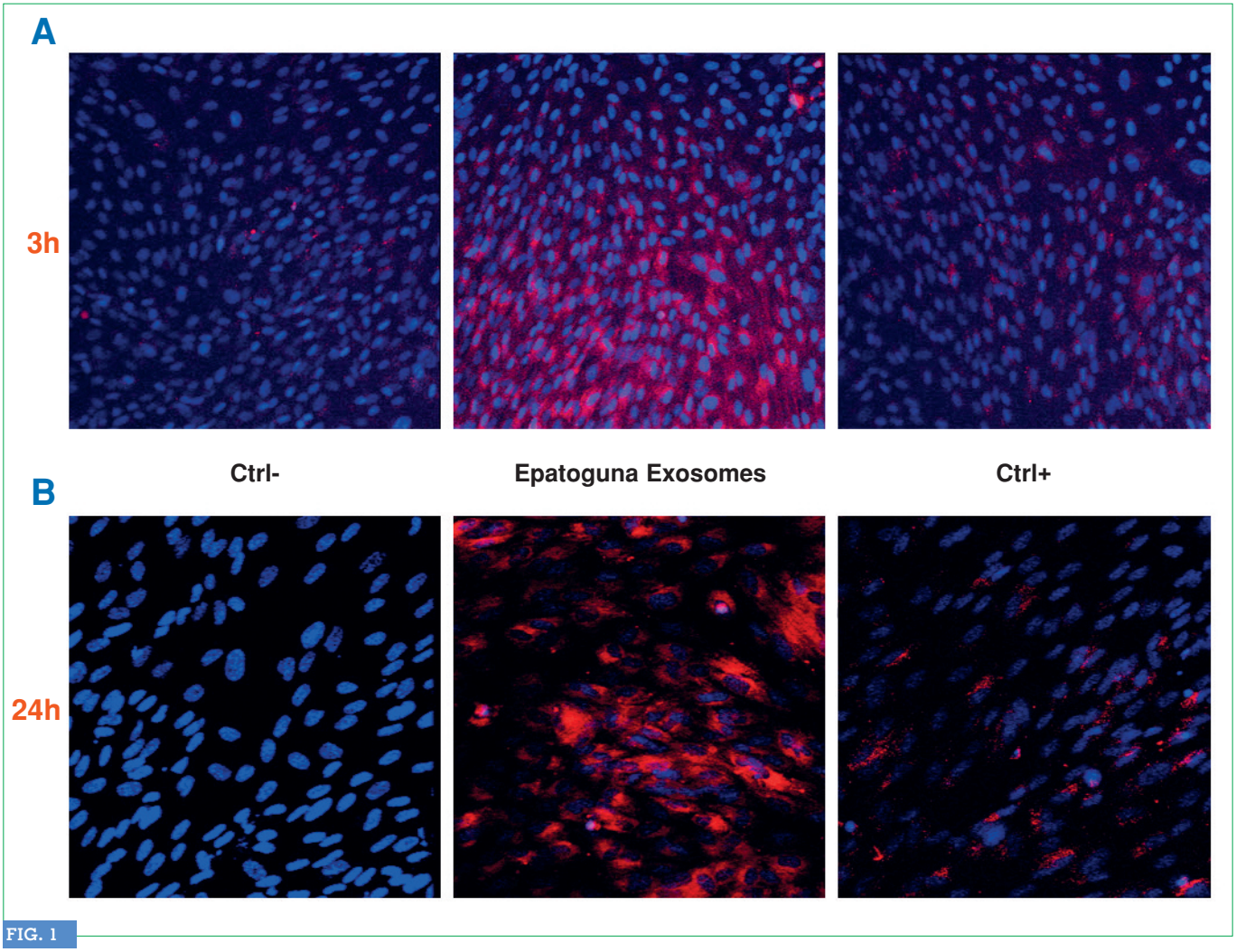


FIG. 1

***In vivo* study of exosome uptake.**

– Photomicrographs of human mesenchymal cells isolated from foetal membranes obtained from full term deliveries (hFM-MSCo), taken 3 hours (A) and 24 hours (B) after the following treatments: Ctrl- = untreated cells; Epatoguna Exosomes = cells placed in contact with exosomes stained with BODIPY; Ctrl+ = positive control consisting of cells placed in contact with BODIPY dye only.

The images were acquired using a Nikon Ti-E microscope and analysed with Nis (Nikon) software.

– Mouse exosomes can be transferred into human mast cells, and the RNA within them is translated into murine proteins in the recipient cell (17).
Some studies have also been conducted on exosomes found in milk (18).
There is some evidence that the exosomal cargo of bovine milk, particularly the nucleic acids (RNA), is transported to circulating immune cells.
– Some types of miRNA and mRNA appear to be able to regulate the expression of human genes or be translated into biologically active proteins, respectively (19).

▶ The aim of the work we have carried out is to investigate the presence of exosomes in Neorland® lyophilized swine liver and, if they are found, to determine whether these nanovesicles could be biologically active on human cells, particularly stem cells.

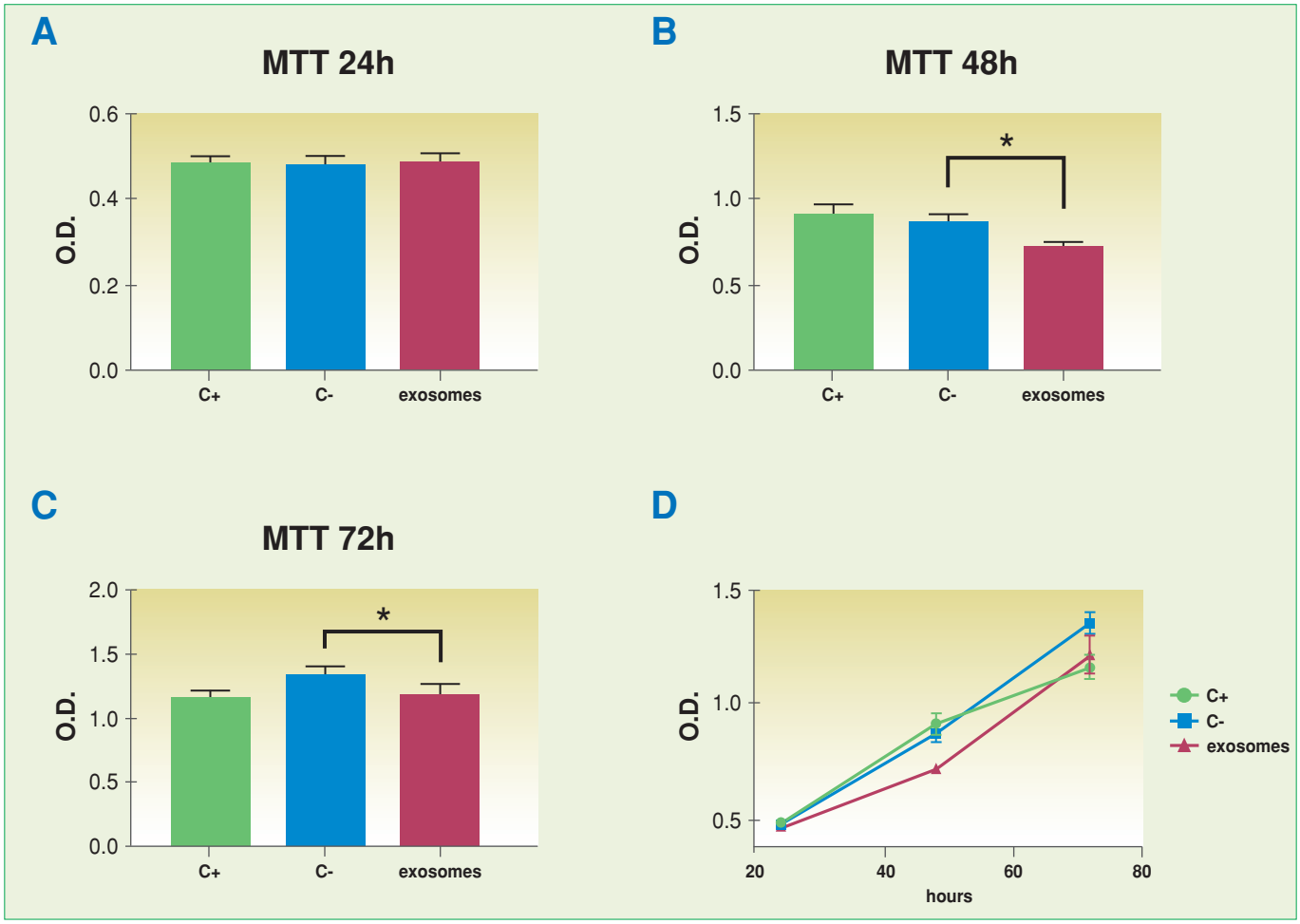
MATERIALS AND METHODS

One milligramme of lyophilized swine liver is resuspended in Ringer-lactate buffer (Krebs-Ringer Solution, Alfa Aesar) for 1 hour, and kept under constant agitation.
Collagenase IA (Sigma-Aldrich) at a concentration of 0.5 mg/ml is then added, and the mixture is kept at 37°C for a further hour, with constant stirring.
At the end of this time, scalar filtrations are carried out, using a cell-grinder (Sigma-Aldrich) and cell strainer (Falcon).
A further 3 filtrations are carried out using syringe filters, until a pore size of 0.2 µm is reached.
– The eluate is added to Exo-Quick-TC (System Biosciences, LLC) and stored overnight at 4°C.
The following morning, the preparation containing the exosomes is centrifuged at 3000 x g for 15 minutes, according to the protocol, and the pellet is resuspended in PBS buffer.
The sample is treated with Rab5b Exo-Flow Capture Kit (System Biosciences, LLC), according to the protocol.
Magnetic beads carrying the Rab5b ex-

osomal antibody are then added to the sample.
Using a magnetic support, the beads with the bound exosomes are held still while the sample is subjected to successive washings.
On completion of the process, the exosomes are released from the beads and eluted in 200 µl of the elution buffer provided in the kit.
Quantification is performed using an ELISA kit (Porcine CD81 antigen ELISA Kit, MyBioSource).
The uptake of porcine exosomes in human cells is investigated using **Mesenchymal Stem Cells** isolated from foetal membranes (FM-MSCs), by viewing them under a fluorescence microscope (Nikon Ti-E).
The exosomes are stained with BODIPY 1 TR ceramide (Molecular Probes™, Life Technologies, Carlsbad, CA, USA), according to the protocol, and purified from the excess dye by means of columns (Exosome Spin Columns, MW 3,000; Invitrogen, Life Technologies). The exosomes stained with BODIPY are added to the culture medium.
The cells are identified by vital staining of the nuclei using NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher).
The proliferation is assessed using the MTT method (Thiazolyl Blue Tetrazolium Bromide, Sigma Aldrich).
The cells are added with MTT, left to incubate at 37°C for 2 hours, the dye solubilised in a lysis buffer, and the absorbance determined using a spectrophotometer (MultiskanGO, Themo Fisher) at 570nm.
A statistical analysis is carried out using the one-way ANOVA method, with Tukey correction.
$p<0.05$ data are considered significant.

RESULTS

The lyophilized swine liver preparation is resuspended in a neutral buffer, Krebs-Ringer Solution, and subsequently digested with collagenase IA in order to release the nanovesicles trapped in the collagen, a protein that occurs extensively in the liver.
Since exosomes are nanovesicles with a diameter of a few nanometres (30 to 150 nm), the digestate undergoes several filtration steps, initially using large-pore filters to remove all coarse compo-



TAB. 1

Cell growth analysis.

A-B-C – On the abscissa axis: C+ (positive control) = cells cultured in standard FBS; C- (negative control) = cells cultured in exosome-free FBS; exosomes = cells cultured in exosome-free FBS with the addition of exosomes derived from swine liver.

On the ordinate axis: absorbance (O.D. – Optical Density) measured at 24hrs (A), 48hrs (B) and 72hrs (C). * = significant differences where $p<0.05$.

D: XY representation of values shown in A, B, C.

nents, then increasingly selective filters, thus enriching the solution in nanovesicles as much as possible.

– Exosomes can be isolated by various methods, for example ultracentrifugation, microfluidics, and many others.
For the purpose of this work, we made use of the ability of some molecules to polymerise, such as polyethylene glycol (PEG), embedding the microparticles present in the solution to make them heavier.
The polymer is added to the solution from which the microvesicles are to be isolated, left to incubate for at least one night at 4°C, and centrifuged the following day.
The sediment (pellet) that formed after centrifugation, containing the exosomes, is washed and resuspended in a sterile isotonic buffer (PBS).

– Exosomes are nano-vesicles that present specific proteins on the membrane that surrounds them (including, for example, CD9, CD81, HSP70, etc.).
The purification method we use is based on magnetic beads, which bind antibodies directed towards one of these proteins, specifically Rab5b.
The beads are placed in contact with the solution that virtually contains exosomes, so that the antibody present on the bead can bind to the exosome to be isolated.

A magnet is used to remove the beads from the solution containing all the other particles co-precipitated by the polymer. Once the beads have undergone several washings, the exosomes are released using the Elution Buffer and remain resuspended in it.

– Once isolated by magnetic separation, the exosomes are quantified in an ELISA assay. The ELISA test is based on specific antigen-antibody recognition.
The kit uses an antibody found on exosomes, CD81, an alternative to Rab5.
This stratagem, i.e. using two different antibodies to isolate and quantify the exosomes, enables us to **eliminate** the possibility of also isolating free proteins with the beads, those not bound to the microvesicles.
In fact, if no exosomes were present, the ELISA would be completely negative for CD81, since there be no single CD81 proteins in solution as they would have been lost during the purification process.

Once the presence of nanovesicles was detected, we investigated whether or not they had a potentially biologically active role.
– To determine whether or not the exosomes derived from lyophilized swine liver were recognised and internalised by human cells, we used mesenchymal stem cells **(MSCs)** isolated from a human placenta during a full term birth (kindly donated by Prof. Laura Bonsi and Prof. Francesco Alviano, University of Bologna).
Foetal membrane-derived MSCs (FM-MSCs) are particularly plastic cells and have all the stem cell markers required to define a population as "mesenchymal" (20).
MSCs can also differentiate into many cell types, including osteblasts, chondroblasts, adipocytes and **hepatocytes** (21).

For the purpose of these experiments, human mesenchymal cells were cultured in exosome-depleted serum (Exosome-depleted FBS one shot, Gibco), in order to avoid confusing them with the swine exosomes administered in culture.

The first matter to be investigated was whether or exosomes isolated from lyophilized swine liver could be **recognised** by human cells and **internalised** by them.

– To test this hypothesis, we used a dye with a high affinity for lipophilic membranes, belonging to a class of fluorescent dyes whose basic structure consists of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene, i.e. the BODIPY 1 TR ceramide, using a published method already practised in our laboratory (22).

More specifically, the exosomes are stained with BODIPY 1 TR ceramide and, after removing the excess dye, i.e. the dye not bound to the exosomes, thanks to the purifying columns (Exosome Spin Columns, MW 3,000; Invitrogen, Life Technologies), they are added to the culture medium.
The cells are viewed under a microscope after 3 and 24 hours.

– As can be seen in FIG. 1A, after 3 hours the cells (highlighted by the blue colour of the nuclei) treated with the exosomes begin to turn red, and already a significant difference between these and both the negative control (without dye) and positive control (dye only) can be seen.

The positive control is treated with a dye solution, with no exosomes, and undergoes the same purification as the sample with exosomes, through the Exosome Spin Columns. This proves that the staining of the analysed sample is **only** caused by the BODIPY retained by the exosomes, and not by the possible contamination of free dye.

The cells are once again observed 24 hours after the process (FIG. 1B).

The images show a striking result: the staining of the cells treated with exosomes (stained with BODIPY) is evident, whereas the two controls, positive and negative, appear the same as the previous day.

• The result of this experiment provide evidence that human cells, in this case stem cells, are able to **recognise** and **internalise** exosomes of porcine origin, **completely eluding the problem of species diversity**.

– Having clarified this very important point, we asked ourselves if, in addition to entering the cells, exosomes had any effect on them.

The first test carried out (many others are currently in progress) assessed the impact on cell growth.

The growth of the cells was assessed us-

ing MTT; MTT is a colorimetric assay that relies on the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) to formazan, a blue coloured substance.

This test is commonly used to assess the toxicity of substances on the mitochondria.

If the substance to be tested has no direct effect on the mitochondria, then, with some approximation, the number of mitochondria can be correlated to the number of cells.
Based on this assumption, the MTT assay is commonly used in the assessment of cell growth.

As in TAB. 1A, the cells treated with a single dose of exosomes show no significant difference when compared with the positive control (cells kept in non-exosome-depleted FBS) and negative (cells kept in FBS purified of exosomes) after 24 hours.

– By contrast, after 48 hours (TAB. 1B), the growth of mesenchymal cells treated with exosomes begins to stop, with an effect that continues for up to 72 hours (TAB. 1C).

As deduced from the graph in TAB. 1D, exosomes have no toxic effect on cells. In fact, the absorbance values (OD) increase steadily, revealing an overall increase in the number of cells in all three samples.

DISCUSSION

The preliminary data presented here provide two very important pieces of information:
1. Neorland® lyophilized swine liver **contains** exosomes; **2.** these are **internalised** by human cells and are **biologically active**.

Recent studies have investigated the effect of lyophilized on the preservation of exosomes, so that they can be biologically active even after a period of storage.
Exosomes are normally stored at -80°C (23), but there is some evidence that this is not an optimal condition for long-term storage.
This is because after 4 days of storage at this temperature, the exosomes are found to be morphologically different to those freshly isolated (24).

– Even storage at 4°C to 37°C changes the morphology of these vesicles, making them smaller, which possibly has a significant effect on their biological action (25).

Lyophilization is a process commonly used to preserve many types of organic material, from proteins to plasma, as it increases long-term stability (26).

In relation to the storage of exosomes, lyophilization has been found to be a technique of choice. Studies have confirmed that this procedure preserves integrity and functionality (27).

Our preliminary data confirm that exosomes are not destroyed by the Neorland® lyophilization procedure, and remain **intact** and **biologically active**.

The cells used in this study are human mesenchymal cells isolated from full term placenta (20).
Mesenchymal stem cells are adult cells that can be isolated from various tissues, since they are found in almost all organs.

They are defined as such by the presence of some surface markers (CD29, CD44, CD73, CD90, CD105) and the absence of others (CD14, CD34, CD45 and HLA-DR), and also their ability to differentiate into other cell types (osteocytes, chondrocytes, and adipocytes).
In addition to their differentiation potential, they also have an intrinsic immunomodulating capacity.

Their function within the organs from which they are obtained is not always known, but the potential demonstrated *in vitro* makes them particularly interesting, not only because of their use in autologous transplant, but above all the possibility of identifying treatments that could improve the regenerative capacity of the organ to which they belong.

The liver also has mesenchymal stem cells (liver-derived human MSCs LHMSCs) which possess specific features that differ from those isolated from other organs; for example greater secretion of protective factors (28).
These cells can differentiate into mature hepatocytes; however, the mechanism by which this occurs *in vivo* is still unknown, although it is clear that they are involved in various physiological and pathological processes (29).

– In this context, it is particularly interesting that exosomes derived from swine liver are able to terminate the growth of mesenchymal stem cells.

Several studies are in progress in our laboratory to investigate this fact.

– Firstly, an assessment of the dose-dependence of this action is taking place.

Moreover, we are analysing the changes in gene and protein expression of the recipient cells, with a view to understanding whether, in addition to the termination of a cycle, there is a differentiation stimulus. It has already been shown that the addition of exosomes extracted from differentiated cells can change the fate of mesenchymal stem cells, and switch the phenotype from adipocytic to osteocytic and vice versa (30).

Regulation could also occur through the miRNA found in the exosome, as already demonstrated in other models, where it was observed that exosomes derived from hematopoietic cells can facilitate the differentiation of embryonic cells *in vitro* by inhibiting a specific pathway (31).

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